Evaluating the Impact of Replication Fork Pausing on Epigenetic Conversions in *Saccharomyces cerevisiae*: A Novel Approach and First Analysis

by

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ABSTRACT

EVALUATING THE IMPACT OF REPLICATION FORK PAUSING ON EPIGENETIC CONVERSIONS IN SACCHAROMYCES CEREVISIAE: A NOVEL APPROACH AND FIRST ANALYSIS

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University of Guelph, 2018
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The subtelomeric regions of Saccharomyces cerevisiae undergo infrequent conversions in the expression state of a gene. The switch in gene expression is thought to be a consequence of a change in the epigenetic information encoded by post-translational modifications on histone proteins. Previous assays used to study epigenetic conversions involved the use of the counter selection drug, 5-FOA, but recent evidence has shown that 5-FOA can lead to unreliable conclusions. Using a new approach involving cellular fluorescence, I developed an assay that circumvents the need for 5-FOA. With flow cytometry, I analyzed the effects of a replication fork pause site on the frequency of epigenetic conversions. The results were cross-validated with microscopy and with the previous assay. However, the data was inconclusive. It is plausible that the design of the reporter protein may be responsible for skewing the results. I have included recommendations for further optimization. Furthermore, I discovered that a combination of serine to alanine point mutations to CDC7 and CDC28 phosphorylation sites, S94A-S238A and S238A-S515A, on Chromatin Assembly Factor I (CAF-I) can significantly alter the morphology of the cell. This morphology is consistent with arrest in the G1 phase of the cell cycle; therefore, this novel observation demonstrates that the deregulation of histone chaperone CAF-I may cause deregulation of the cell cycle.
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<th>Abbreviation</th>
<th>Description</th>
<th>Symbol</th>
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<tr>
<td>5-FOA</td>
<td>5-fluoroorotic acid</td>
<td>mM</td>
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<td>°C</td>
<td>degrees Celsius</td>
<td>OD</td>
<td>Optical Density</td>
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<td>facilitates chromatin transcription</td>
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<td>Luria-Bertani broth</td>
<td>SIR</td>
<td>silent information regulator</td>
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<td>microliter</td>
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<td>MCM</td>
<td>mini-Chromosomal Maintenance</td>
<td>YPD</td>
<td>Rich yeast media</td>
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Chapter 1: Review of Literature

1.1 What is Epigenetics?

Epigenetics is the study of changes heritable gene expression patterned that are not encoded within the DNA sequence, but rather through transmissible chromatin structures. Epigenetic mechanisms regulate gene expression by modifying DNA and histone proteins through the addition or removal of certain chemical groups (1). These modifications, known as epigenetic marks, determine the chromatin structure and the transcriptional state of a locus (2). Epigenetic marks determine if a gene is transcriptionally active or transcriptionally silent, and its reversibility can create tissue specific-expression patterns or adaptations to the environment. Several epigenetic modifications to histone proteins have been identified and their effects on gene expression have been well characterized. However, epigenetic modifications are still being discovered. Altogether these patterns make up the “epigenetic landscape” or “epigenome” of the cell. Notably, the patterns of gene expression are heritable; therefore, they are passed from a mother to a daughter cell. The faithful transmittance of these patterns is critical because epigenetics plays a key role in many processes, such as development, X-inactivation, and gene imprinting. Furthermore, the dysregulation of these epigenetic processes has been linked to many diseases and disorders in humans.

1.2 Chromatin and Gene Expression

Epigenetic modifications regulate the transcriptional activity of a gene by influencing its chromatin structure. There are two general types of chromatin: euchromatin and heterochromatin. Euchromatin is an extended form of chromatin with irregularly spaced
nucleosomes and linker DNA that is susceptible to nuclease degradation (3). Euchromatin favours transcription due to the accessibility of transcription factors.

Heterochromatin confers a compact chromatin structure that prevents the access of transcription factors to the locus, thus transcriptionally silencing the gene. This chromatin contains regularly spaced nucleosomes and inaccessible linker DNA (3). Heterochromatin can be further divided into two subtypes, constitutive and facultative. Constitutive heterochromatin contains repetitive DNA, such as the chromosome centromeres and telomeres, and localizes at the nuclear periphery (4). In contrast, facultative heterochromatin mainly comprises promoters of specific genes of a certain cell type that are regulated (4).

1.3 Epigenetic Marks on Histone Proteins

Eukaryotic DNA is efficiently condensed into chromatin using nucleosome proteins. The nucleosome is made of an octamer of histones proteins: H2A, H2B, H3, and H4. Two H3-H4 dimers are assembled as a core and form a tetramer, and two H2A-H2B dimers flank each side of the tetramer (5). Histones contain a highly conserved fold motif that allows for the formation of the structure and the protrusion of N-terminal tails (6). Certain amino acids of the N-terminal tails of histones are subjected to various post-translational modifications (PTMs), or epigenetic marks. Modifications such as acetylation, ubiquitylation, or methylation can occur to lysine (K) and arginine (R) residues, while phosphorylation takes place at some serine (S) and threonine (T) residues (7). Combinations of PTMs of histone proteins can activate or silence gene transcription by remodeling the chromatin structure. Modifications can alter the binding affinity of the positively charged histones to the negatively charged DNA. For example, when an acetyl group is added to a lysine residue, it neutralizes
the positive charge of the amino acids on histone tails, which relaxes the chromatin and exposes it to transcription factors (8). In *S. cerevisiae*, the acetylation of H3K9 (histone H3 at lysine 9), H3K18, and H3K27 are known as epigenetic markers of euchromatin (9). Moreover, the trimethylation of H3K4 is an important marker of active transcription and has been investigated for its role in activating developmental genes (10, 11). The deacetylation of H4K16 is an important mark of heterochromatin because it is involved in the binding of silent information regulator (SIR) proteins. This will be discussed in detail below (12).

PTMs to histones proteins can also provide docking sites for protein-protein interactions. For instance, acetylated lysine residues on histones are recognized by bromodomains on proteins (13). As a result, PTMs can recruit protein complexes which carry out various functions, such as recruiting transcription factors. The PTMs to histone protein are reversible, which allows for dynamic changes within the cell. Histone acetyltransferases (HATs) are the enzymes that transfer an acetyl group to specific lysine residues and histone deacetylases (HDACs) can remove the acetyl groups. Likewise, histone methyltransferases (HMTs) and histone demethylases (HDMs) have inverse functions. It has been proposed that the coordination of PTMs of histones proteins may act as a “code” for the cell to mediate chromatin-based processes, such as replication, DNA-repair, and transcription (14).

1.4 Silencing in *S. cerevisiae*

In eukaryotes, heterochromatin can be found at most repetitive DNA elements, including the telomeres, the centromeres, and most transposable elements (4). However, in *S. cerevisiae*, heterochromatin is also formed at another region, the donor mating type loci. Studies of these loci have been instrumental in our understanding of how heterochromatin silences gene
expression. *S. cerevisiae* can exist in a haploid state or in a diploid state. Haploids express one of two mating types, *a* or *α*, determined by the allele at the mating type (*MAT*) locus on chromosome *III* (15). The mating type genes, *a* and *α* cassettes encode regulatory proteins that control the expression of surface proteins and pheromones used during the mating process (16). Adjacent to the *MAT* locus, there is a copy of each of the alleles, the *HML-a* and the *HMR-α*, also termed donor mating type loci. The *HML-α* and the *HMR-α* serve as donors in the process of mating type switching and are permanently repressed with heterochromatin (Figure 1) (16).

**Figure 1. The structure of the mating type loci in *S. cerevisiae*.** The presence of the *a* or *α* allele at the *MAT* locus determines the mating type in the haploid state in *S. cerevisiae*. The *HML-α* and *HMR-α* cassettes flank the *MAT* locus and the SIR proteins establish and maintain heterochromatin at these loci. The *E* and *I* elements act as silencers, which aid in the formation of heterochromatin.

1.5 Establishment of Silencing: SIR Proteins

The *SIR* genes were identified through a series of knockdown studies for their role in the silencing of the *HML* and *HMR* donor loci in *S. cerevisiae* (17, 18). The SIR proteins are responsible for the establishment and maintenance of the heterochromatin structure in order to repress the transcription of underlying loci (19). The establishment of heterochromatin at the mating type loci begins with the *cis*-acting silencer elements, *E* and *I*, which flank the loci (Figure 1) (4). Silencer elements are DNA sequences that contain binding sites for proteins involved in the formation of heterochromatin. The *E* and *I* elements contain an *ARS* consensus site (*ACS*), which is a highly conserved 11 base pair sequence that functions as an origin of
replication in yeast (20). The ACS is a binding site for the Origin Recognition Complex (ORC) (21). The E and I elements also contain binding sites for Rap1p and Abf1p (16). In S. cerevisiae, Rap1p and Abf1p belong to a family of multifunctional DNA-binding proteins termed general regulatory factors, and have roles in chromatin based processes, such as gene activation and repression, DNA replication, and telomere structure and function (22). Rap1p binds to poly(C1-3A) sites, and Abf1p recognizes the sequence ‘5-TnnCGTnmmnTGAT-3’ which is typically found at promoters, silencers, and telomeres (23, 24). The binding sequences for these proteins in the E and I silencers are important for the formation of heterochromatin, as deletion mutations for these binding sites within these elements can lead to the depression of the donor mating type loci (25).

During the establishment of heterochromatin at the donor mating type loci, ORC, Rap1p, and Abf1p bind their respective sites on DNA and recruit the SIR proteins. The trimeric SIR complex, comprised of Sir2p, Sir3p, and Sir4p, is responsible for the nucleation and spreading of the heterochromatic domain in yeast (26). The SIR proteins are recruited in a stepwise manner. The Orc1p of the ORC recruits Sir1p, and Sir1p can recruit Sir4p, Sir4p forms a stable dimer with the Sir2p, a class III histone deacetylase (27, 28). Sir2p removes an acetyl group on lysine 14 on histone H4, which is a hallmark of heterochromatin in S. cerevisiae (29). The deacetylation of H4K16 by Sir2p recruits Sir3p to bind (29). Subsequently, based on the affinity of Sir3p for Sir4p, this attracts additional Sir4p-Sir2p dimers (29). Alternatively, Sir3p can be recruited by Rap1p or Abf1p, but preferentially binds nonacetylated histones (12, 29, 30). This step-wise process permits a spreading of the SIR complex and propagation of the deacetylation of H3K16 and thus heterochromatin.
The establishment of heterochromatin at the telomeres also involves the SIR proteins. However, the TG1-3 repeats act as the principal silencer. Rap1p binds TG1-3 repeats and recruits Sir3p, which recruits Sir4p-Sir2p, and the signal is spread towards the subtelomeric region (29). In the subtelomeric region lies an ORC binding site that acts as a proto-silencer (Figure 2 A) (31). A proto-silencer is defined as an element that can propagate the silencing signal (31). The indefinite spreading of the SIR complexes is prevented by the limiting amounts of Sir3p and Sir4p present in the cell, and by the anti-silencing activity of Sas2p. Sas2p is a histone acetyltransferase that adds an acetyl group to H3K16, thus directly counteracting the deacetylation activity of Sir2p (32). The opposing forces of Sir2p and Sas2p are believed to create a fluctuating boundary of heterochromatin and euchromatin (Figure 2 B). The Sas2p anti-silencer is an example of a mechanism that maintains a negotiable border. Negotiable borders are not established at a specific DNA sequence but rather by the balance of factors that promote heterochromatin and euchromatin (33). In contrast, fixed borders involve a chromatin boundary, this prevents the spreading of heterochromatin over a region. An example of this is the protein Bdf1p; Bdf1p interacts with histone H4 tails to form a physical barrier to prevent histone deacetylation and thereby prevents the spread of heterochromatin (34).
Figure 2. The spreading of SIR proteins at the telomere. A: Multiple Rap1p proteins bind the TG(1-3) repeats. Rap1p recruits Sir3p and Sir4p-Sir2p dimers. Sir2p deacetylates histones promoting the formation of heterochromatin. Subtelomeric ACS binds ORC and Sir1p to boost the silencing signal of the telomeres. B: Sas2p counteracts the silencing signal by acetylating histones at H4K16. The balance between the Sir2p and Sas2p activity create a fluctuating boundary.

1.6 Position Effect Variegation

Position effect variegation (PEV) is an epigenetic phenomenon that occurs when a gene is translocated near a region of heterochromatin and its transcription becomes reversibly repressed. Transcriptional repression is a consequence of the spreading of the heterochromatic domain over the gene (35). The negotiable euchromatin and heterochromatin boundary is variable from cell to cell, which causes expression of the gene in some cells and repression in others, generating a variegated phenotype. In S. cerevisiae, PEV can be visualized using the ADE2 as a reporter gene that is juxtaposed to the heterochromatic domain of the telomere (Figure 3). When ADE2 is not expressed a non-lethal red pigment builds up in the cell (36). Colonies with a variegated expression of ADE2 contain red and white sectors. This is a visual representation of a colony that began with a single progenitor cell that has an epigenetic
conversion from an active \textit{ADE2} locus to silent \textit{ADE2} locus. It was calculated that the number of generations that the epigenetic state of \textit{ADE2} was stably inherited to be approximately 15-20 cell divisions (36). This indicates that an epigenetic switch occurs every 15-20 cell divisions. Other reporter genes, such as \textit{URA3}, \textit{TRP1}, \textit{HIS3}, were found to have the same variegated transcription patterns as \textit{ADE2} when positioned near the telomere (36).

