2015

Mitotic Recombination in *Saccharomyces cerevisiae* Results from Genetic Instability at Fragile Site FS2

Shaylynn Delaney Miller

Mitotic Recombination in *Saccharomyces cerevisiae* Results from Genetic Instability at Fragile Site FS2

Abstract
Common fragile sites (CFSs) are present in a number of species and are considered a normal structural feature of the eukaryotic chromosome. CFSs are regions of genetic instability prone to breaking under DNA replication stress. Cancer is a possible result of DNA breaks that lead to a loss of heterozygosity (LOR) in the cell. Mitotic homologous recombination is a method of DNA repair following a break from DNA instability. Our experiments investigated the role of mitotic recombination in the development of LOH. Replication stress was induced in *Saccharomyces cerevisiae* through the use of the *GAL*l/lO promoter; this modified promoter is dependent on the galactose concentration in the media for the expression of polymerase alpha. We examined a strain with an intact fragile site (FS2 of the yeast chromosome III) as well as a control strain with FS2 interrupted by a *NAT* gene. We hypothesized that DNA breaks at FS2 during mitosis, under conditions of replication stress, would be repaired by homologous recombination. Higher frequencies of mitotic recombination events leading to LOH were observed for the strain with an intact FS2 region, when subjected to replication stress and compared with the control strain in conditions of unstressed growth (9.83 fold increase). Our data adds further support to LOH resulting from mitotic recombination following the formation of fragile site breaks as the experimental strain under replication stress had higher frequencies of mitotic recombination events than the control strain.

Degree Type
Open Access Senior Honors Thesis

Department
Biology

First Advisor
Anne Casper

Second Advisor
Aaron Liepman

Keywords
homologous chromosome, homologous recombination, yeast, galactose

MITOTIC RECOMBINATION IN *SACCHAROMYCES CEREVISIAE* RESULTS FROM GENETIC INSTABILITY AT FRAGILE SITE FS2

By

Shaylynn Delaney Miller

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in ____Biology___ (include the department name)

Approved at Ypsilanti, Michigan, on this date April 17, 2015

Mitotic Recombination in *Saccharomyces cerevisiae* Results from Genetic Instability at Fragile Site FS2

By

Shaylynn Delaney Miller

Submitted to the Department of Biology and the Honors Program for Departmental Honors

April, 2015

Ypsilanti, Michigan

Acknowledgements

I would like to thank Dr. Anne Casper for her instruction and guidance throughout my undergraduate career, as well as for providing me with the opportunity to study as a student in her genetics lab. I would also like to acknowledge Dr. William Fennel for his four years of help supporting my research project through the SURF program, and also my fellow members of the Casper Lab, both past and present. Financial support for the lab was mostly provided the NIH grants Dr. Casper has received (NIH 1R15-GM0932901).

Abstract

Common fragile sites (CFSs) are present in a number of species and are considered a normal structural feature of the eukaryotic chromosome. CFSs are regions of genetic instability prone to breaking under DNA replication stress. Cancer is a possible result of DNA breaks that lead to a loss of heterozygosity (LOH) in the cell. Mitotic homologous recombination is a method of DNA repair following a break from DNA instability. Our experiments investigated the role of mitotic recombination in the development of LOH. Replication stress was induced in *Saccharomyces cerevisiae* through the use of the $GAL1/10$ promoter; this modified promoter is dependent on the galactose concentration in the media for the expression of polymerase alpha. We examined a strain with an intact fragile site (FS2 of the yeast chromosome III) as well as a control strain with FS2 interrupted by a *NAT* gene. We hypothesized that DNA breaks at FS2 during mitosis, under conditions of replication stress, would be repaired by homologous recombination. Higher frequencies of mitotic recombination events leading to LOH were observed for the strain with an intact FS2 region, when subjected to replication stress and compared with the control strain in conditions of unstressed growth (9.83 fold increase). Our data adds further support to LOH resulting from mitotic recombination following the formation of fragile site breaks as the experimental strain under replication stress had higher frequencies of mitotic recombination events than the control strain.

TABLE OF CONTENTS

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Background Information on Common Fragile Sites

Recent estimates of the prevalence of cancer, among the world's adult population, included 28.8 million people as of 2008 (Bray et al., 2013). Although numerous types of abnormal cell functions can induce a cancerous state in the surrounding tissues, the study of fragile sites has been gaining interest in recent years; this is partially due to the associations that have been found between common fragile sites and cancer development (Arlt et al., 2003; Bester et al., 2011). Common fragile sites (CFS) are regions of a chromosome that show enhanced instability during periods of stressed replication (Arlt et al., 2006; Glover et al., 2005; Durkin and Glover, 2007). It is also hypothesized that CFSs exhibit a tendency for genetic recombination to repair DNA breaks during metaphase of the cell cycle (Wang, 2006).

Fragile sites are categorized as being either rare or common. This distinction is drawn based on prevalence in the population as well as the context of structural characteristics on the chromosomal and cellular levels (Durkin and Glover, 2007). Rare fragile sites are present in less than 5% of the human population and result specifically from massive expansion (increased copy number) of a di- or tri-nucleotide repeat (Schwartz et al., 2006). Expansion of these repeats can lead to the onset of various genetic disorders in humans (Pearson and Sinden, 1998). In contrast, common fragile sites (CFS) are present in all mammals studied to date and are thought to be a naturally occurring structural feature of mammalian chromosomes (Durkin and Glover, 2007; Glover et al., 1984). CFSs are large stretches of DNA up to 1Mbp long and have been found to contain high concentrations of AT rich sequences. Normally CFSs exhibit stable

behavior; it is under conditions of replication stress that DNA breaks occur at CFSs (Arlt et al., 2006; Glover et al., 2005; Durkin and Glover, 2007).

The early stages of cancer development especially have been linked to instability at common fragile sites (Arlt et al., 2006; Bester et al., 2011; Ozeri-Galai et al., 2012). It is thought that the replicative environment of DNA is somewhat stressed and slowed during the early stages of cancer progression, thus long stretches of single stranded DNA (ssDNA) are present (Ozeri-Galai et al., 2012). Replication stress refers to an impaired state of DNA replication in the cell. The preferred method of experimentally inducing replication stress in mammalian cells involves exposing the cells to a compound such as aphidicolin (Arlt et al., 2003). Aphidicolin hinders the elongation of DNA during the replication process; this is accomplished through the inhibition of DNA polymerases α and δ (Glover et al., 1984). When the cell has insufficient replicative machinery, a state of replication stress is induced; a common component of current explanations for fragile site breakage has been the collapse of replication forks during DNA synthesis under stress (Walsh et al., 2013). Once broken, several routes can be taken by the cell to minimize the harmful effects of the DNA damage that ensues.

1.2 Formation and Repair of Breaks at Common Fragile Sites

A. Common fragile site break formation mechanisms

There have been numerous hypotheses proposed to explain the underlying mechanisms leading to breaks at human common fragile sites. The primary mechanisms currently found in the literature are concerned with the sequence and its context. Context here refers to epigenetic aspects, specifically, the cell's usage of origins of replication where DNA synthesis begins (Letessier et al., 2011; Ozeri-Galai et al., 2011). In mammalian cells, origin use is epigenetically controlled, and both Ozeri-Galai et al.

(2011) and Letessier et al. (2011) reported that increase in origin activation when the cell is under replication stress allowed for the completion of DNA synthesis despite the less than ideal conditions; they reported that fragile regions did not activate extra origins needed under stressed conditions (Figure 1). The lack of origins, resulting in incomplete replication during stressed conditions, could therefore be a contributing factor to break formation in the stretches of DNA sequence deemed common fragile sites.

Epigenetic regulation is responsible for the activation of origins of replication during DNA replication in human cells. The "origin paucity hypothesis" adresses how break formation at common fragile sites could result from a lack of activated origins of replication in the cell (Figure 1). CFS fragility can be partially attributed to the cell type, *i.e.*, that fragile site stability is in part epigenetically determined (Letessier et al., 2011). FRA3B is a well characterized CFS that has been shown to be the most frequently broken CFS in human lymphocytes (Glover et al., 1984). However, FRA3B is not the most frequently broken CFS in human fibroblast cells (Letessier et al., 2011). Therefore, FRA3B has varying levels of fragility depending on the cell type. In lymphoblasts, activated origins of replication are relatively scarce in the FRA3B region, and in fibroblasts, there are numerous active origins of replication in the FRA3B region (Letessier et al., 2011).

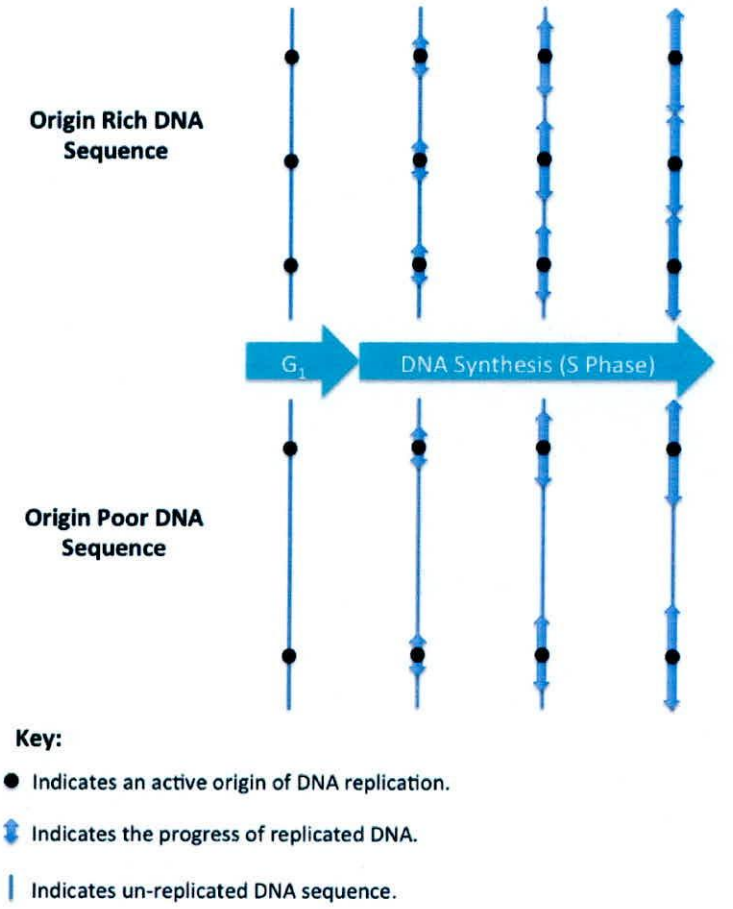


Figure 1:Origin Paucity Hypothesis Example

The lower half of the above figure shows the possibility of incomplete DNA replicaiton in a cell that lacks sufficient activated origins of replicaiton; this is contrasted with the sufficient origins of replication in the upper half that led to completed DNA replication.

The DNA sequence within CFS regions has also been examined. There are several components of the sequence that have been previously targeted for further investigation of fragility. Among them are di- and trinucleotide repeats, inverted transposable elements, and predicted usceptibility for secondary structure formation of palindromic regions (Casper et al., 2009). Excess single stranded DNA is frequently cited as a prerequisite to the formation of secondary structures (Figure 2) often as a result

of a disconnected replisome (Feng et al., 2011). DNA Polymerase alpha inhibition is among the known methods of inducing fragile site instability (Casper et al., 2008). Inhibition of polymerase alpha can be acomplished by various methods depending on the cell type, i.e., human cells respond to the drug aphidicolin for inhibition of polymerase alpha but yeast cells do not as the yeast cell wall is impermeable to aphidicolin (Arabshahi et al., 1988). In yeast cells another method of inhibition using the regulation of DNA polymersase alpha through galactose concentration of the growth media is implemented that will be discussed futher in Chapter 2.