Figure 3. Visualization of telomere position effect variegation using a \textit{ADE2} reporter gene. The heterochromatin domain can affect the transcription of an adjacent gene. The spreading of heterochromatin can cause transcriptional silencing.

1.7 Subtelomeric Elements of \textit{S. cerevisiae}

Telomeres consist of simple, non-coding, tandemly repeated sequences important for the structural integrity of the chromosome. In \textit{S. cerevisiae}, telomeres consist of repeats of \(300 +/- 75\) base pairs of \(TG_{(1-3)}\) repeats followed by a 3’ single-stranded overhangs rich in
guanine (37). The telomeric repeats do not form a T-loop but they do have a similar fold back mechanism (37). This telomeric structure helps to prevent the fusion of linear ends and promotes the proper replication of the entire linear chromosome (38).

Adjacent to the telomeres lies the subtelomeric region. In *S. cerevisiae*, the subtelomeric regions are made up of the X element core sequence, the X element combinatorial repeats, the Y’ element and the telomeric repeats (39). Subtelomeric elements display a degree of polymorphism between chromosomes and strains (40). The X core element is a small conserved 475 base pair sequence that is found at all telomeres (41). The X core element contains the proto-silencing elements, ACS and Abf1p binding site (42). The X combinatorial repeats, when present, lie proximal to the telomeric repeats and can be found in different combinations of small elements termed A, B, C, or D (39, 41). The A type X combinatorial element has been shown to contain a Tbf1p binding site, which has anti-silencing activity (41, 42). The Y’ element when present is found between the X combinatorial elements and the X core element in up to four tandem copies, it is only found on 50%-60% of yeast telomeres (43). There are two subclasses of Y’ elements which are distinguished by their size, 6.7 kb and 5.4 kb (41). The Y’ element contains proto-silencing and anti-silencing activity due to the presence of ACS and Tbf1p binding sites, respectively (44). In addition, the Y’ element includes an open reading frame for an ATP-dependent helicase, Y’-Help1 (44, 45).

1.8 Inheritance of Epigenetic Marks

In simplified terms, epigenetics is the ability to transmit information of specific gene regulation patterns to daughter cells. However, the mechanism of transmission of post-translational modifications on histones is still unclear. This process is tightly linked to
DNA replication when chromatin is disassembled and reassembled on the daughter strands. Eukaryotic cells have evolved an elaborate system of histone chaperones, nucleosome remodeling factors, and epigenetic reader and modifier molecules, all of which ensure the epigenetic state is maintained in the genomes after the passage of the fork. A model of this process is described in Figure 4.

1.9 Chromatin Disassembly

Chromatin disassembly involves the DNA replication helicase, Minichromosome Maintenance Protein Complex (MCM), which is a key component of the pre-replication complex (46). The MCM complex works with the histone chaperone Facilitates Chromatin Transcription (FACT), to disassemble nucleosomes ahead of the fork to allow it to progress. FACT disrupts the interactions between the nucleosome and DNA by removing one H2A-H2B dimer (47, 48). Asf1p is a histone chaperone that interacts with MCM complex and has been shown to interact with H3-H4 histones (49). FACT and Asf1p are not tethered to the replication fork and can ferry parental histones behind the fork to be recycled into the daughter strands.

1.10 Nucleosome Re-assembly Behind the Fork

Currently, the molecular mechanism of how parental H3-H4 histones are incorporated into newly replicated DNA is still largely unknown. However, the process of how newly synthesized H3-H4 histones are deposited onto DNA is better understood. Histone chaperones, Asf1p, Chromatin Assembly Factor-1 (CAF-1) and Rtt106p work collectively to assemble H3-H4 tetramers on to newly replicated DNA in replication-coupled nucleosome assembly (50). During S phase, histones are synthesized in the cytoplasm and transported to
replication forks. Asf1p complexes with H3-H4 dimer at the interface that forms the tetramer (51). Before transport to the nucleus, newly synthesized H3 histones are marked by H3K56ac by Rtt109p, and Asf1p is essential for this process (52). Asf1p transports newly synthesized H3-H4 dimers to the nucleus where it can transfer the H3K56ac-H4 dimers to CAF-1 or Rtt106p (50, 52). Asf1p was found to directly interact with the Cac2p subunit on CAF-1 (53). On the other hand, Rtt106p directly interacts with the H3-H4 dimer, via a double pleckstrin-homology domain that interacts with the region containing K56 on H3 (54). Interestingly, data in recent years suggests that CAF-1 and Rtt106 can promote the formation of an H3-H4 tetramer prior to deposition onto daughter strands (55, 56). Once the H3-H4 tetramer is assembled, H2A-H2B dimers are incorporated by FACT to complete the formation of the nucleosome (50, 57). After the assembly of nucleosome onto nascent daughter strands, epigenetic reader proteins recognize the parental mark on the old histones, then recruit a chromatin modifier protein or writer protein, to reinstate the modification onto the new histone (58).
1.11 Nucleosome reconstitution and H3-H4 tetramer splitting

The two daughter strands produced during DNA synthesis includes half of the histones from the parental strands and half of the newly synthesized histones (59). Currently, one of the gaps in knowledge is how the new and old histones are distributed. Evidence has shown that the H2A-H2B dimers are disassembled from the H3-H4 tetramer in front of the fork (48). However, there is conflicting evidence regarding the disassembly of the parental H3-H4 tetramer. There are three proposed models, to describe the distribution of old and new H3-H4 tetramers: conservative, semi-conservative, and a random model. In the conservative model, the H3-H4 parental tetramers stay intact and one daughter strand contains only parental H3-H4 tetramers and the other daughter strand contains only new tetramers. In the semiconservative model, the parental H3-H4 tetramer is split into two H3-H4 dimers, and a
new tetramer is formed with one parental H3-H4 dimer and one new H3-H4 dimer. Thus, the nucleosomes in the daughter strands consist of a tetramer with one old and one new H3-H4 dimer. In the random distribution model, old H3-H4 tetramers remain intact, and nucleosomes with old and new H3-H4 tetramers are randomly deposited on each of the daughter strands.

Recently, one study used fluorescently labeled nucleosomes to determine if H3-H4 tetramers are split into dimers. In this study, *Physarum* H3 was labelled with acetoxyrylene, which fluoresce blue, but when two pyrene residues are within a few angstroms, they fluoresce green. Following a pulse-chase analysis, authors reported only green fluorescence for 90 minutes, thus providing evidence for the conservative distribution model (60). Crystallization studies revealed that Asf1p interacts with the H3-H4 heterodimer at the interface where these dimers interact to form the tetramer; therefore, Asf1p interacts with an H3-H4 heterodimer and not an H3-H4 heterotetramer (51). Interestingly, recent data pointed to the possibility that the type of histone H3 variants may determine if the H3-H4 pairs are split into dimers or remain as a tetramer during assembly. Histone variants have high sequence similarity but are not alleles of one another. In eukaryotes, histone H3 has two primary variants, H3.1 and H3.3, which only differ at three to four amino acids (61). Histones H3.1 and H3.3 were found to have different distribution patterns. H3.1 was typically found enriched in silenced regions, whereas H3.3 was typically found at actively transcribed genes (62).

1.12 CAF-1 Histone Chaperone Complex

The histone chaperone complex CAF-1 is composed of three subunits Cac1p, Cac2p, and Cac3p (63). CAF-1 possesses a single consensus PIP box sequence that allows it to interact with replication processivity factor, PCNA. The interaction with PCNA allows CAF-1
to carry out its aforementioned role in the assembly of H3-H4 tetramers onto newly synthesized daughter strands (64). Other roles for CAF-1 have recently emerged; CAF-1 has been shown to have a role in the repair of single-stranded breaks in DNA and nucleotide excision repair (65). The factors that regulate the activity of CAF-1 are still not well understood. Mass spectrometry analysis reported putative phosphorylation sites for Cyclin-Dependent Kinases (CDKs), Cdc7p and Cdc28p. CDKs regulate the cell cycle by phosphorylating residues on substrates to alter their activity and coordinate it with the cell cycle (66). Cdc7p is important for the cell cycle transition from G1 phase to S phase. Cdc7p interacts with Dbf4p cyclin to form a DDK-complex, which targets pre-replication complexes to initiate replication (67). CDK1, encoded by CDC28, also promotes the transition from G1 phase to S phase through the activation of specific transcription factors. Cdc28p also works cooperatively with Clb cyclins to prevent the formation of pre-replication complexes until after the completion of mitosis (66). Based on site-directed mutagenesis (SDM) studies on Cac1p, serine residues 94 and 515 were putative substrates for Cdc28p, and serine residues 238, 501, 503 are suspected Cdc7p phosphorylation sites (68). These modifications were found to contribute to the affinity between Cac1p with chromatin and PCNA. SDM of Cac1p to serine 94 to alanine (S94A) and S515A showed a reduced association with chromatin but the ability to bind PCNA was unaffected, compared to wildtype CAF-1 (68). The mutations S238A, S501A, and S503A were able to bind DNA, but the S238A mutation had a modest decrease in association with PCNA (68). Further research is needed to better understand the effect of phosphorylation by CDKs on the regulation of CAF-1.
1.13 Replication Fork Pausing

During the normal progression of replication, the replication machinery pauses, or stalls, at programmed sites throughout the genome, termed replication fork pause sites (69). In *S. cerevisiae*, approximately 1,400 discrete replication fork pause sites have been characterized (70). These sites are concentrated at the centromere, the tRNA genes, the silent mating type donor loci, the rDNA repeats, the telomeres, and inactive replication origins (71).

A well-characterized fork pausing site is located within the ribosomal RNA array (rRNA) genes (72). In *S. cerevisiae*, the genes encoding rRNA is located on chromosome XII and consists of approximately 220 copies organized into tandem repeats (73). Each copy contains two transcriptional units encoding for the 5S rRNA and the 35S precursor rRNA, and two non-transcribed spacers (NTS), *NTS1* and *NTS2* (74). In S phase, transcription and replication of the rRNA genes occur simultaneously (75). Replication begins in *NTS2* which contains an *autonomously replicating sequence* (*ARS*) (76). This poses a problem for the cell because transcription of the 35S transcript can occur in the opposite direction of replication, which could result in collisions between replication and transcription machinery.
Figure 5. The structure of rRNA repeats in *S. cerevisiae*. The rRNA genes are present in multiple copies on the chromosome XII. Each rRNA copy contains the 5S gene, 35S gene, ARS, and RFB. During replication, every fifth ARS is active. The replication fork proceeds in both directions; however, the rightward moving fork is stalled once it arrives at the RFB in the NTS2. The pausing of the replication fork prevents the collision between replication machinery and the 35S machinery.

The replication of the rRNA gene repeats occurs in a unidirectional manner. Leftward moving replication forks are arrested at a specific sequence in the NTS1, termed a *replication fork barrier* (RFB) (Figure 5) (77). The RFB only impedes replication forks proceeding in the opposite direction of transcription of the 35S gene (77). Not every ARS within the repeats is active during replication; an ARS in approximately every fifth repeat is utilized (75). Therefore, the rightward moving fork can continue through neighboring repeats until it reaches a replication fork paused at RFB. This allows for the continuity and termination of DNA replication in a way that will not clash with the active transcription of the rDNA. The RFBs in the rDNA loci are highly conserved from yeast to humans (78). A study using atomic force microscopy revealed that the mechanism of pausing was due to the binding of Fob1p at the RFB (79). Fob1p can pause a replication fork by wrapping the RFB around the protein, similar to that of a nucleosome (79). Mutations to Fob1p lead to an increased recombination
rate in rDNA, which may be a result of increased DNA damage because of the collapse of the replication fork (80). *RFB* and Fob1p are an example of replication fork pausing through tightly bound protein complexes. Replication forks can also pause due to complex DNA secondary structures, called G-quadruplexes.

The prolonged pausing of replication forks can lead to their collapse and result in DNA breaks (81). In *S. cerevisiae*, there is a small family of 5’ to 3’ DNA helicases that have a role in replication fork pausing, Rrm3p and Pif1p (82). Rrm3p can dissolve stable DNA-protein complexes, including Fob1p at the *RFB* (70, 82). The mechanism of Rrm3p to remove Fob1p to facilitate replication progression is not well understood. Rrm3p has been implicated as a replication factor because of its interaction with PNCA (83). Pif1p can remove proteins bound to DNA but can also resolve RNA-DNA hybrids and G-quadruplex structures (84).