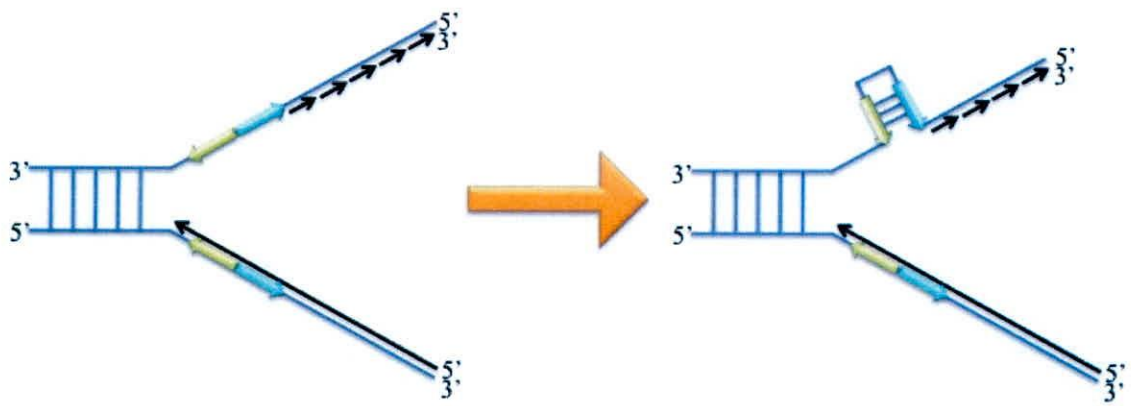


Figure 2: Example formation of a secondary, "hair-pin" structure.
The above figure displays a possible result of the presence of two inverted transposable elements under replication stress. In this case, as well as in our study, limiting polymerase alpha expression induced replication stress, as polymerase alpha-primase complexes control the availability of primers for DNA replication. The large golden arrow indicates the difference between the two replication forks shown in the figure. The first fork has not had time to form secondary structures between the two palindromic regions, while the other replication fork shows what happens when the two transposable elements have had time to bond together. Notice the leading strand requires fewer primers to replicate, where the lagging strand must continue to lay down primers as the replication fork opens further. The small black arrows represent primers; the slightly larger green and blue block arrows are the inverted transposable elements and the directionality has been indicated to emphasize the impact of limiting primase for a given direction during DNA replication.

B. Common fragile site break repair mechanisms

Once a break in a CFS has occurred, there are several possibilities open to the cell for response to the damage. The cell can repair double-strand breaks (DSBs) through homologous recombination, stabilize the break through telomere capping, or it could fail to address the break, which could lead to chromosome loss (Yim et al., 2014). There are several types of homologous recombination repair pathways (Lambert et al., 2010). A reciprocal crossover (RCO), as the name implies, is the reciprocal exchange of genetic information by two homologs. RCOs are one of the DNA repair mechanisms that a cell might implement to restore genetic stability. A BIR (Break Induced Replication) event entails a broken chromosome being repaired by copying off of the homologous chromosome's DNA. According to Mitchel et al. (2010) the probability of a double-stranded break being repaired through a cross-over (CO) event or a non-crossover (NCO) event has to do with the length of the invading 3' end during the formation of the Holiday Junction (HJ). They proposed that a longer 5' end resection, which would result in a longer 3' end available for invasion of the homolog, in conjunction with the correct direction of HJ resolution, is more likely to produce a CO event than when re-sectioning is shorter. Outcomes of DSB repair are often, break induced replication (BIR), reciprocal cross-over (RCO), or loss of the chromosome (distal to the break) entirely (Lemoine et al., 2005).

Once a chromosome arm has been lost, or genetic material exchanged, the heterozygosity of that chromosome is lost; the implications of this loss are described below.

C. Loss of heterozygosity

Chromosome loss and mitotic homologous recombination can both result in loss of heterozygosity (LOH). LOH occurs when a section of the chromosome no longer

contains both of the original sets of DNA, but instead contains a region with two copies of the same sequence; this is often the result of repaired DNA damage. LOH at a chromosomal locus has ties to cancer development. In part, the maintenance of normal cellular division can be attributed to the protection provided by tumor suppressor and proto-oncogenes in the chromosomes. When a region of a chromosome is lost or genetic information in that region is copied from the homologous chromosome, the genetic information in that region enters a state of LOH (Casper et al., 2012). The cell then has fewer defenses to prevent the development of cancer if the aberration occurs in a region possessing tumor suppressor genes and heterozygosity is lost (Kobayashi et al., 2003).

The current thought on the mechanism behind CFS involvement in cancer development suggests that instability at a CFS drives LOH during the early stages of cancer progression in the cell. It is important to note that CFSs are typically stable in a cell that is not experiencing replication stress (Glover et al., 2005). In a cell that has begun to experience delays in DNA replication, as frequently occurs in the early stages of cancer, the fragile sites would become increasingly susceptible to breakage under these more inhibited conditions (Arlt et al., 2006; Bester et al., 2011). It is in this way that the initial replicative problems associated with early cancer development could stall the DNA replication process enough to lead to further genetic aberrations, as the fragile sites become more unstable and cancer progresses (Barlow et al., 2013). Homologous recombination that occurs during mitosis to repair the DNA damage induced by replication stress in an early stage cancer could lead to LOH in the affected cell, thus yielding further genetic problems.

D. *Saccharomyces cerevisiae* as a Model Organism

Yeast and mammalian fragile sites contain both similarities and differences; however, enough common traits are present for yeast, such as *Saccharomyces cerevisiae*, to be used as excellent model organisms for the study of fragile site breakage. Mammalian common fragile sites contain base pair lengths of approximately 250 kb to 1 Mb, which are significantly longer than yeast CFSs (Durkin and Glover, 2007). Mammalian fragile sites can contain greater than 100 kb of often AT-rich sequence, particularly in rare fragile sites. Yeast fragile sites do not typically contain a significant amount of AT-rich sequence (Lemoine et al., 2005; Zhang and Freudenreich, 2007). However, the DNA sequence in both mammalian CFS and yeast fragile sites appear to have the ability to form hairpin loops and other DNA secondary structures (Casper et al., 2009; Lukusa and Fryns, 2008).

Yeast fragile sites, like human common fragile sites, are particularly unstable under conditions of replication stress. However in yeast, replication stress cannot be controlled with aphidicolin. Mammalian cells do not have cell walls; *S. cerevisiae* does possess a cell wall that blocks the entry of aphidicolin from gaining access to the intracellular environment. For this reason, an alternate system of inducing replication stress in yeast has been well established in previous studies (Lemoine et al., 2005 and Rosen et al., 2013). In this system, a galactose-regulatable promoter region is linked to the yeast *POL1* gene, which acts to induce replication stress on the system. A more detailed description of the galactose-regulatable promoter region can be found below in the "Materials and Methods" chapter.

E. Overview of hypothesis and experimental design

LOH can have deleterious implications for an affected cell. We examined the relationship between mitotic recombination and the fragility of the fragile site, FS2. We hypothesize that DNA breaks at FS2 during mitosis, under conditions of replication stress, will be repaired by homologous recombination. Cultures containing both stressed (no [galactose]) and unstressed (high [galactose]) were examined. The *GAL-POL1* genetic system controlled the cell's *POL1* (primase) production to induce a state of replication stress in the cell. Without adequate primase levels the replicative machinery fails to function properly and replication forks can collapse along the chromosomes. Homologous recombination has been shown to restart stalled replication forks (Lambert et al., 2010). Both strains Y383 and AMC301 are homozygous for the *GAL-POL1* genetic system (Figure 3).

Both strains used in this experiment were homozygous for the *GAL-POL1* genetic system (Figure 3). The *POL1* gene codes for the catalytic subunit of the primase holoenzyme complex of *S. cerevisiae*; with this system, the amount of primase, and therefore the level of replication stress, is controlled by the amount of galactose in the growth medium (Table 1) (Lemoine et al., 2005). The abundance or lack of primase in the cell, as controlled by high or low galactose concentration, results in lack of replication stress or replication stress, respectively. In wild-type yeast cells (without *GAL-POL1*) the expression of *POL1* is unaffected by the concentration of galactose in the medium.

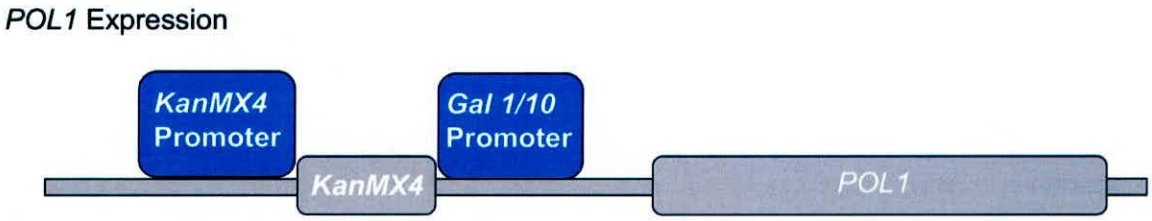


Figure 3: Control of *POL1* expression with the *GAL*1/10 promoter
Replication stress can be regulated through the use of a galactose regulatable promoter region. In this way the expression of POL1, the gene for the catalytic subunit of the yeast primase enzyme, is controlled by the concentration of galactose in the growth media. KanMX4 is a drug marker from the parental strain that provides resistance to kanamyacin.

We were able to successfully implement the previously published red-white colony sectoring system to facilitate the identification of cells that have undergone possible mitotic recombination events resulting from the replication stressed induced instability (Ronne and Rothstein, 1988; Rosen et al., 2013; Zimmermann et al., 1975). Selected colony halves were purified separately to ensure identical isolates were used throughout the later stages of the experiment. Chromosomes were examined through the use of restriction enzyme associated single nucleotide polymorphisms (SNPs) that were present due to the 0.5% sequence divergence of the two homologs of chromosome III. SNP testing was done to determine if LOH had occurred at the SNP in question. In this way, LOH could be roughly mapped by examining SNPs along the length of chromosome III.

Chapter 2: Materials and Methods

2.1 Overview of Yeast Strain Characteristics

A. Components on chromosomes other than chromosome III

In order to study mitotic recombination events at FS2, experiments were performed on two different *S. cerevisiae* yeast strains; both strains were created by Rosen et al. (2013). The experimental strain will be referred to as AMC310, and the control strain, Y383. Y383 is an identical isolate of the strain Y382 described by Rosen et al. (2013). Construction details for both strains can be found in the corresponding paper. To allow for the visualization of red-white colonies, the strains were homozygous for the mutant gene *ade2-1* as well as other important features such as FS2 and *SUP4-o*. The presence of the mutant *ade2-1* gene, instead of the wild-type copy, allowed for the build-up of a red product in the sixth step of the adenine biosynthesis pathway (Figure 4), when *SUP4-o* was not present due to fragile site activity. The build-up of a red product is due to the *SUP4-o* tRNA's ability to suppress the ochre stop codon's mutation; this suppression allows for the completion of adenine production and a phenotypically off-white colony. However, if the *SUP4-o* gene is inactivated, or simply not present, then the red precursor product develops and a sectored colony is formed (Figure 4).

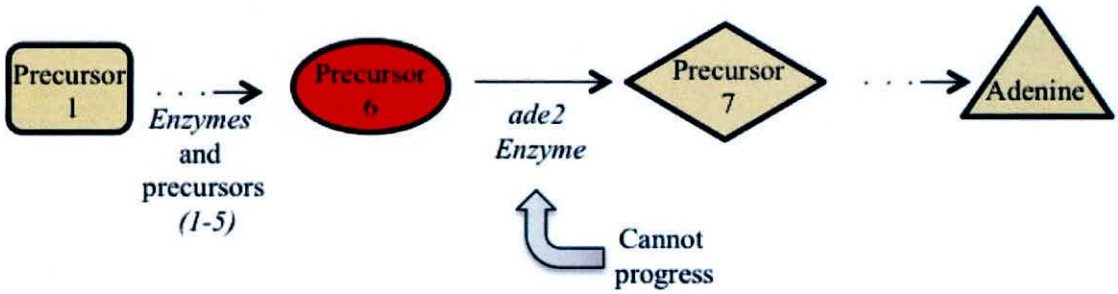


Figure 4: Adenine Biosynthesis Pathway and *SUP4-o* Regulation.
The SUP4-o tRNA suppresses the mutant version of the ade2 enzyme by continuing through the ochre stop codon following the ade2 gene sequence. Colonies will appear red in the absence of the SUP4-o gene product (SUP4-o tRNA) and colonies will appear white when the SUP4-o gene is present to bypass the stop codon. The color change was used as an indicator of a DNA recombination event in mitosis.