Similarly to the rDNA, the subtelomeric regions can cause replication forks to pause due to the tight binding of Rap1p to the TG(1-3) repeats (85). Rrm3p also plays a role in the recommencement of paused forks in the subtelomeric region. In *rrm3Δ* strains, replication fork pausing in the subtelomeric region was exacerbated (70). The deletion of Rrm3p was also found to have an effect on epigenetic conversions and histone turnover at the subtelomere (86).
1.14 Epigenetic Conversion Factors

The FOA\textsuperscript{R} variegation assay can be used to observe and quantify epigenetic conversions in \textit{S. cerevisiae} (86–88). The principal reporter gene, \textit{URA3}, has been used to study telomeric silencing for the last decade (36). The \textit{URA3} gene is routinely used because the active expression state of \textit{URA3} can be selected or counter selected using different media. Ura3p is an Orotidine5’-Phosphate Decarboxylase (ODCase), which is required for the synthesis of pyrimidine ribonucleotides (89). The active state of \textit{URA3} can be selected for with media deficient in uracil and counter selected for with media supplemented with 5-Fluoroorotic acid (5-FOA) because Ura3p converts 5-FOA to a toxic metabolite, fluorodeoxyuridine (90).

In the FOA\textsuperscript{R} variegation assay, an artificial telomere is generated by targeting \textit{URA3} through homologous recombination to the left telomere of chromosome \textit{VII} of \textit{S. cerevisiae} (36). Due to TPE, the spreading of the telomeric silencing \textit{URA3} can undergo conversions from the active to silent state and vice versa. Certain DNA elements have been studied using this technique and can be classified as having proto-silencing or anti-silencing activity based in their effect on epigenetic conversions. Similarly, the role of certain proteins on epigenetic conversions have been analyzed using deletion strains. The FOA\textsuperscript{R} variegation assay has identified the haploid yeast strains \textit{cac1Δ}, \textit{rrt106Δ}, and later \textit{rrm3Δ} and \textit{sas2Δ} to have a reduced frequency of epigenetic conversions (68, 86). Strains with reduced epigenetic conversions are described as a loss of variegation phenotype. This phenotype shows that most of the cells remain in their initial selection state, indicating that their ability to switch the expression of the \textit{URA3} reporter has been lost, compared to their \textit{wildtype} counterparts.
Therefore, it can be concluded that the deletion of a gene that results in a loss of variegation phenotype has an essential role in the mechanism of epigenetic conversions.

The mechanism for epigenetic conversions behind TPE is still unclear. It has been hypothesized that the disassembly and reassembly of nucleosomes could provide a window of opportunity for epigenetic change (91). The recently observed phenotypes of cac1Δ mutants support this idea (68). The finding that Rrm3p has a role in epigenetic conversions is consistent with the presence of many replication fork pausing sites in the subtelomeric region. Altogether, these lines of evidence suggest a model of epigenetic conversions. When the replication fork reaches a barrier, such as a tightly bound protein, the parental nucleosomes ahead of the fork are not being disassembled and are not recycled into the daughter strands. However, newly synthesized histones are still being delivered. Operating under the semi-conservative model of histone reassembly, if a nucleosome is reassembled with only newly synthesized histones then this could lead to a loss of epigenetic information because the parental histone, which acts as a template for epigenetic marks, is absent.

A limitation of the FOA<sup>R</sup> variegation assay is the use of the drug, 5-FOA. In recent years it was revealed that 5-FOA has an inhibitory effect on the ribonucleotide reductase (RNR) enzyme, which can result in the dis-balanced abundance of the cellular dNTPs (92). RNR is an enzyme that generates the four deoxyribonucleotide triphosphates. In keeping with this observation, the use of 5-FOA has been linked to unreliable assays in certain mutants. For example, dot1Δ and pol30Δ were falsely concluded to be involved in gene silencing (93). This suggests that the decreased growth in the presence of 5-FOA may not always indicate increased expression of a telomeric URA3 reporter but can rather reflect an increase in RNR.
activity. For this reason, the authors have cautioned against the use of 5-FOA as it is a questionable indicator for the silencing of the \textit{URA3} reporter gene.
1.15 Summary

Silencing at the subtelomeric regions is dynamic, it can be simplified and summarized as the culmination of two independent processes. First, the opposing activities of the Sir2p and Sas2p. Sir2p promotes heterochromatin by deacetylation of H4K16; in contrast, Sas2p promotes euchromatin by acetylating H4K16. The type of chromatin is dependent on the acetylation status of H4K16. The opposing activities of Sir2p and Sas2p will eventually reach an equilibrium and a heterochromatin-euchromatin dynamic boundary is created during the cell cycle. Second, the presence and coordination of specific subtelomeric DNA elements can impact the state of chromatin. Silencer and proto-silencers can enhance and relay the signal of heterochromatin and anti-silencers can act as a barrier to prevent the spreading of heterochromatin. Together, the level of histone acetylation and the presence of subtelomeric elements determine telomeric silencing.

The FOA\textsuperscript{R} variegation assay was created in the Yankulov lab to quantify epigenetic conversions of a reporter gene at the subtelomeres in \textit{S. cerevisiae}. Previously, factors have been identified to have a role in epigenetic conversions at the subtelomeres. First, the Cac1p, a subunit of the histone chaperone CAF-1 was found to have a loss of variegation phenotype. More recently, Rrm3p a protein that relieves replication forks paused sites within the genome, had a similar loss of variegation phenotype. The role of replication fork pause sites in epigenetic conversions has not been investigated.
Chapter 2: Research Proposal

Hypothesis: replication fork pausing impacts the frequency of epigenetic conversions at the subtelomeres of *S. cerevisiae*.

Research objectives:

1. Establish a 5-FOA free variegation assay using cellular fluorescence.
2. Assess the impact of replication fork pausing on epigenetic conversions using new techniques: flow cytometry and microscopic scoring.
3. Cross-validate the results with the new techniques with results from the old technique, FOA\textsuperscript{R} variegation assay.
Chapter 3: Materials and Methods

3.1 Growth Media and Conditions

3.1.1 *Saccharomyces cerevisiae*

Yeast cells used in the study were grown in non-selective Yeast Peptone Dextrose (YPD; 1% w/v yeast extract, 2% w/v peptone, and 2% w/v pre-sterilized glucose). The cells were routinely cultured on a rotating wheel at 30˚C. The doubling time of *S. cerevisiae* is typically 90 minutes. If selection for a strain or plasmid was required, yeast cells were grown in Synthetic Complete (SC) media with the appropriate amino acid(s) dropout(s). SC media is prepared with 2.32 g/L of amino acid free yeast nitrogen base, 2% w/v filter-sterilized glucose, and amino acids and nucleobases as outlined in Table 1. Amino acids and nucleobases were made in a solution at a 100X final concentration and filter-sterilized with a 0.22 µm nylon membrane. SC media supplemented with 5-FOA was prepared with all amino acid and nucleobases and supplemented with 1g/L of 5-FOA, which was dissolved in water and boiled. YPD and SC plates were occasionally supplemented with the antibiotic, geneticin or G418, at a concentration of 400 µg/mL.

3.1.2 *Escherichia coli*

DH5α was grown in Luria-Bertani broth (LB; 1% w/v NaCl, 1% w/v tryptone and 0.5% w/v yeast extract) on a rotating wheel at 37˚C for 16-20 hours. To maintain the expressing plasmid, ampicillin was added to liquid and solid media at a concentration of 100 µg/mL.
Table 1: Amino acid and nucleobase concentration in SC media.

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* Amino acid or nucleobase omitted for drop-out SC media.

3.2 Phenol Chloroform Plasmid Isolation

The *E. coli* containing the plasmid of interest was inoculated into 3 mL of LB broth supplemented with appropriate antibiotic. The cells were grown at 37°C with shaking for 15-18 hours. The culture was pelleted at 10000g for two minutes and resuspended in 100 µl of resuspension solution (50 mM glucose, 10 mM EDTA and 25 mM Tris, pH 8.0). The cells were lysed, and the DNA was denatured using 100 µl of freshly prepared solution II (1% SDS, 200 mM NaOH, MQ water), next the solution was neutralized with 150 µl of ice-cold solution III (11.5% acetic acid, 3 M potassium acetate). The tubes were placed on ice for 7 minutes and the cell debris were removed by centrifugation at 10000g for 5 minutes. The supernatant containing the plasmid was transferred to a fresh microfuge tube and 400 µl of phenol chloroform (50:50 v/v) was added to remove cellular proteins. The solution was vortexed, centrifuged at 10000g for two minutes, and the top layer was removed and placed into a fresh tube. The DNA was precipitated by adding 700 µl of 95% ethanol and 40 µl of 5M NaCl and the tube was placed at -20°C for one hour. The DNA was pelleted at 12000g for 10 minutes and the supernatant was aspirated. Pellet was washed with 70% ethanol, aspirated and resuspended in 40 µl of sterile mili-Q water.

3.3 Cloning of PCR Amplicon into Vectors

The insert fragment was prepared by using primers designed to amplify the precise region with restriction enzyme cut sites at the 3’ end. The DNA in the PCR reaction was
purified using phenol chloroform and ethanol precipitation protocol. The reaction was diluted to 200 μl with miliQ water and an equal volume of phenol chloroform (1:1; v/v) was added. The solution was vortex vigorously, centrifuged at 13000g for 2 minutes, the top aqueous layer was removed and transferred to a fresh microfuge tube. 700 μl of 95% ethanol and 40 μl of 5M NaCl was added and the tubes were placed at -20°C for 1 hour. The DNA was pelleted at 13000g for 10 minutes and the pellet was washed with 70% ethanol. The liquid was aspirated, and the DNA was resuspended in 20 μl of water. The quality and quantity of the DNA was assessed by electrophoresing 2.5 μl on a 1% agarose gel and run at 120 V for 40 minutes. The insert and the vector were digested with restriction enzymes, and shrimp alkaline phosphatase treatment was used when appropriate. The digested DNA was purified by phenol chloroform and concentrated by ethanol precipitation. The prepared insert and vector were combined in a 20 μl reaction at an insert to vector ratio of 3:1, 5:1, and 7:1. The reaction was incubated for one hour at room temperature or at 15°C overnight. Chemically competent DH5α cells were transformed with 5 μl of each reaction, described in section 3.4. Successful transformants were cultured overnight in appropriate media and plasmids were isolated, described in section 3.2. DNA sequencing was performed at the Advanced Analysis Center, University of Guelph. DNA and primer concentrations were followed as per their instructions located online: (http://www.uoguelph.ca/aac).

3.4 CaCl₂ Chemically Competent E. coli DH5α

A single colony of DH5α was used to inoculate a 3 mL culture of LB medium and was incubated overnight at 37°C with vigorous shaking. A 1 mL aliquot of the grown culture was added to 100 mL of LB medium in a baffled flask and was placed at 37°C with shaking. The O.D₆₀₀ was monitored until the cells reached a density of 0.3-0.4. The culture was chilled on
ice for 15 minutes. The culture was transferred to a sterile centrifuge tube, and the cell pellets were collected by centrifugation at 3,000g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 30 mL of ice-cold 100 mM CaCl$_2$, and the solution was incubated on ice for 30 minutes. The cells were pelleted by centrifugation as previously mentioned. The cells were gently resuspended in 6 mL of 100 mM CaCl$_2$ with 15% v/v glycerol. The cell suspension was aliquoted into 100 μl volumes into sterile microcentrifuge tubes. Cells were snap frozen on dry ice and stored at -80°C.

3.5 *E. coli* Transformation by Heat Shock

Chemically competent DH5α cells were carefully thawed on ice for 10 minutes. Approximately 2μl-5μl of DNA (concentration of 1 μg/μl) was gently mixed into 50 μl of CaCl$_2$ chemically competent cells DH5α, and the mixture was incubated on ice for 30 minutes. The cells were moved to a 42°C water bath for 2 minutes, quickly moved to ice for 1 minute, and 1 mL of pre-warmed LB medium was added. The cells recovered at 37°C with shaking for 1-1.5 hours. The cells were gently centrifuged at 6,000g for 2 minutes and the pellet was resuspended in 100 μl. The cells were spread on an LB plate supplemented with the appropriate antibiotic. The plates were incubated overnight at 37°C.

3.6 Yeast Transformation

3.6.1 Electroporation Yeast Transformation

The yeast strains of interest were inoculated into 3 mL of YPD and grown for approximately 12-16 hours at 30°C on a rotating wheel. The starter culture was diluted to approximate O.D$_{600}$ of 0.1 and was grown until it reached an O.D$_{600}$ of 0.8-1. The cells were
centrifuged at 13,000g for 3 minutes and the media was decanted. The pellet was washed twice with ice-cold sterile milli-Q water and resuspended in sorbitol (1M) to an O.D$_{600}$ of 1.0. 40 µl of cells were mixed with 5 µl of DNA. The solution was added to a 2 mm gap electroporation cuvette. Cells were pulsed using a Bio-Rad Micropulser set to “Fungi Sc2” and 1 mL of recovery media (YPD supplemented with 1 M sorbitol) was immediately added. The cell suspension was placed at 30°C for 1.5-2 hours to recover. The cells were gently pelleted at 6000g and approximately 900 µl of the supernatant was removed and the cells were resuspended in the residual liquid by mixing with a sterile pipette tip. 5 µl and 50 µl of the cells were spread onto the appropriate SC drop out plates. Plates were incubated at 30°C for 3 to 5 days. Successful transformants were confirmed by a re-streaking onto a plate with appropriate media.