Media Type	YPR+HG		YPD		SR+HG (-ade -arg)	SR+HG (-His)
	Plate	Liquid	Plate	Liquid	Plate	Plate
Raffinose (%)	3	3	N/A	N/A	3	3
Peptone (%)	2	2	N/A	N/A	N/A	N/A
Dextrose (%)	N/A	N/A	2	2	N/A	N/A
Yeast Extract (%)	1	1	1	1	N/A	N/A
Agar (%)	3	N/A	3	N/A	3	3
Galactose (%)	0.05	0.05	N/A	N/A	0.05	0.05
His Dropout mix (%)	N/A	N/A	N/A	N/A	N/A	0.14
ade & arg Dropout Mix (%)	N/A	N/A	N/A	N/A	0.14	N/A
Yeast Nitrogen Base (%)	N/A	N/A	N/A	N/A	0.17	0.17
Ammonium Sulfate (%)	N/A	N/A	N/A	N/A	0.5	0.5

Table 1: Media Compositions by Reagent Percentage.
The above table shows the ingredient percentages for all the media types used in our experiments (other than deionized water, which makes up the remaining percentage for any given media type listed above). High galactose (HG) and Low galactose (LG) indicate the concentration of galactose used in the media and control the amount of replication stress experienced by the cell.

B. Experimental system components of yeast chromosome III

AMC310 contained the intact version of FS2, however, in Y383, a *NAT* gene, which provides resistance to the drug nourseothricin, was inserted in between the two transposable elements that make up FS2 (Goldstein and McCusker, 1999). The purpose of the insertion described was to inactivate the fragile site, resulting in a control strain that lacks a fragile FS2, while still retaining the interrupted sequence of the fragile site itself. The method of inactivating a fragile site by way of physically distancing the

transposable elements through the insertion of a drug resistance marker has been previously established by Lemoine et al. (2005).

The diploids Y383 and AMC310 were made by mating together haploids that are distantly related; one haploid is derived from strain NPD1 (Lemoine et al., 2005; Wei et al., 2007a), which is closely related to the sequenced yeast strain in the *Saccharomyces* Genome Database (SGD), therefore the chromosome III contributed by this haploid is labeled "SGD". The other haploid is derived from strain YJM789, therefore the chromosome III contributed by this haploid is labeled "YJM" (Wei et al., 2007b). The SGD and YJM homologous chromosomes III utilized in the experiment possess a 0.5% sequence divergence. This divergence results in many single nucleotide polymorphisms (SNPs between the two homologs. Many of these SNPs affect restriction enzyme sites, such that at a particular SNP site, one homolog of chromosome III is cut by an enzyme, and the other homolog is not cut. These SNP differences are employed in our analysis of events that occur on chromosome III.

The Y383 and AMC310 strains also had certain distinct structural features on their chromosomes III. The SGD chromosome of AMC310 held the intact version of FS2; the YJM chromosome of AMC310 had the *SUP4-o* gene and did not have FS2. Instead, the YJM chromosome has a region of homology composed of the centromere distal Ty1 transposable element of which FS2 is composed. As the other Ty1 element is not present, there is no palindromic sequence with which the distal transposable element can bind with to form a DNA secondary structure. Secondary structure formation by the inverted pair of Ty1 elements is required for breaks to form at FS2 (Lemoine et al, 2005). The presence of the non-fragile region of homology on the YJM homolog of

chromosome III allows the cell to have a sequence to implement homologous recombination DNA repair mechanisms without being inherently fragile itself. The SGD chromosome of Y383, on the other hand, had an inactivated FS2 (the *NAT* insert between the two Ty1 elements of FS2) and also supported the *SUP4-o* gene. The YJM chromosome of Y383 only has the region of homology. Diagrams indicating the relevant structural features can be seen below in Figure 5.

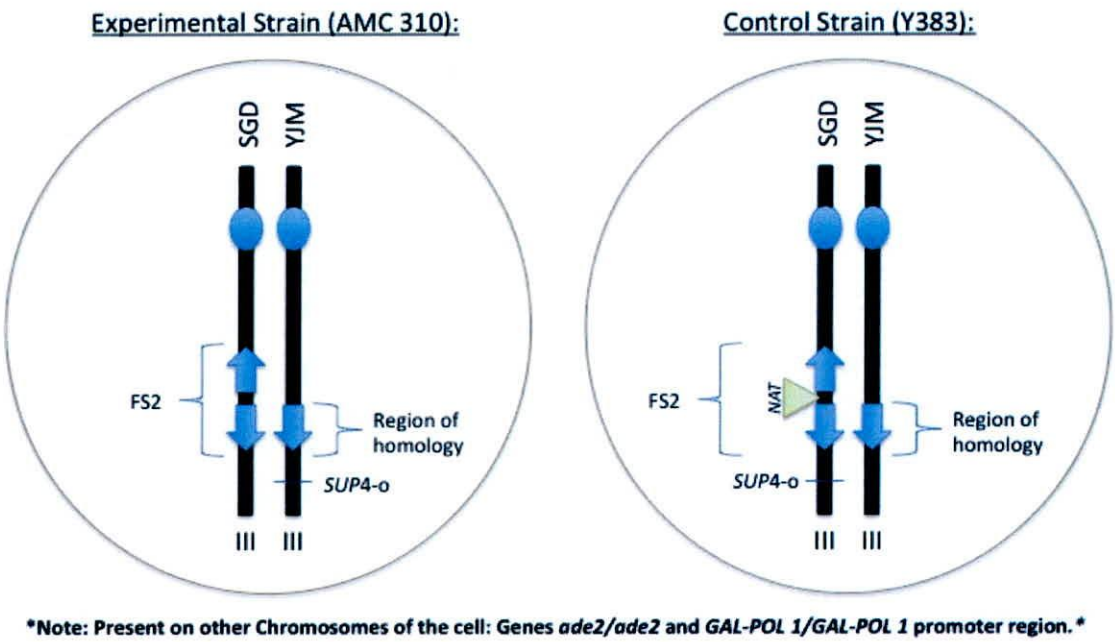


Figure 5: Diagrams of Y383 and AMC310 Strain Features.
The experimental strain (pictured on the left), AMC 310, contains several key features, including an intact fragile site at FS2, on the SGD chromosome, and a region of homology for the lower transposable (Ty1) element on the other chromosome (YJM). The blue ovals represent the centromeres present. The blue arrows are the transposable elements that make up the FS2 and the region of homology. SGD and YJM refer to the respective chromosomal names of the cromosomes present in this study. The two chromosomes pictured for both the experimental and control strains represent only the third chromosome of the 16 linear chromosomes of S. cerevisae. Note that SUP4-o is present on the distal end of the YJM chromosome. All chromosomes pictured are the third (III) chromosome of S. cerevisiae. The control strain (right), Y383, also has the region of homology on chromosome YJM to allow for repair off of the homologous chromosome. The location of SUP4-o in the control strain Y383 is on the SGD chromosome instead of the YJM chromosome. The fragile site in the control strain contains both of the original Ty1 elements, but the fragile nature of FS2 has been subdued through the insertion of the NAT gene. Present on other chromosomes are the GAL-POL1 construct and the mutant genes ade2/ade2. The purpose of the GAL-POL 1 construct is to allow the control of replication stress through our experimental mechanism. The mutant genes ade2/ade2 are present for red-white colony sectoring that defines our method of colony selection. There is also a 0.5% sequence divergence between the two homologs of chromosome III that permit single nucleotie polymorphism (SNP) testing, which has been described in more detail below.

2.2 Experimental Procedure

A. Strain growth conditions

All initial inoculations, for both Y383 and AMC 310, were grown for approximately 20-24 hours in a 30^{O}C test tube shaker at 300 rpm, in 5ml of YPR+HG media to ensure proper and un-stressed growth for all strains involved (regardless of the treatments that they would be assigned to later on for the experiments themselves). All media compositions are listed in Table 1. Cells were sedimented by centrifugation (5,000 x g) of the inoculated culture 1 mL at a time; this was followed by a thorough wash of each cell pellet with 1 mL sterile, distilled H_2O (mixing was accomplished by vortexing the samples for 10 seconds) to ensure the previous media would not remain to influence further treatments of the cell pellet. The washed cell pellet was re-suspended in either 5 mL of YPR+HG or 5 ml of YPD liquid media for 6 hours in a 30^{O}C test tube shaker. The YPD liquid media contained no galactose, and through our system, this imposed a state of replication stress on the cells within that treatment. The cell pellets re-suspended in the YPR+HG liquid media, however, had galactose present in more than sufficient levels (300% expression of POL1 through the *GAL-POL1* genetic system) to prevent excessive replication stress on the replicating cells (Lemoine et al., 2005). At the six-hour time point, cell density was calculated through the use of a DU 800 Spectrophotometer with the optical density of the culture at 600nm (OD_{600}). Samples were initially diluted through 1:5 dilutions prior to obtaining the OD_{600} readings. Only OD_{600} readings within the range of 0.2 and 1.2 were used. Serial dilutions were done in order to plate the cell cultures in such a way that the cell density yielded 250-300 colonies per plate. However, more than 250-300 cells were place on each plate due to the diminished viability of the cells. A frequent target was 900 cells per plate for the YPD treatment and 600 for the

YPR+HG treatment. The higher cell number for the YPD treatment was due to the lower viability of the cells grown in a cellular environment that was under stress (due to the absence of galactose in the media). Plates were kept in a 30^{O}C incubator for 4-5 days to permit colony growth, followed by 1-2 days in a 4 ^{O}C refrigerator to allow time for further definition of the red color to develop.

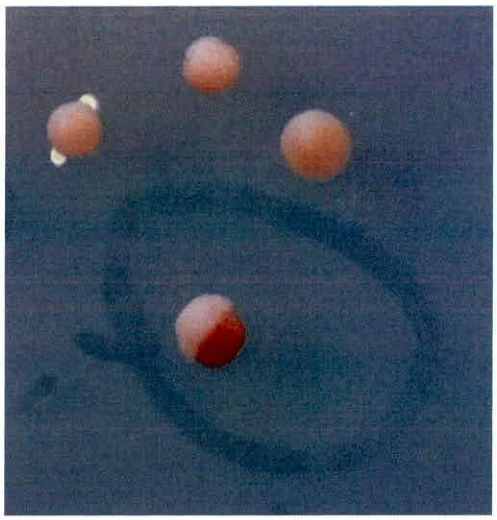


Figure 6: A Sectored Colony Example
Note that the red side of the sectored is smaller than the white side, as growth is slightly hindered for the red cells due to the inhibited adenine biosynthesis pathway. The red and white sides were purified separately, so the respective genetic alterations could be identified independently. The other three (pink) colonies pictured would not be selected for further analysis, because their coloration does not indicate that a genetic change has occurred on chromosome III.

B. Colony screening and DNA extraction

The total number of colonies on each plate was determined, and then colony screening was accomplished through the use of our red-white sectoring system. In this system, sectoring can result from mitotic recombination events of interest, as explained in section 2E, below. An example sectored colony and three un-sectored colonies can be seen in Figure 6. Other possibilities for red-white sectoring include the presence of a

wedge-shaped alteration in color as opposed to the more evenly distributed split down the middle. If at least half or one-fourth of the colony presented as red, these colonies were chosen for preliminary analysis. If the red portion of a colony was less than one-fourth, or if the red portion of the colony was completely centered on the white portion below and not in contact with the media itself, these were not chosen for further analysis.

The red and white sides of identified sectored colonies were analyzed separately. To obtain a pure isolate from each side, the red and white halves were streaked out (to streak for single colonies) on SR+HG-Ade-Arg plates (see Table 1 for media composition). SR+HG-Ade-Arg plates had high concentrations of galactose to prevent undue replication stress; they also contained an amino acid drop-out mix (-Ade-Arg) which was used to force the cells to implement the adenine biosynthesis pathway (Figure 4) that allows for better visualization of the sectored colonies present. One red colony and one white colony from each streak were patched on YPR+HG plates. Further screening was done at both of the stages described (streaking for single colonies and red-white sector patches). If the red and white halves of a sectored colony failed to demonstrate markedly different colors at each stage, then they were not included in the remainder of the analysis.

C. PCR amplification

DNA was extracted from the purified red and white halves of each sectored colony using the previously published a boil-freeze method (Ward, 1992). Colony-sized cell clusters were immersed in 20-50µl sterile distilled H_2O. Samples were incubated for 6 minutes at 100°C followed by incubation for a minimum of 10 minutes at -80°C. The

cells in each sample were pelleted by centrifugation at 6,000 rpm, and the supernatant was used to supply template DNA for the process of PCR.