3.6.2 Lithium Acetate Yeast Transformation

A single colony was used to inoculate a 3 mL YPD seed culture and was grown overnight at with shaking at 30°C. The seed culture was used to sub-inoculate a 50 mL flask of YPD to an O.D$_{600}$ of approximately 0.3. Cells were harvested once the culture reached an O.D$_{600}$ of 0.8-1. The cells were centrifuged at 3000g for 5 minutes, the pellet was resuspended in 25 mL of sterile water, and the cells were pelleted again by centrifuging again at 3000g for 5 minutes. The cells were resuspended in 1 mL of sterile water and the cell suspension was transferred to a 1.5 mL microcentrifuge tube. The cells were centrifuged at 12000g and the supernatant was decanted. The pellet was resuspended in 1 mL of sterile water and 100 µl was aliquoted into a 1.5 microcentrifuge tube, 1 for each transformation. The cells were pelleted by centrifugation for 30s at 12000g and the supernatant was removed. For each transformation, 336 µl of transformation master mix (240 µl of PEG3350 (50% w/v), 36 µl of
1.0 M LiAc, and 50 μl of single-stranded carrier DNA (2.0 mg/mL)) was added to the cell pellet. The tubes were placed at 42°C for 40 minutes. 5 μl and 50 μl of cells were spread onto the appropriate SC drop out plates. Plates were incubated at 30°C for 3 to 5 days. Successful transformants were confirmed by a second passage on appropriate media.

3.7 Yeast genomic DNA isolation

Yeast cells were grown in 3 mL of YPD medium overnight at 30°C until saturation. The cells were pelleted by centrifugation at 12000g for 2 minutes and washed with 500 μl of water. The supernatant was decanted, and the cells were resuspended in the remaining liquid. The cells were lysed using 200 μl of lysis buffer, 0.3 g of glass beads, and 200 μl of phenol chloroform (1:1 v/v), the solution was vortexed for 20 minutes at 4°C. 200 μl of 1X TE was added and the supernatant was removed and placed into a fresh tube. The DNA was concentrated by adding 700 μl of 95% ethanol was added and tubes were placed at -20°C for 1 hour. The DNA was pelleted at 12000g for 10 minutes and the pellet was washed with 70% ethanol. The DNA pellet was resuspended in 400 μl of 1X TE and the RNA was degraded by adding 2 μl of RNase A and incubating at 37°C for 1 hour. The DNA was concentrated again using ethanol precipitation, 700 μl of 95% ethanol was added and tubes were placed at 37°C for 1 hour. The DNA pellet was resuspended in 40 μl of water. The quantity and quality of genomic preparation was assessed by electrophoresing 2.5 μl on a 1% agarose gel at 120V for 40 minutes.
3.8 Polymerase Chain Reaction for Cloning

Polymerase chain reaction (PCR) was conducted with standard reagents in a total 25 µL reaction. Each reaction contained, 2.5 µL of BioShop PCR buffer mix (-MgCl$_2$), 2.5 µL of 25 mM MgCl$_2$, 0.5 µL of 10 mM dNTPs, 0.5 µL of forward and reverse primer, 1 µL of the template genomic DNA (10-100 ng/µL), 0.13 µL of standard Taq polymerase. A master mix was made with all the components except the template and primers, which were added individually. The primers used are listed in the appendix. The PCR was conducted using a Thermofisher Simipliamp thermocycler.

3.9 FOA$^R$ variegation assay

The stains used with this assay were always freshly transformed. Respective plasmids were digested with *Eco*RI and *Sal*I in ThermoFisher FastDigest buffer for 4 hours. The DNA was purified using phenol-chloroform and ethanol precipitation, described in section 3.2. The linearized constructs were transformed into required strains and the integration was confirmed via PCR, the methods are described in section 3.5-3.7. An overview of the assay is visualized in Figure 6.

Once successful integration was confirmed, three colonies were selected and re-streaked on YPD medium and grown at 30°C for 2 days. Within each technical replicate, a large single colony is divided and placed on an SC-Ura and SC+5-FOA media and grown at 30°C for 3 to 5 days. A colony from each plate was chosen and inoculated into a 3 mL of YPD media and grown at 30°C overnight, which is approximately 10 cell divisions. 10 µl of the overnight culture was sub-inoculated into 3 mL of YPD and grown again at 30°C overnight.
for another 10 cell divisions. The O.D$_{600}$ of each culture was recorded and the number of cell divisions was calculated using the formula, cell divisions= $(\log(\text{final O.D}_{600}/\text{initial O.D}_{600})) / \log(2)$. The final cultures were serially diluted by a factor of 10 in a 95-well plate, and 5 µl of each dilution was spotted onto a YPD, SC-Ura, SC+5-FOA plates. The plates were grown at 30°C for 3-5 days. The percentage of cells expressing $URA3$ (%URA+) were calculated by counting the number of colonies on the SC-Ura and dividing by the total number of colonies grown on YPD. Conversely, the percentage of cells silencing $URA3$ (%FOA$^\text{R}$) was calculated by the dividing the number of colonies grown on SC+5-FOA by the total number of colonies grown on YPD.
Figure 6. A flowchart of the 5-FOA<sup>R</sup> assay used to study telomere position variegation. Constructs were transformed into strains. A single colony is re-streaked onto another SC-Ura plate to confirm successful integration of the URA3 containing construct. Three colonies were from each strain was re-streaked onto a YPD to remove the selective pressure. Three colonies were chosen, and half was re-streaked onto an SC-Ura plate and the other half was re-streaked on an SC-FOA plate. SC-Ura provides the selective pressure for the transcriptionally active state of URA because of the absence of uracil in the media. Whereas, SC-FOA selects for cells with the transcriptionally inactive state of URA because if URA is expressed it will lead to a lethal intermediate. Three colonies from each type of selection were inoculated into a 3 mL culture of YPD and grown for approximately 10-12 generations. Yeast cells were sub-cultured into a fresh tube of YPD and grown for a total of 25-30 generations. The culture was serially diluted by a factor of 10 and spotted onto a YPD, SC-Ura, and SC+5-FOA plate. The proportion of cells that have undergone an epigenetic switch can be calculated by dividing the number of cells grown on the SC-Ura and SC+5-FOA plate by the number of cells grown on the YPD plate.
3.10 Microscopy

Microscopy was performed using a Leica DM 6000B microscope according to the manufacturer’s manual. The cells were prepared by growing in appropriate media, diluted and grown again until the OD\textsubscript{600} reached 0.7. The cells were diluted again into 50 mL of medium and grown from an O.D\textsubscript{600} of 0.1 to 0.4. On microscope slide, 20 µl 0.1% poly-l-lysine solution was spotted and dried for 20 minutes, any excess liquid was aspirated. 1 µl of cells were spotted onto the slide and cells were allowed to settle by incubation for 1 hour at room temperature. A coverslip was placed over the sample and sealed with nail polish. Images were taken using brightfield and GFP settings with a 40x objective.

3.11 Flow Cytometry

The stains used with this assay were always freshly transformed. Respective plasmids were digested with \textit{EcoRI} and \textit{SalI} in ThermoFisher FastDigest buffer for 4 hours. The DNA was purified using phenol-chloroform and ethanol precipitation, described in section 3.2. The linearized constructs were transformed into required strains and the integration was confirmed via PCR, the methods are described in Section 3.5-3.7.

Once successful integration was confirmed, three colonies were selected and re-streaked on YPD medium and grown at 30°C for 2 days. Cells were re-streaked onto an SC-Ura plate and grown at 30°C for 3 to 5 days. A colony was inoculated into a 3 mL of YPD media and grown at 30°C overnight, which is approximately 10 cell divisions. 10 µl of the overnight culture was sub-inoculated into 3 mL of YPD and grown again at 30°C overnight, for approximately 10 cell divisions. The O.D\textsubscript{600} of each culture was recorded and the number
of cell divisions was calculated using the formula, cell divisions= \( \frac{\log(\text{final O.D}_{600}/\text{initial O.D}_{600})}{\log(2)} \).

The cells were inoculated into 3 mL of YPD and grown to an O.D of approximately 0.5. The cells were fixed with formaldehyde (1% final concentration) for 15 minutes and quenched with 125 mM of glycine for 5 minutes. The cells were washed twice with PBS and the cell concentration was adjusted to \(~2\times10^7\) cells with PBS. The cells were sonicated with Misonix\textsuperscript{®} XL-2020 for 15 seconds at 30\% output. Flow cytometry was performed using BD LSRII (BD Biosciences) according to the manufacturer’s manual. The gates were set to acquire only single cells. The FITC channel was used to analyze fluorescence intensity. Approximately 12,000 events were acquired for each strain. The raw data was analyzed using the program Flow Jo. A threshold based on the negative control was used to determine proportions of GFP\(^+\) and GFP\(^-\) cells.
Chapter 4: Results

My first objective was to create a novel assay to study epigenetic conversions without the use of 5-FOA. As previously mentioned, 5-FOA can be supplemented into the media to determine the transcriptional state of the reporter gene URA3. However, it was recently reported that 5-FOA can antagonize the enzyme RNR, which can lead to unbalanced nucleotide pools in the cell (93). As an alternative, I attempted to use a fusion of a Ura3p-GFP to eliminate the need for 5-FOA to monitor the transcriptional state of URA3. The Ura3-GFP fusion protein can be used to measure epigenetic conversions with the established FOA\textsuperscript{R} assay and other techniques using cellular fluorescence, such as microscopy and flow cytometry. Therefore, this provides the opportunity to compare and cross-validate new assays. The cellular fluorescence approach provides benefits beyond the circumvention of 5-FOA, it provides more comprehensive analysis of epigenetic conversions of single cells and real-time detection of live cells. Moreover, this new approach can be used to monitor epigenetic conversions at different time points, which provides new opportunities in this line of research.

To address my second research objective, to find a correlation between replication fork pausing and epigenetic conversions, I introduced a replication fork pause sites into the URA3-GFP reporter construct. The RFB was inserted at 2 different positions and both orientations in the reporter construct. The constructs were transformed into three strains of \textit{S. cerevisiae}: the routinely used laboratory haploid \textit{BY4742}, and its derivatives \textit{cac1Δ} and \textit{rrm3Δ}, which are missing \textit{CAC1} and \textit{RRM3}, respectively. The latter strains have been reported to have lost the ability to undergo epigenetic conversions (68, 86). I analyzed any effects of the presence
of an RFB on the frequency of epigenetic conversions with three different methods: the FOA\textsuperscript{R} assay, flow cytometry, and microscopic scoring.

In a separate set of experiments, I investigated the effects of mutations to Cdc7p and Cdc28p putative phosphorylation sites on Cac1p in a \textit{cac1}Δ strain. These sites are hypothesized to regulate CAF-1 during the cell cycle. I tested the effects of mutations to individual sites of phosphorylation and combinations of mutations to Cdc7p and Cdc28p phosphorylation sites on Cac1p on cellular growth.

4.1 Experimental Strategy

Previously, the construct used in the FOA\textsuperscript{R} assay contained \textit{ADH4-P\textsubscript{URA3}-URA3-TG\textsubscript{(1-3)}} and was targeted to the \textit{VII}-L telomere to analyze the frequency of epigenetic conversions (88). A fusion of Ura3p and GFP was optimized by another group, and the construct contained \textit{AHD4-P\textsubscript{URA3}-URA3-G8-yEGFP1-NLS-CLN2PD-TG\textsubscript{(1-3)}} (94). The distal portion of \textit{ADH4} and the \textit{TG\textsubscript{(1-3)}} repeats are used to target the construct to the \textit{VII}-L telomere. (95). The \textit{URA3} and \textit{GFP} ORFs were separated by 8 glycine residues (G8) that act as a linker, which prevents GFP from interfering with Ura3p function (96). The \textit{yEGFP1} is a codon-optimized yeast specific enhanced GFP gene. The Ura3p-GFP fusion protein gene is regulated by the \textit{URA3} promoter. The Ura3p-GFP fusion protein is tagged with the PEST domain of the G1 cyclin, Cln2p, which is a sequence that targets proteins for rapid degradation. The stability of the GFP reporter protein interferes with dynamic changes in gene expression. The addition of the Cln2p PEST domain destabilizes GFP, which decreases the half-life of GFP from approximately 7 hours to approximately 30 minutes (95). Therefore, destabilizing the fusion reporter protein it more accurately conveys the transcriptional state of the construct, and thus epigenetic changes (96).
The Ura3p-GFP fusion protein is also tagged with an NLS, which concentrates the Ura3p-GFP in the nucleus to increase its signal intensity for detection because transcription at the subtelomeres is characteristically low.