Colony PCR was used to amplify regions of chromosome III surrounding SNPs. All colony PCR was done in a 10µl total reaction volume (Ward, 1992). The concentration of each component for colony PCR was as follows: 5µl of 2X GoTaq® from Promega (Fitchburg, WI, USA), 4µl of boil-freeze supernatant, 0.5µl of 10mM primer 1, and 0.5µl of 10mM primer 2. All primer sequences used in our analysis are listed in Table 2. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The following thermocycler parameters were applied to all samples for colony PCR: 1 X (94.0°C for 2'), 35 X (94.0°C for 30", 50.0°C for 30", and 72.0°C for 30"), 1X (72.0°C for 7').

D. Restriction enzyme digests and agarose gel electrophoresis

Amplified products from colony PCR reactions were subjected to restriction enzyme (RE) digests for SNP analysis. The RE digest solution contained 10µl PCR product, 0.5 µl of the respective restriction enzyme (Table 3) for a given cut site with approximately 0.38 to 0.77 units of enzyme activity per PCR reaction, 1.25µl buffer 4 from New England BioLabs (Ipswich, MA, USA), and 1.25µl sterile distilled H_2O. All REs were purchased from New England BioLabs (Ipswich, MA, USA). Agarose gel electrophoresis in 2% agarose gels containing 0.3X GelRed from VWR International (Radnor, PA, USA) was used to separate the products of restriction digests. To visualize the banding patterns, agarose gels were viewed under UV light. A 100bp DNA ladder

SNP #	PCR Forward Primer	PCR Reverse Primer	RE	Chromosome RE Cuts	Full Product size (bp)	Cut Products Sizes (bp)	Polymorphic Site (bp)	Units of RE activity per 0.5 µl
1	AMC 281	AMC 282	*HinfI*	SGD	452	282/170	120340	5
2	AMC 301	AMC 302	*MspI*	YJM	442	288/154	164273	10
3	AMC 305	AMC 306	*BanI*	YJM	298	192/106	175324	10
4	AMC 313	AMC 314	*HinfI*	YJM	351	229/122	193671	5
5	AMC 317	AMC 318	*HinfI*	SGD	428	250/178	201157	5
6	AMC 337	AMC 338	*HinfI*	YJM	355	233/100	246475	5
7	AMC 353	AMC 354	MspI	YJM	466	284/182	289633	10
8	AMC 357	AMC 358	RsaI	YJM	353	226/127	298875	5

Table 3: Restriction Enzyme Information

The restriction enzymes (REs) in this table correspond to the forward and reverse AMC primers in the same row. The SNP site is listed in the rightmost column. The "SNP #s" listed in the second to left most column are arbitrary numbers; they simply signify the order of the RE cut sites along the chromosome III, from centromere proximal to centromere distal. The chromosome that the RE cuts in AMC 310 is listed in the right middle column. In the Y383 strains note that the chromosome cut is opposite; for example, in Y383 the RE HinfI cuts on YJM instead of SGD. The units of enzyme activity per 0.5µl are shown in the left most column.

E. Determination of the event responsible for red/white colony sectoring

Red/white sectored colonies can result from a variety of events that affect the *SUP4-o* locus, including chromosome loss (Figure 7), reciprocal cross over (RCO) (Figure 8), break-induced replication (BIR) (Figure 9), gene conversion (Figure 10), and point mutation at *SUP4-o,* which would produce results similar to those shown in GC of the *SUP4-o* locus.