To analyze the effects of pausing of replication forks on epigenetic conversions I used the replication fork barrier, RFB, from the rDNA. The RFB is a small DNA element with a well-characterised mechanism of action. A RFB was inserted at two different locations and in two different orientations to produce four different constructs (Figure 7). I hypothesized that the RFB integrated near the promoter of URA3 would have a different effect on epigenetic conversions compared to the effect of a RFB integrated near the telomeric repeats. The RFB sequence was obtained through polymerase chain reaction (PCR) by amplifying the sequence in the rRNA repeats from genomic DNA of S. cerevisiae. The primers for the PCR of RFB contained BglII restriction enzyme cut sites in the 3’ overhangs and are listed the Appendix. The RFB was integrated near the promoter of URA3 and near the telomeric repeats in both orientations (Figure 7). The 3’ to 5’ orientation BFR can induce replication fork pausing, while the 5’ to 3’ RFB orientation cannot pause replication forks and was used as a control (79). All cloned vectors were sequenced for verification of the orientation of the RFB. These constructs were introduced through homologous recombination into the laboratory wildtype strain BY4742, cac1Δ, and rrm3Δ strains. Successful integration of the construct was confirmed by PCR.
Figure 7. Schematic of cloned RFB constructs used in this study. Each construct contains a partial ADH4 sequence and telomeric repeats (black triangles) to target the construct to the VII-L telomere. A single RFB was integrated at a site near the URA3 promoter in both orientations and integrated at a site near the telomeric repeats in both orientations. Only the BFR orientation is able to pause replication forks.

4.2 Impact of Replication Fork Barrier: FOA\(^R\) Variegation Assay

Initially, I used the FOA\(^R\) variegation assay to assess the impact of an RFB on the frequency of epigenetic conversions at VII-L telomere. I transformed the RFB constructs into the routinely used BY4742 wildtype, cac1\(\Delta\), and rrm3\(\Delta\), listed in Table 3. BY4742 was used as a wildtype control. The cac1\(\Delta\) and rrm3\(\Delta\) were used to test if an RFB was able to elicit epigenetic conversions in these strains, which are characterized by a loss of epigenetic conversions (68, 86). The technical details of the FOA\(^R\) variegation assay are found in Section 3.7 of the Materials and Methods.
Table 2. Strains used in the FOAR variegation assay.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BY4742</td>
</tr>
<tr>
<td></td>
<td>cac1Δ</td>
</tr>
<tr>
<td></td>
<td>rrm3Δ</td>
</tr>
<tr>
<td>URA3-Tel</td>
<td>URA3-GFP-Pest-NLS-Tel</td>
</tr>
<tr>
<td>RFB-URA3-GFP-Pest-NLS-Tel</td>
<td>RFB-URA3-GFP-Pest-NLS-Tel</td>
</tr>
<tr>
<td>BFR-URA3-GFP-Pest-NLS-Tel</td>
<td>BFR-URA3-GFP-Pest-NLS-Tel</td>
</tr>
<tr>
<td>URA3-GFP-Pest-RFB-NLS-Tel</td>
<td>URA3-GFP-Pest-RFB-NLS-Tel</td>
</tr>
<tr>
<td>URA3-GFP-Pest-BFR-NLS-Tel</td>
<td>URA3-GFP-Pest-BFR-NLS-Tel</td>
</tr>
</tbody>
</table>

First, I determined the proportions of URA3+ and FOAR cells with the established URA3-Tel construct and compared it to the new URA3-GFP-Pest-Tel construct. The data is presented in Figure 8 and listed in Table 3. In the BY4742 strain, the URA3-Tel produced approximately 70% URA3+ cells and 30% FOAR cells regardless of the initial selections (87). This means that in the SC-Ura initial selection, 30% of cells undergo an active to silent epigenetic switch. This is in agreement with other reported values for BY4742 URA3-Tel (68, 86). However, in BY4742 URA3-GFP-Pest-NLS-Tel, under the initial selection of SC-Ura produced approximately 18% URA3+ and 87% FOAR cells. Approximately 87% of cells had undergone an active to silent epigenetic conversion, which deviates significantly from the expected proportion of 30% in a wildtype strain.

The cac1Δ and rrm3Δ strains have been reported to have a reduced frequency of epigenetic conversions, which is demonstrated by the majority of the cells remaining in the initial selection state (86). I reproduced the expected percentages of URA3+ and FOAR cells
for the cac1Δ and rrm3Δ cells with the URA3-Tel construct (68, 97). In the cac1Δ strain, in the SC-Ura selection, the URA3-Tel construct, approximately 2% of cells underwent an epigenetic conversion from the active to silent state. In comparison, the URA3-GFP-Pest-NLS-Tel construct 11% of cells had switched from active to silent state.

In rrm3Δ, in the initial selection SC-Ura, the URA3-Tel construct had approximately 13% of cells underwent an active to silent epigenetic switch. However, the URA3-GFP-Pest-NLS-Tel approximately 65% of cells had undergone an active to silent epigenetic switch.

It was expected that the frequency of epigenetic conversions using the URA3-Tel and URA3-GFP-Pest-NLS-Tel constructs would produce similar results. However, it is evident that the URA3-GFP-Pest-NLS-Tel construct in BY4742 and rrm3Δ strains did not produce proportions of URA⁺ and FOAᴿ cells. In both strains, there is an increase in active to silent epigenetic conversions in the SC-Ura selection. But the data from the SC+FOA selection shows that there is not an increase in silent to active epigenetic conversions. Overall, it appears that the URA3-GFP-Pest-NLS-Tel construct may produce a large population of FOAᴿ or silent cells.
Figure 8. Comparison of the FOA<sup>R</sup> variegation assay results for the *URA3-Tel* reporter versus the *URA3-GFP-Pest-NLS-Tel* reporter. Cells were initially selected on either SC-Ura or SC+5-FOA. After growing for 20-30 generations the proportion of URA3<sup>+</sup> cells (white bars) and proportion of FOA<sup>R</sup> cells (black bars) were counted. This graph shows the results of two independent experiments performed in triplicate. Error bars represent standard deviation.

The effect of the position and orientation of *RFB* on epigenetic conversions was measured in *BY4742, cac1Δ*, and *rrm3Δ* using the strains in Table 2. The results of the FOA<sup>R</sup> assay were plotted in Figure 9 and summarized in Table 3. I performed a two-tailed unpaired T-test assuming unequal variance was used to determine statistical significance between the experimental *RFB* constructs and the no *RFB* control construct in the same strain. Strains with a statistically significant difference, a p<0.05, were marked with an asterisk (*). The SC-Ura initial selection, which measures the conversions from active to silent state are plotted in
Figure 9 (A) and the SC+5-FOA initial selection, which measures the conversions from silent to active state are plotted in Figure 9 (B).

In the BY4742 wildtype, overall there is no significant effect of the presence of an RFB on the frequency of epigenetic conversions compared to the no RFB control, URA3-GFP-Pest-NLS-Tel. It is important to note the pattern of a large proportion of FOA$^R$ or silent cells in these strains, even in the Sc-Ura selection. In SC-Ura selection for the cac1Δ strain, compared to the no RFB control, URA3-GFP-Pest-NLS-Tel, the RFB in the pausing orientation near the URA3 promoter, BFR-URA3-GFP-Pest-NLS-Tel, had a significant increase of 64% of cells that underwent an active to silent epigenetic conversion. In the rrm3Δ strain, overall there was not a significant effect due to the presence of a RFB on the frequency of epigenetic conversions. The rrm3Δ strains also show a pattern of a large proportion of silent cells.

Overall, this data shows that there is no major effect due to the presence of RFB at different positions and orientations on epigenetic conversions in BY4742 wildtype. However, because the proportions of active, URA3+, and silent, FOA$^R$, cells in the BY4742 wildtype with the URA3-GFP-Pest-NLS-Tel construct, these results need to be interpreted with caution.
Figure 9. Comparison of the effect of an RFB in BY4742, cac1Δ, and rrm3Δ using the FOA\textsuperscript{R} variegation assay. Cells were initially selected on their respective initial media and grown in non-selective media for 20-30 generations. The culture was serially spotted, and the proportion of URA3\textsuperscript{+} cells (white bars) and proportion of FOA\textsuperscript{R} cells (black bars) were counted. This experiment was done with two biological replicates with three technical replicates each. A two-tailed unpaired T-test assuming unequal variance was used to determine statistical significance between the construct and the no RFB control in the same strain. A $p<0.05$ was marked with an asterisk (*). The error bars show standard deviation.
Table 3. The average %URA3⁺ (± SD) and %FOAR⁺ (±SD) cells in the FOAR⁺ variegation assay.

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Initial Selection SC-Ura</th>
<th>Initial Selection SC+5-FOA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%URA3⁺</td>
<td>%FOAR⁺</td>
</tr>
<tr>
<td><strong>BY4742 URA-Tel</strong></td>
<td>4</td>
<td>73% ± 25%</td>
<td>33% ± 15%</td>
</tr>
<tr>
<td><strong>cac1Δ URA-Tel</strong></td>
<td>4</td>
<td>95% ± 6%</td>
<td>2% ± 2%</td>
</tr>
<tr>
<td><strong>rrm3Δ URA-Tel</strong></td>
<td>4</td>
<td>87% ± 8%</td>
<td>13% ± 7%</td>
</tr>
<tr>
<td><strong>BY4742 URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>18% ± 9%</td>
<td>87% ± 33%</td>
</tr>
<tr>
<td><strong>BY4742 RFB-URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>13% ± 10%</td>
<td>113% ± 13%</td>
</tr>
<tr>
<td><strong>BY4742 BFR-URA-GFP-Pest-NLS-Tel</strong></td>
<td>3</td>
<td>28% ± 19%</td>
<td>76% ± 27%</td>
</tr>
<tr>
<td><strong>BY4742 URA-GFP-Pest-NLS-RFB-Tel</strong></td>
<td>4</td>
<td>22% ± 14%</td>
<td>76% ± 23%</td>
</tr>
<tr>
<td><strong>BY4742 URA-GFP-Pest-NLS-BFR-Tel</strong></td>
<td>4</td>
<td>39% ± 32%</td>
<td>58% ± 38%</td>
</tr>
<tr>
<td><strong>cac1Δ URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>73% ± 6%</td>
<td>11% ± 8%</td>
</tr>
<tr>
<td><strong>cac1Δ RFB-URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>63% ± 23%</td>
<td>30% ± 32%</td>
</tr>
<tr>
<td><strong>cac1Δ BFR-URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>31% ± 22%</td>
<td>75% ± 9%</td>
</tr>
<tr>
<td><strong>cac1Δ URA-GFP-Pest-NLS-RFB-Tel</strong></td>
<td>4</td>
<td>69% ± 23%</td>
<td>23% ± 9%</td>
</tr>
<tr>
<td><strong>cac1Δ URA-GFP-Pest-NLS-BFR-Tel</strong></td>
<td>4</td>
<td>81% ± 19%</td>
<td>13% ± 13%</td>
</tr>
<tr>
<td><strong>rrm3Δ URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>29% ± 19%</td>
<td>67% ± 12%</td>
</tr>
<tr>
<td><strong>rrm3Δ RFB-URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>40% ± 12%</td>
<td>44% ± 12%</td>
</tr>
<tr>
<td><strong>rrm3Δ BFR-URA-GFP-Pest-NLS-Tel</strong></td>
<td>4</td>
<td>16% ± 8%</td>
<td>62% ± 16%</td>
</tr>
<tr>
<td><strong>rrm3Δ URA-GFP-Pest-NLS-RFB-Tel</strong></td>
<td>4</td>
<td>19% ± 8%</td>
<td>85% ± 16%</td>
</tr>
<tr>
<td><strong>rrm3Δ URA-GFP-Pest-NLS-BFR-Tel</strong></td>
<td>4</td>
<td>42% ± 26%</td>
<td>75% ± 28%</td>
</tr>
</tbody>
</table>
4.3 Optimizing a Flow Cytometry Protocol

To utilize cellular fluorescence to characterize epigenetic conversions, I used a Ura3p-GFP fusion protein. The presence or absence of GFP can convey the transcriptional state of the construct.

I validated that the construct could undergo variegation, meaning that a subset of cells the reporter construct is transcriptionally active and a subset of cells the reporter construct is transcriptionally silenced. Cells with the transcriptionally active construct are GFP\(^+\) and cells with the transcriptionally silenced construct are GFP\(^-\). In addition, I verified that the different transcriptional states of the construct can be selected for by using specific media. SC-Ura media selects for the active transcriptional state and cells are GFP\(^+\) and SC+FOA media selects for the silent transcriptional state and all cells are GFP\(^-\). The plasmid containing the construct \textit{AHD4-PURA3-URA3-G8-URA3-yEGFP1-NLS-CLN2PD-TG\(_{1-3}\)}, was digested with \textit{EcoRI} and \textit{SalI} to produce a linearized construct and was transformed into BY4742. Colonies were screened with PCR for successful integration. These cells were grown under different media conditions and analyzed for GFP activity (Figure 10).

The cells were inoculated into SC, SC-Ura, and SC+FOA, grown over-night and imaged using a confocal Leica DM 6000B microscope. In non-selective SC media, the cells showed a variegated expression of GFP, some cells were GFP\(^+\) and some cells were GFP\(^-\), which reflects the two different transcriptional states of the construct. SC-Ura media was able to select for cells with the active transcriptional state of the construct, indicated by the presence of GFP. SC+FOA media was able to select for cells with the silenced transcriptional state of the construct, indicated by GFP absence. Over time, I found that cells with the \textit{URA3}-
GFP-Pest-NLS-Tel construct at a high density had a substantially different GFP profile, compared to the same cells at a lower density. I did a time-course analysis and determined that cells at an O.D$_{600}$ 0.6 and lower, cells had a clear sharp localization of GFP in the nucleus (Figure 11). Therefore, I strongly suggest that all analysis of Ura3p-GFP should be diluted and grown to obtain a low-density culture.

I also analyzed the effects of the fusion of the Pest domain and NLS to the Ura3p on growth rate. I performed growth curve analysis with a Ura3p-GFP fusion protein without a Pest domain or a NLS and compared it to the Ura3p-GFP fusion protein with a Pest domain and NLS (Figure 12). I found the addition of a Pest domain and NLS had impacted the growth of the cells under SC-Ura conditions.
Figure 10. Analysis of the transcriptional state of the \textit{URA3-GFP-Pest-NLS-Tel} reporter in \textit{BY4742} with different media conditions. The \textit{BY4742 URA3-GFP-Pest-NLS-Tel} strain was grown overnight at 30°C in non-selective media (SC), SC-Ura, and SC+5-FOA media. The cells were prepared for microscopy and visualized with a confocal Leica DM 6000B microscope under brightfield and GFP conditions.
Figure 11. The Relationship between GFP localization and cell density. The strain BY4742 URA3-GFP-Pest-NLS-Tel was grown in SC-Ura media with shaking at 30°C. Samples of the culture were taken at different time points, and the O.D$_{600}$ was recorded. The cells were fixed with ethanol and stained with DAPI. A confocal Leica DM 6000B microscope was used to visualize the cells, under brightfield and GFP conditions.
Figure 12. Comparison of growth rates of *URA3-GFP-Tel* and *URA3-GFP-Pest-NLS-Tel* in *BY4742*. The saturated cultures of the strains were diluted to an O.D$_{600}$ of 0.1 in SC-Ura in a 96-well plate. The cells were incubated at 30°C with shaking and O.D$_{600}$ was recorded every hour with a Multiskan™ FC Microplate Photometer.

4.4 Flow Cytometry using Model Strains

To measure the efficacy of the *URA3-GFP-Pest-NLS-Tel* construct as a reporter, I transformed the construct into *BY4742, sir3Δ, cac1Δ, and rrm3Δ* strains. The *sir3Δ* was a positive control as it is strain devoid of silencing; thus, all cells are GFP$^+$. The proportions of active and silent cells in *BY4742, cac1Δ, and rrm3Δ with URA3-Tel* were already reported, and were used as a comparison for the validity of the *URA3-GFP-Pest-NLS-Tel* construct (68, 86).

The respective yeast strains were inoculated into SC-Ura media and grown overnight to saturation to generate a 100% GFP$^+$ population of cells. The cells were sub-inoculated into non-selective media, grown overnight to saturation, sub-inoculated into non-selective media,
and grown overnight to saturation. After approximately 20-30 cell divisions in non-selective media, the cells were diluted and grown to obtain a low-density culture with an O.D of approximately 0.5. The cells were fixed with 1% formaldehyde and prepared for flow cytometry (see section 3.11 in Materials and Methods). The cells were acquired with a flow cytometer, the forward scatter and side scatter was collected. The GFP intensity was analyzed using the FITC filter. Approximately 12,000 events were counted, and cells were gated to acquire only single cells.

The data was analyzed using the program FlowJo, and histograms for each sample depicting the distribution of GFP intensity were generated (Figure 13). The GFP- strain BY4742 URA-Tel was used as a negative control (Figure 13, A). The sir3Δ URA3-GFP-PEST-NLS-Tel strain was a positive control (Figure 13, B). I analyzed subpopulations of GFP+ and GFP- by creating a gate using the negative control, the gate was applied to classify the negative control cells as GFP-. The estimates of GFP+ and GFP- cells of the experimental strains are based on a cut-off value determined by the negative control. In the positive control, there is a slight shift in the distribution of GFP intensity, which suggests that the GFP is detected by the flow cytometer. Evidently, there is not a clear distinction between GFP+ and GFP- cells. This may be a result of two compounding factors. Firstly, the low expression of the reporter due to the position in the subtelomeric region. Secondly, the destabilization of GFP by the Pest domain and the subsequent lag in accumulation.

The proportions GFP+ and GFP- cells for each strain are listed in Table 4. In the positive control, sir3Δ URA3-GFP-Pest-NLS-Tel, 63.7% of cells were GFP+ and 36.3% of cells were GFP-. Although it was expected that 100% sir3Δ URA3-GFP-Pest-NLS-Tel cells would be GFP+; however, the addition of the Pest domain could explain why a portion of the
population was GFP-. Based on the FOA<sup>R</sup> assay results with BY4742 URA3-Tel, the BY4742 URA3-GFP-Pest-NLS-Tel was expected to have approximately 60% of cells to be GFP<sup>+</sup> and 40% of cells to be GFP-. However, the results of the flow cytometry show that only 17.5% of BY4742 URA3-GFP-Pest-NLS-Tel were GFP<sup>+</sup>. The population of GFP<sup>+</sup> in cac1Δ URA3-GFP-Pest-NLS-Tel were similar to sir3Δ, this indicates the majority of cells were in their initial selection state. This is in agreement with the cac1Δ URA-Tel data (68). The rrm3Δ URA-GFP-Pest-NLS-Tel strain was expected to be similar to cac1Δ URA3-GFP-Pest-NLS-Tel; however, approximately 25.4% of cells were GFP<sup>+</sup> cells and 74.6% were GFP<sup>-</sup> cells (86). These findings are similar to the findings of these strains analyzed by the FOA<sup>R</sup> variegation assay, both indicate a large proportion of FOA<sup>R</sup> or GFP<sup>-</sup> cells. These discrepancies between the previously reported results using the URA3-Tel and these results using the URA3-GFP-Pest-NLS-Tel may be due to the design of the Ura3-GFP reporter protein.
Figure 13. Flow cytometry analysis of GFP of model strains. The strains BY4742 URA3-Tel (black), sir3Δ URA3-GFP-Pest-NLS-Tel (green), BY4742 URA3-GFP-Pest-NLS-Tel (blue), cac1Δ URA3-GFP-Pest-NLS-Tel (red), and rrm3Δ URA3-GFP-Pest-NLS-Tel (orange) were initially selected in SC-Ura media and grown for approximately 25 generations in non-selective media. The cells were gated appropriately and histograms for the FITC channel are shown for each strain. The proportions of GFP+ and GFP− cells were determined using the negative control.

Table 4. Percentage of GFP+ and GFP− populations for model strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% GFP+</th>
<th>% GFP−</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742 URA3-Tel</td>
<td>1.85%</td>
<td>98.2%</td>
</tr>
<tr>
<td>sir3Δ URA3-GFP-Pest-NLS-Tel</td>
<td>63.7%</td>
<td>36.3%</td>
</tr>
<tr>
<td>BY4742 URA3-GFP-Pest-NLS-Tel</td>
<td>17.5%</td>
<td>82.5%</td>
</tr>
<tr>
<td>cac1Δ URA3-GFP-Pest-NLS-Tel</td>
<td>63.4%</td>
<td>36.6%</td>
</tr>
<tr>
<td>rrm3Δ URA3-GFP-Pest-NLSTel</td>
<td>25.4%</td>
<td>74.6%</td>
</tr>
</tbody>
</table>
4.5 Analyzing the Effect of RFB on Epigenetic Conversions: Flow Cytometry

I followed up the FOA\textsuperscript{R} variegation assay with flow cytometry to investigate the effects of a replication fork pause site on epigenetic conversions. The \textit{BY4742} strain was transformed with the \textit{RFB} containing constructs and integration was confirmed with PCR (Figure 7). The strains were originally selected in SC-Ura media to generate a population of 100\% GFP\textsuperscript{+} cells. The cells were sub-inoculated into non-selective media, grown overnight to saturation, and sub-inoculated into non-selective media again and grown overnight to saturation. The O.D\textsubscript{600} was recorded for each overnight culture and the number of cell divisions were calculated using the formula: number of cell divisions= \((\log(\text{final O.D}_{600}/\text{initial O.D}_{600}))/\log(2)\). The cells were diluted and grown up to obtain a low-density culture, fixed with 1\% formaldehyde and prepared for flow cytometry (see section 3.11 in Materials and Methods). The samples were acquired by the flow cytometer, and the forward scatter, the side scatter, the GFP intensity using FITC filter of each cell was analyzed. The cells were gated appropriately and approximately 12,000 events were acquired.

The data was analyzed using FlowJo and the subpopulations of GFP\textsuperscript{+} and GFP\textsuperscript{-} were gated in a similar way as section 4.3. Histograms of the experimental strains are plotted with the negative control (gray) and positive control (green) (Figure 14). The proportions of GFP\textsuperscript{+} and GFP\textsuperscript{-} cells for each strain are found in Table 5. Overall, the presence of a \textit{RFB} did not have a significant effect on epigenetic conversions. Of note, the placement of the \textit{RFB} near the telomere had a different effect depending on the orientation. The no pausing, \textit{RFB}, had a reduced proportion of GFP\textsuperscript{+} cells by 5\% compared to the control. In the pausing, \textit{BFR}, there was an increase of 11.8\% in GFP compared to the control.
Figure 14. Distribution of GFP for RFB experimental strains in non-selective media. Experimental strains, BY4742 RFB-URA3-GFP-PEST-NLS-Tel (red), BY4742 BFR-URA3-GFP-PEST-NLS-Tel (purple), BY4742 URA3-GFP-PEST-NLS-RFB-Tel (turquoise), and BY4742 URA3-GFP-PEST-NLS-BFR-Tel (orange) are plotted with the negative control BY4742 URA-Tel (gray) and positive control sir3Δ URA3-GFP-PEST-NLS-Tel (green). Cells were initially selected in SC-Ura media and grown for approximately 25 generations in non-selective media. The cells were gated appropriately and histograms for the FITC channel are shown each strain. The proportions of GFP+ and GFP− cells were determined using the negative control.
Table 5. Percentage of subpopulations from flow cytometry results for each strain. The O.D$_{600}$ was recorded before each sub-inoculation and the number of generations were calculated.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Generations</th>
<th>%GFP+</th>
<th>%GFP-</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742 URA3-GFP-Pest-NLS-Tel</td>
<td>23.98</td>
<td>17.5%</td>
<td>82.5%</td>
</tr>
<tr>
<td>BY4742 RFB-URA3-GFP-Pest-NLS-Tel</td>
<td>25.05</td>
<td>17.3%</td>
<td>82.7%</td>
</tr>
<tr>
<td>BY4742 BFR-URA3-GFP-Pest-NLS-Tel</td>
<td>24.41</td>
<td>17.1%</td>
<td>82.9%</td>
</tr>
<tr>
<td>BY4742 URA3-GFP-Pest-NLS-RFB-Tel</td>
<td>23.78</td>
<td>11.6%</td>
<td>88.2%</td>
</tr>
<tr>
<td>BY4742 URA3-GFP-Pest-NLS-BFR-Tel</td>
<td>24.99</td>
<td>29.3%</td>
<td>70.7%</td>
</tr>
</tbody>
</table>

4.6 Impact of Replication Fork Barrier: Microscopic Scoring

The impact of a RFB on the frequency of epigenetic conversions was also assessed using direct observation using fluorescence microscopy. The BY4742 strain was transformed with the experimental RFB constructs and integration was confirmed via PCR (Figure 7). The cells were selected with SC-Ura to generate a population of 100% GFP$^+$ cells. The cells were subinoculated into non-selective medium and grown overnight to saturation. The cells were sub-cultured into non-selective media and grown again overnight. The O.D$_{600}$ was recorded to monitor the number of cell divisions. The cells were prepared for microscopy and imaged using a confocal Leica DM 6000B microscope. This experiment was done in duplicate and at two time-points, at least 100 cells were scored as GFP$^+$ or GFP$^-$. Cells that had a clear GFP signal isolated in the nucleus were counted as GFP$^+$ cells. An exhibit of the BY4742 URA3-GFP-Pest-NLS-Tel, no RFB control cells can be seen in Figure 15. The % GFP$^+$ subpopulation of cells for all strains was plotted in Figure 16 and listed in Table 6.

These results of the microscopic scoring show in the wildtype strain there is a preference for the inactive reporter ie. GFP$^-$ cells. In this experiment, I measured the change in GFP$^{+/-}$ cells at two different time points. There was an increase in GFP$^+$ cells, which is indicative of epigenetic conversions. The no RFB control, URA-GFP-Pest-NLS-Tel, had an
increase of 3% of GFP\(^+\) cells from day one to day two. The experimental strains all had an increase in GFP\(^+\) cells; however, they were not statistically significant compared to the control.

**Figure 15.** Visualization of the change in GFP expression in cells initially selected for GFP\(^+\) with SC-Ura media followed by non-selective media. The strain *BY4742 URA3-GFP-Pest-NLS-Tel* was inoculated into Sc-Ura media and grown overnight. The culture was sub-inoculated into non-selective media and grown overnight again. A sample of cells were taken after each overnight culture. A confocal Leica DM 6000B microscope was used to visualize the cells, under brightfield and GFP conditions.
Figure 16. Proportions of GFP\(^+\) and GFP\(^-\) cells at different time points in non-selective media. The cells were initially selected using SC-Ura media to select for a 100% GFP\(^+\) cells. The cultures were sub-inoculated into non-selective media and grown for two consecutive overnights. Cells were collected after each overnight and at least 100 cells were scored for GFP activity. This experiment was done with two biological replicates. The error bars represent standard deviation.

Table 6. Proportions of GFP\(^+\) and GFP\(^-\) cells after 18-20 hours of growth in non-selective media. The O.D\(_{600}\) was used to calculate the number of generations (g) each culture underwent.
5.0 Mutations to CDC7 and CDC28 Phosphorylation Sites on Cac1p Result in a Decreased Growth Rate

In a separate set of experiments, I did preliminary work to characterize genetic interactions with mutants that lack CDK1 and DDK sites on Cac1p and histone H3 (98, 99). During S phase of the cell cycle, the pre-replication complex is assembled and activated by phosphorylation by CDK1 and DDK. Cac1p has two serine residues, 95 and 515 that are putative substrates for CDK1, and three serine residues 238, 501, and 503 are speculated to be phosphorylated by DDK (98). The CDK1 and DDK are thought to coordinate the activity of CAF-1 with the cell cycle. DDK also targets Thr 45 on histone H3 during DNA replication, it is suspected that replication stress can result in a loss of phosphorylation at this site (99).

In the Yankulov lab, SDM experiments generated Cac1p serine to alanine mutations at CDK1 and DDK sites, some contain double mutations in different combinations, listed in Table 7. Plasmids carrying these mutations were transformed into BY4742, cac1Δ, H3T45, and H3T45A. The isogenic BY4742 and cac1Δ provide data on the effect of these mutations on CAF-1 in the absence of wildtype Cac1p. The H3T45 WT and H3T45A, in this pair of strains both copies of histone H3, HHT1, were knocked out and a plasmid carrying one copy of histone H3 was introduced. In the H3T45 WT contains a non-mutated histone H3 and H3T45A contains a T to A point mutation at amino acid position 45. I tested the effects of the mutations in Cac1p are altered by a T45A substitution on histone H3.

Initially, I looked for growth defects in BY4742 and cac1Δ transformed with the Cac1p phosphorylation mutants listed in Table 7. The strains were inoculated into appropriate media and grown overnight to saturation, the cultures were diluted to an O.D600 of 0.1 into a 96-well
plate and the O.D\textsubscript{600} was recorded every hour in a plate reader. The data is plotted in Figure 17 and the growth rate constant was calculated for each strain and listed in Table 8. In the \textit{cac1A}, with \textit{wildtype} Cac1p had a growth rate of approximately 0.093. The single mutants S94A, S238A, S501A, S503A, and S515A which had similar growth rates at the \textit{wildtype} Cac1p. The mutations to only DDK sites, S238A-S501A and S238A-S503A also had a similar growth rate with \textit{wildtype} Cac1p.

However, in the \textit{cac1A} the Cac1p mutants S94A-S238A and S238A-S515A, contain double mutations to CDK1 and DDK sites, mutants had the slowest growth rates, 0.022 and 0.029, respectively. The S94A-S515A contains a double mutation to CDK1 sites, had a growth rate of 0.05, and grew at about half the rate of the \textit{wildtype}.

### Table 7. Cac1p phosphorylation mutants used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation(s) to regulatory site</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEG202 Cac1</td>
<td>-</td>
</tr>
<tr>
<td>pEG202-Cac1-S94A</td>
<td>CDK1</td>
</tr>
<tr>
<td>pEG202-Cac1-S238A</td>
<td>DDK</td>
</tr>
<tr>
<td>pEG202-Cac1-S501A</td>
<td>DDK</td>
</tr>
<tr>
<td>pEG202-Cac1-S503A</td>
<td>DDK</td>
</tr>
<tr>
<td>pEG202-Cac1-S515A</td>
<td>CDK1</td>
</tr>
<tr>
<td>pEG202-Cac1-S94A-S238A</td>
<td>CDK1, DDK</td>
</tr>
<tr>
<td>pEG202-Cac1-S94A-S515A</td>
<td>CDK1</td>
</tr>
<tr>
<td>pEG202-Cac1-S238A-S501A</td>
<td>DDK</td>
</tr>
<tr>
<td>pEG202-Cac1-S238A-S503A</td>
<td>DDK</td>
</tr>
<tr>
<td>pEG202-Cac1-S238A-S515A</td>
<td>DDK, CDK1</td>
</tr>
</tbody>
</table>

The \textit{H3T45WT} and \textit{H3T45A} strains contain a knockout deletion to both copies of histone H3 gene and has the insertion of one copy of non-mutated (H3T45) and mutated (H3T45A) histone H3 (99). I attempted to perform a knockdown of the \textit{CAC1} gene in the \textit{H3T45A WT} and \textit{H3T45A} strain but was unsuccessful. Hence, in this situation, the assays were performed in the presence of a genomic copy of \textit{CAC1} and the observed effects should
be deemed dominant negative phenotypes. In the *H3T45 WT* strain, the addition of a mutated copy of Cac1p did not cause a change in rates (Figure 18). In the *H3T45A* strain, the least effect on the growth rate were S94A-S238A and S238A-S501A. On the other hand, the S501A mutant has reduced the growth dramatically.
Figure 17. Comparison of growth rates for *BY4742* and *cac1Δ* transformed with the Cac1p phosphorylation mutants. Saturated cultures of the cells were diluted in appropriate media to an O.D$_{600}$ of 0.1 in a 96-well plate. The cells were incubated at 30°C with shaking and O.D$_{600}$ was recorded every hour with a Multiskan™ FC Microplate Photometer.
Figure 18. Comparison of growth rates for H3T45 WT and H3T45A transformed with the Cac1p phosphorylation mutants. Saturated cultures of the cells were diluted in appropriate media to an O.D$_{600}$ of approximately 0.3 in a 96-well plate. The cells were incubated at 30°C with shaking and O.D$_{600}$ was recorded every hour with a Multiskan™ FC Microplate Photometer.
5.1 Mutations to CDC7 and CDC28 Phosphorylation Sites on Cac1p have an Altered Morphology

Interestingly, I discovered that in the cac1Δ with certain Cac1p phosphorylation mutants had an altered morphology. The slowest growing strains, cac1Δ S94A-S238A and cac1Δ S238A-S515A had large elongated cells with frequent protrusions often called a “shmoo” (Figure 19). This morphology was more severe at a higher cell density (Figure 20).

The shmoo morphology occurs during yeast mating, a yeast cell recognizes pheromones released by the opposite mating type, and the yeast cell begins to form a protrusion in that direction to initiate the mating process (15). Laboratory strains usually contain cells with the same mating type, the shmoo phenotype could be indicative of defects in the silencing mechanism at the mating type loci (100). This lead me to the hypothesis that mutations to Cac1p could cause defects in structural integrity of the heterochromatin at the mating type loci. To test this, a cac1Δ strain with the GFP integrated at the HMR was transformed with Cac1p phosphorylation mutants listed in Table 7, the detection of GFP would be indicative of depression at the HMR locus (Figure 21). The double deletion mutant

| Table 8. The growth rate constant calculated for each strain. |
|-----------------|--------|--------|--------|--------|
|                 | BY4742 | cac1Δ  | H3T45 WT | H3T45A |
| pEG202 Cac1     | 0.153  | 0.093  | 0.05    | 0.042  |
| pEG202-Cac1-S94A| 0.147  | 0.095  | 0.051   | 0.014  |
| pEG202-Cac1-S238A| 0.143 | 0.080  | 0.056   | 0.030  |
| pEG202-Cac1-S501A| 0.140 | 0.094  | 0.052   | 0.010  |
| pEG202-Cac1-S503A| 0.154 | 0.093  | 0.057   | 0.021  |
| pEG202-Cac1-S515A| 0.161 | 0.100  | 0.057   | 0.028  |
| pEG202-Cac1-S94A-S238A| 0.156 | 0.022  | 0.050   | 0.040  |
| pEG202-Cac1-S94A-S515A| 0.157 | 0.050  | 0.048   | 0.020  |
| pEG202-Cac1-S238A-S501A| 0.159 | 0.096  | 0.048   | 0.041  |
| pEG202-Cac1-S238A-S503A| 0.148 | 0.105  | 0.051   | 0.016  |
| pEG202-Cac1-S238A-S515A| 0.144 | 0.029  | 0.054   | 0.017  |
cac1Δasf1Δ was used as a positive control, and its variegated expression of GFP was detected (101). All the Cac1p phosphorylation mutants in the cac1Δ GFP::HMR strain did not show a defect in silencing at the HMR loci. This result demonstrates that the shmoo morphology of the Cac1 S94A-S238A and Cac1 S238A-S515A were not due to the derepression of the mating type loci. An alternative hypothesis is the elongated morphology depict G1 arrest, and the mutations to Cac1p antagonize the cell cycle regulators CDK and DDK; however, this will need further investigation.
Figure 19. Morphology of Cac1p phosphorylation strains in the cac1Δ. Cells from each strain were grown overnight in non-selective media and sub-inoculated the next morning and grown to an O.D$_{600}$ of 0.4 and visualized with light microscopy.

Figure 20. Morphology of cac1Δ S94A-S238A and S238A-S515A mutants. Strains were grown to an O.D$_{600}$ of 0.8 and visualized with light microscopy.
**Figure 21. Analysis of repression of the HMR loci using GFP.** The \( \textit{cac1}\Delta \text{GFP::HMR} \) strain was transformed with the Cac1p phosphorylation mutants. The cells were grown up in appropriate media and imaged using confocal microscopy. The \( \textit{cac1}\Delta asf1\Delta \text{GFP::HMR} \) was a positive control.
Chapter 6: Discussion

6.1 The Impact of \textit{RFB} on Epigenetic Conversions: TPE Variegation Assay

The inheritance of epigenetic marks on histone proteins and DNA is a central question in the field of epigenetics. Recent findings into the mechanistic role of histone chaperones and nucleosome disassembly and reassembly have produced a model of how the epigenetic marks on old histones are transferred to the new histones (91). However, the mechanisms that govern epigenetic change remain elusive. The underlying mechanism that causes an epigenetic conversion between the active and silent state of a gene in at the \textit{VII-L} telomere in \textit{S. cerevisiae} is still unknown.

There are a few lines of evidence that suggest that a pause in replication fork progression may have a role in epigenetic conversions. First, regions that are highly repetitive, such as the subtelomeres, contain many replication fork pause sites (69). Moreover, the deletion of Rrm3p, which is involved in the recommencement of replication forks, impairs the cells’ ability to undergo an epigenetic conversions at the subtelomeres (86). Based on these observations, I hypothesized that the addition of a replication pause site would influence epigenetic conversions.

The effect of \textit{RFB} on epigenetic conversions was first addressed with the FOA\textsuperscript{R} variegation assay. The \textit{RFB} strains were cloned into a construct, \textit{URA3-GFP-Pest-NLS-Tel}, that could later be used with flow cytometry to cross-validate the results. I did a direct comparison with the established \textit{URA3-Tel} construct and the new \textit{URA3-GFP-Pest-NLS-Tel} construct. The new construct did not behave in the \textit{wildtype} as expected. The results show that in the SC-Ura initial selection, in \textit{BY4742 URA3-GFP-Pest-NLS-Tel} approximately 87%
of the cells had an epigenetic switch compared to approximately 33% in BY4742 URA3-Tel. This deviates significantly from the hypothesized rate of 3% of epigenetic conversions (88). Initially, it was hypothesized that this was due to an insufficient concentration of 5-FOA. I tested this by carrying out the FOA<sup>R</sup> variegation assay at increasing concentrations of 5-FOA, and found the colony size was reduced, but the proportions remained the same. Another hypothesis for the large populations of cells with the silent reporter could be due to the construct. This will be addressed further in the next section.

Overall, these results suggest that the presence of an RFB element does not have an effect on epigenetic conversions. However, these results need to be interpreted with caution. Since the *wildtype* proportions were not reproduced, conclusions cannot be made on the impact of a RFB on epigenetic conversions. These experiments should be followed up with the a RFB cloned into a URA3-Tel construct and evaluated with the FOA<sup>R</sup> variegation assay. Alternatively, these experiments could be done with the Ura3-GFP fusion protein, but the large proportion of silent cells must be addressed and corrected.

6.2 A Novel Assay for the Analysis of Epigenetic Change in *S. cerevisiae*

The main goal of this research was to establish a new method to study epigenetic conversions without the use of 5-FOA. Traditionally, epigenetic inheritance and TPE in *S. cerevisiae* has been studied by the FOA<sup>R</sup> variegation assay (36). More recently, the Yankulov lab modified this assay to specifically address the regulators of epigenetic change. Both assays use 5-FOA as a selecting agent to determine the expression state of the *URA3* reporter. However, it has been shown that 5-FOA reduces the activity of RNR, which distorts the
nucleotide pools in the cell. For this reason, I attempted to establish an assay without the use of 5-FOA to evaluate epigenetic conversions.

The proposal made use of a new construct that produced a Ura3-GFP fusion protein that was tagged with a PEST domain and an NLS. I measured epigenetic conversions in *wildtype* BY4742, *sir3Δ, cac1Δ*, and *rrm3Δ* strains using flow cytometry (Figure 13). The proportion of active and silent cells from flow cytometry were not as expected but were similar to the proportions from the FOA<sup>R</sup> variegation assay (Table 3, Table 4). Specifically, the *wildtype* BY4742 typically has 60% active cells and 40% of silent cells; however, the flow cytometry reported 17.5% active cells and 82.5% silent cells using the *URA3-GFP-Pest-NLS-Tel* construct (86, 88).

The pattern of a large proportion of silent or GFP<sup>−</sup> cells in *BY4742* is evident in the FOA<sup>R</sup> variegation assay and in the flow cytometry data. I suspect that the reporter protein may not be accurately reflecting the transcriptional state of the reporter construct. If the locus is in an active state and the reporter protein is transcribed but is targeted for degradation, it would produce a silent state phenotype. I hypothesize that the design of the construct and reporter protein may produce false negatives, and there are a few factors that may contribute to this including the fusion of the Pest domain, the fusion of the NLS, and the expression of the reporter protein.

6.2.1 The Effect of the Pest Domain and the NLS

GFP is a widely used reporter molecule in gene expression and protein localization studies. However, the stability of GFP can interfere with dynamic changes in gene expression.
A Pest domain is a small basic sequence that is associated with rapid protein degradation, which can be tagged to GFP to reduce its half-life (95). In the \textit{URA3-GFP-Pest-NLS-Tel} construct, the GFP is tagged with a \textit{CLN2} Pest domain; however, the GFP is fused with Ura3p, thus the addition of the Pest domain destabilizes the GFP and Ura3p. The Ura3p is an enzyme in the essential pyrimidine pathway. As mentioned, different media can be used to select and counter-select for the activity of Ura3p. I examined this with a growth curve in different selection media and found that growth in media that requires Ura3p activity, Sc-Ura, was severely inhibited in the \textit{URA3-GFP-Pest-NLS-Tel} construct (Figure 12). However, the \textit{URA3-GFP-Tel} construct, which encodes a Ura3p-GFP fusion protein, but it does not contain a Pest domain or a NLS, the growth rate of \textit{BY4742 URA3-GFP-Tel} was comparable to the \textit{wildtype}. Therefore, this indicates that the Pest domain or NLS has an effect in certain growth conditions. This finding has several implications. First, the FOA\textsuperscript{R} variegation assay determines proportion of active cells by growth on Sc-Ura plates; therefore, this inhibited growth may bias the results of active cells. The growth in SC+FOA may also be affected, if a cell is active and expressing Ura3p-GFP but it is targeted for degradation it still may be able to grow, it would produce a phenotype of a silent cell. Although I examined for cellular fluorescence in the different media, and I found the cells in SC+FOA to be GFP\textsuperscript{-}, a better analysis would be with a Western blot. A Western blot is more sensitive and would be able to detect levels of Ura3p that may be present but not be detectable by fluorescence microscopy.

6.2.2 The Effect of the NLS and the Expression of the Reporter Protein

The Ura3p-GFP fusion protein was also tagged with a NLS in order to concentrate the GFP signal. The subtelomeric region is generally low in gene density and low in transcriptional activity (44). Low expression of the reporter protein influences the distribution
of the intensity of the fluorescence when analyzing with flow cytometry. The distribution of GFP intensity in the positive control (sir3Δ URA3-GFP-Pest-NLS-Tel) has only a slight shift compared to the negative control (BY4742 URA3-Tel) (Figure 14).

Ura3p normally functions in the cytoplasm and the targeting of the Ura3p with the NLS could interfere with its efficacy. The addition of the Pest domain and NLS means that Ura3p is targeted for degradation. In the growth curve experiment, I was not able to test the effects of the Pest domain and the NLS alone (Figure 12). Therefore, I was not able to distinguish if the effects were exclusively from the Pest domain or the NLS. However, a simple experiment to test this would involve an SDM with PCR primers to disrupt each sequence to test the effects of each with a growth curve. If this experiment is carried out and the NLS had an impact on the enzymatic activity of Ura3p, a strong or medium constitutive promoter such as CYC or ADH, can be used to circumvent the use of the NLS.

6.2.3 Proposal of a New Construct

I observed high proportions of silent cells with the URA3-GFP-Pest-NLS-Tel construct. I hypothesized that the high proportions of silent cells may be due to compounding variables introduced by the Ura3p-GFP fusion protein, but this needs further investigation. I have proposed a few experiments that can be done to continue the validation of the URA3-GFP-Pest-NLS-Tel construct. If this construct was redesigned, I would have a few recommendations. First, I would eliminate the use of Ura3p as a marker. The use of this marker such as Ura3p means the assay relies on cellular growth, which can be influenced by several different variables. Ura3p was incorporated to allow for the selection for the active or silent state. Alternatively, fluorescent associated cell sorting (FACS) can be used to generate active and silent populations, GFP+ and GFP- respectively.
Moreover, I would revisit the use of the Pest domain. The rates of epigenetic conversions have been calculated to occur 1/20 generations or about 3%, which is a rather rare occurrence (68). The Pest domain was added to destabilize eGFP, which has a normal half-life of approximately 7 hours (102). Given that epigenetic conversions are infrequent, the GFP reporter may not need to be destabilized to reflect the infrequent changes in transcription. If the construct still contained a Pest domain, a no Pest domain construct should be made to use as a control. Instead of the addition of the NLS to compensate for the low transcriptional activity at the subtelomeres, I would use a strong or medium constitutive promoter, such as CYC or ADH.

The new construct would include the distal end of ADH4 and TG(1-3) repeats to target the construct to the VII-L telomere. Possible controls would include the wildtype BY4742, sir2Δ as a positive control, and cac1Δasf1Δ as a variegation control. The proposed ADH4-pCyc-eGFP-Tel construct would need to be validated through many of the experiments done here. Moreover, the stability of this protein can be determined using cycloheximide analysis to determine protein degradation. Cycloheximide inhibits protein translation; therefore, no new proteins are being synthesized, and the concentration of the protein of interest should decrease at the rate it is being degraded. Timepoints can be taken and a Western blot preformed to determine the concentration of the protein of interest.

6.3 GFP Based Analysis of the Impact of RFB on Epigenetic Conversions

The investigation of the impact of RFB on epigenetic conversions was designed as a three-pronged approach. The results of the FOA^n suggest that the presence of an RFB did not have a major impact on the frequency of epigenetic conversions. These results also revealed
that the URA3-GFP-Pest-NLS-Tel construct produces a different distribution of cells with the active reporter versus the cells with the silent reporter. Approximately 10% of cells had the active reporter and 90% of cells had the silent reporter, which was not expected. In the flow cytometry analysis and microscopic scoring, I saw a similar distribution of active and silent cells as the FOA\textsuperscript{R} assay. The flow cytometry data showed a similar frequency of conversions in the RFB experimental controls as the no RFB control. Overall, the data from the microscopic scoring showed small but not significant changes due to the presence of a RFB. Based on my experiments, the presence of a RFB did not have a major effect on the frequency of epigenetic conversions. However, due to the variation in proportions of active and silent cells in the wildtype using URA3-GFP-Pest-NLS-Tel from previously reported proportions, these results are inconclusive.

6.4 Comparison of the Techniques Used

The use of cellular fluorescence to assess the frequency of epigenetic conversions was unprecedented. Therefore, this novel technique was cross-validated with the previous method. To compare the three techniques used to study RFB on epigenetic conversions, the data from the FOA\textsuperscript{R} variegation assay, microscopic scoring, and flow cytometry experiments in BY4742 was plotted together (Figure 22). Only the initial active selection in SC-Ura is shown. The results from all three experiments show a similar distribution of active and silent cells for each construct. All three techniques show there was no significant change in epigenetic conversions when an RFB is present.

The variability within the FOA\textsuperscript{R} variegation assay versus the variability within microscopic scoring is evident. Based on the standard deviation from each experiment, the
microscopic scoring is a more precise experiment. The large variability in the FOA<sup>R</sup> variegation assay may be due to the small sample size used. In this assay, a saturated culture is serially diluted and spotted onto a plate, and the 5<sup>th</sup> or 6<sup>th</sup> dilution is counted and used to calculate the proportions of cells. Typically, these dilutions typically contain 5-10 yeast colonies. Consequently, small deviations in the number of colonies can produce a large amount of variation. Moreover, there is an assumption made using this assay, the proportion of active cells and the proportion of silent cells are equal to 100%. However, I have found this is not always the case in practice. Perhaps it may be due to the calculation in the proportion of cells, which is calculated by three different aliquots of cells on different plates. The proportion of active cells and silent cells is sometimes more than the total number of cells, that is more then 100%. The sample size for microscopic scoring was approximately 100 cells and the sample size for flow cytometry was approximately 12,000 cells.

The approach of cellular fluorescence has additional benefits beyond the circumvention of 5-FOA. Microscopic scoring and flow cytometry provides single-cell analysis. This provides a more accurate assessment of the state of the reporter protein. It also abrogates the use of proportions to determine the number of cells with an active versus a silent reporter, which decreases the amount of variability within the experiment. Moreover, both techniques assess a larger sample size which also contributes to a decreased variability. In addition, this approach involves growth in liquid media, which can be analyzed for the number of cell divisions. Whereas, growth on plates in the FOA<sup>R</sup> variegation assay operates under the assumption that it takes approximately 25 cell divisions to form a colony. These two techniques also have a significantly faster procedure time. The FOA<sup>R</sup> variegation assay takes
approximately four weeks to complete, while the microscopic scoring and flow cytometry may take up to a week to complete.

For future studies on epigenetic conversions, I would recommend the use of a fluorescent reporter and flow cytometry. I would also suggest that fluorescent microscopy should be supplemented for visual confirmation and subcellular localization. The microscopic scoring was more cumbersome in comparison to flow cytometry. Moving forward, the use of flow cytometry to analyze epigenetic conversions will provide more quantifiable and reproducible results in comparison to the FOA$^R$ variegation assay.
Figure 22. Comparison of results of the FOA$^R$ variegation assay, microscopic scoring, and flow cytometry. The same data from Figure 9, Figure 14, and Figure 16 were plotted to directly compare the results of the impact of a $RFB$ on epigenetic switching for each technique. The error bars represent standard deviation.
6.5 Cac1p CDK and DDK mutants

The histone chaperone CAF-1 plays a key role in histone turnover (55). The largest subunit of CAF-1, Cac1p was identified to be highly phosphorylated at several serine residues (103). Phosphorylation at these residues by CDK and DDK are suspected to regulate the activity of CAF-1 with the cell cycle. I discovered that a double mutation to a CDK and DDK site had a significant deleterious effect on cell growth and morphology. This is an interesting finding that opens an avenue for detailed mechanistic studies on CAF-1. My findings suggest that the altered morphology of these mutants is not attributed to the derepression of the silent mating type loci. Continuation on the study on the regulation of CAF-1 should focus on if the phosphorylation mutants to Cac1p deteriorate the cell cycle.
Chapter 7: Conclusion

The aim of this study was to use a novel approach to studying epigenetic conversions at the subtelomeres of *S. cerevisiae*. I have shown that cellular fluorescence can be used as an alternative to 5-FOA to monitor the expression of *URA3*. This approach provides a better avenue to produce quantifiable and reproducible results and opens new opportunities to address research questions regarding epigenetic conversions. I investigated the impact of replication fork pausing on the frequency of epigenetic change with three different techniques. However, the results are inconclusive. However, the current state of the construct may produce false negatives. This construct needs further optimization, I have made recommendations for follow-up experiments and new construct design.
References


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9.0 Appendix

Appendix A:

Primer sequences (5’ to 3’) and respective melting temperatures for all PCR reactions used in this study.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT1 R</td>
<td>GATGAAGCTCAATCCAAAGAGG</td>
<td>65.1</td>
</tr>
<tr>
<td>ACT1 F</td>
<td>AGTGGTGAGAAAGAGTAACCACG</td>
<td>66.3</td>
</tr>
<tr>
<td>RFBgenforBg</td>
<td>TGCCGAGATCTGCTACGGACTTCCACTGTCGAAAGAGG</td>
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</tr>
<tr>
<td>RFBgenrevBg</td>
<td>TGCGAGATCTGATGGGTTGAAAGAGGAGG</td>
<td>79</td>
</tr>
<tr>
<td>pTK023seqURA3RFB</td>
<td>GCCATACCTGCAAGTGCTTTAGCTGCG</td>
<td>71</td>
</tr>
<tr>
<td>pTK023seqTgRFB</td>
<td>CGCGCTGTACTCCAAACAAAGAG</td>
<td>70.7</td>
</tr>
<tr>
<td>BglIIinsertfor</td>
<td>GGCCCTTCTTGTCTTTAGATCTACCGT</td>
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</tr>
<tr>
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<tr>
<td>Rg3BglIIinsert2rev</td>
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