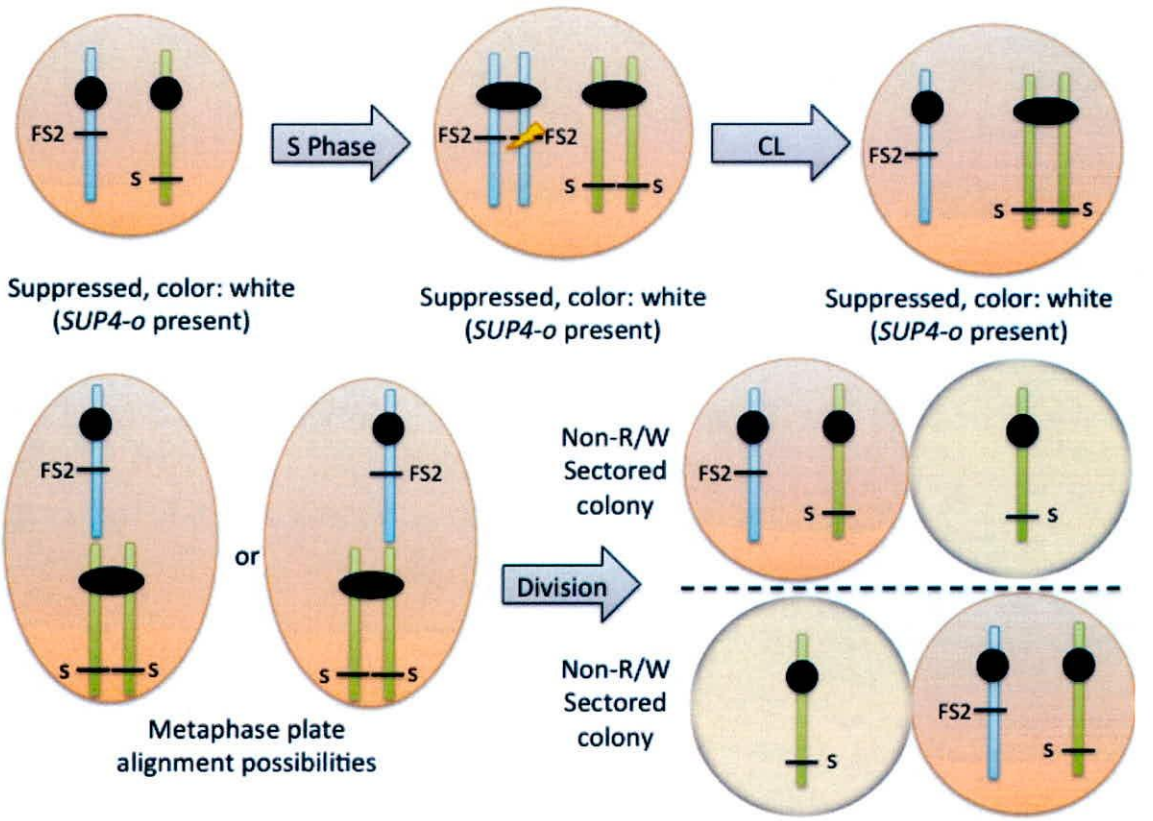


Figure 7: Formation of a Sectored Colony as a result of Chromosome loss (CL).
In the case of chromosome loss, the extent of DNA repair seen with RCOs, BIRs, and GC events is not actualized. Instead, following DNA synthesis in S-phase, the break occurs, but there is no strand invasion that would lead to the other types of homologous recombination of interest in this study. For the two strains used in this study only the strain with FS2 and SUP4-o can form sectored colonies from both metaphase alignments present for the third chromosome. Recall that the absence of a functional SUP4-o gene produces the red coloring of a red/white sectored colony under our experimental conditions. Also note that ade2/ade2 was present, on another un-pictured chromosome. The black circles/ovals represent centromeres present on the third S. cerevisiae chromosome. A sectored colony would most probably result from a chromosome containing both FS2 and SUP4-o, with the other chromosome lacking both of those features; Y383, our control strain, most closely follows this chromosomal layout. The example pictured is a model of the experimental strain, AMC 310, as is shown in the other DNA repair and sectored colony formation examples. In a case such as the one pictured in this figure, it might appear that a sectored colony has formed because of the color difference between the pinkish-white and bright-white sides of the heterozygous and homozygous sides, respectively.

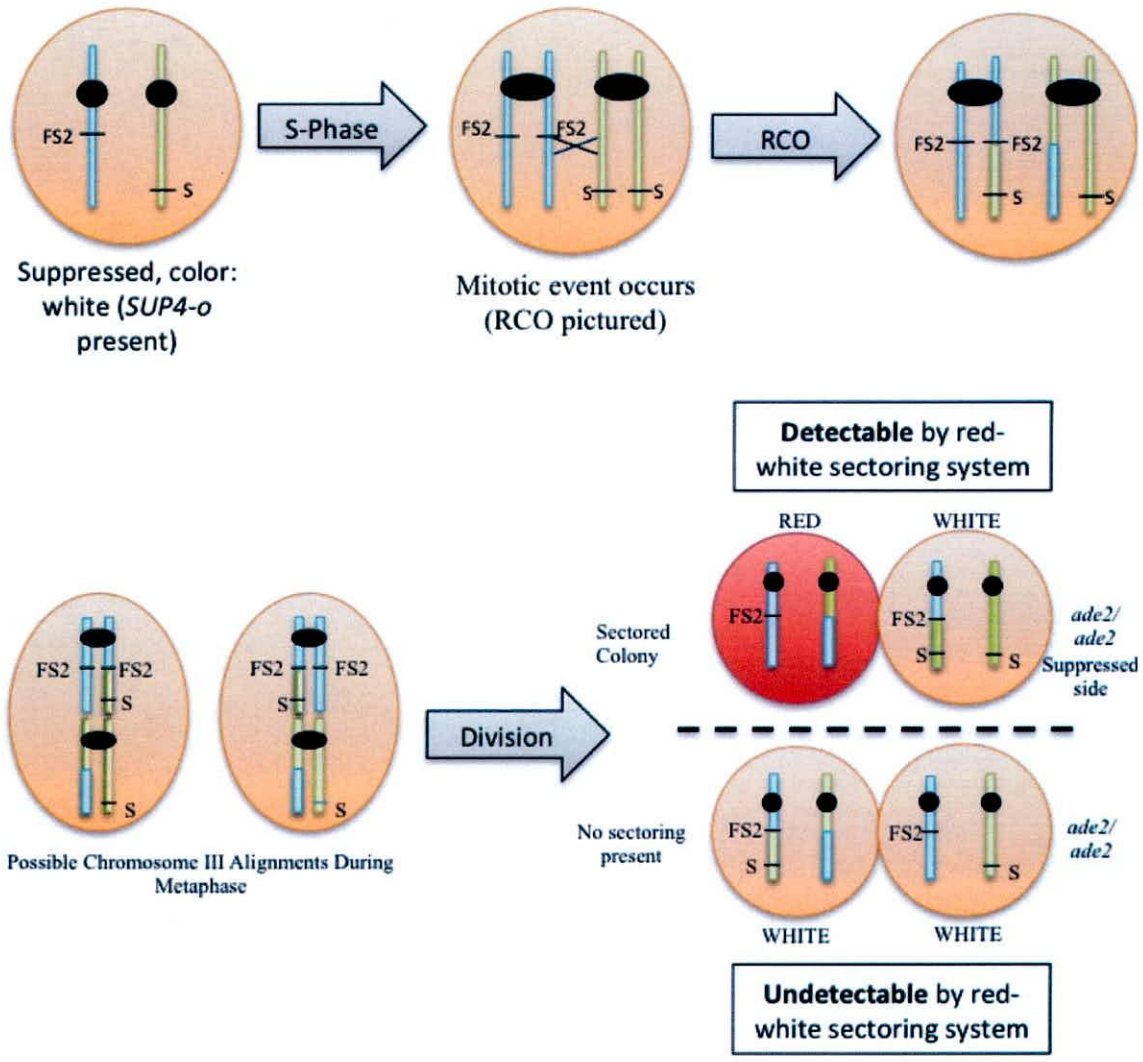


Figure 8: Formation of a Sectored Colony as a result of Reciprocal Crossover (RCO). *After DNA synthesis in S-phase a break is repaired by reciprocal cross over (RCO) in the figure above. The way the chromosomes line up along the metaphase plate, (only referring to chromosome III) can lead to two possible results. One of these results is undetectable by our methods of event selection, as both sides retain a copy of the SUP4-o gene. The half red color of a sectored colony can only arise through our experimental system with the absence of a functional SUP4-o gene. For this reason, the observed RCO event frequencies were multiplied by two, in order to account for the other unidentified half of the events. The ade2/ade2 is present on another chromosome (not pictured) to yield the development of red color in the absence of the SUP4-o gene.*

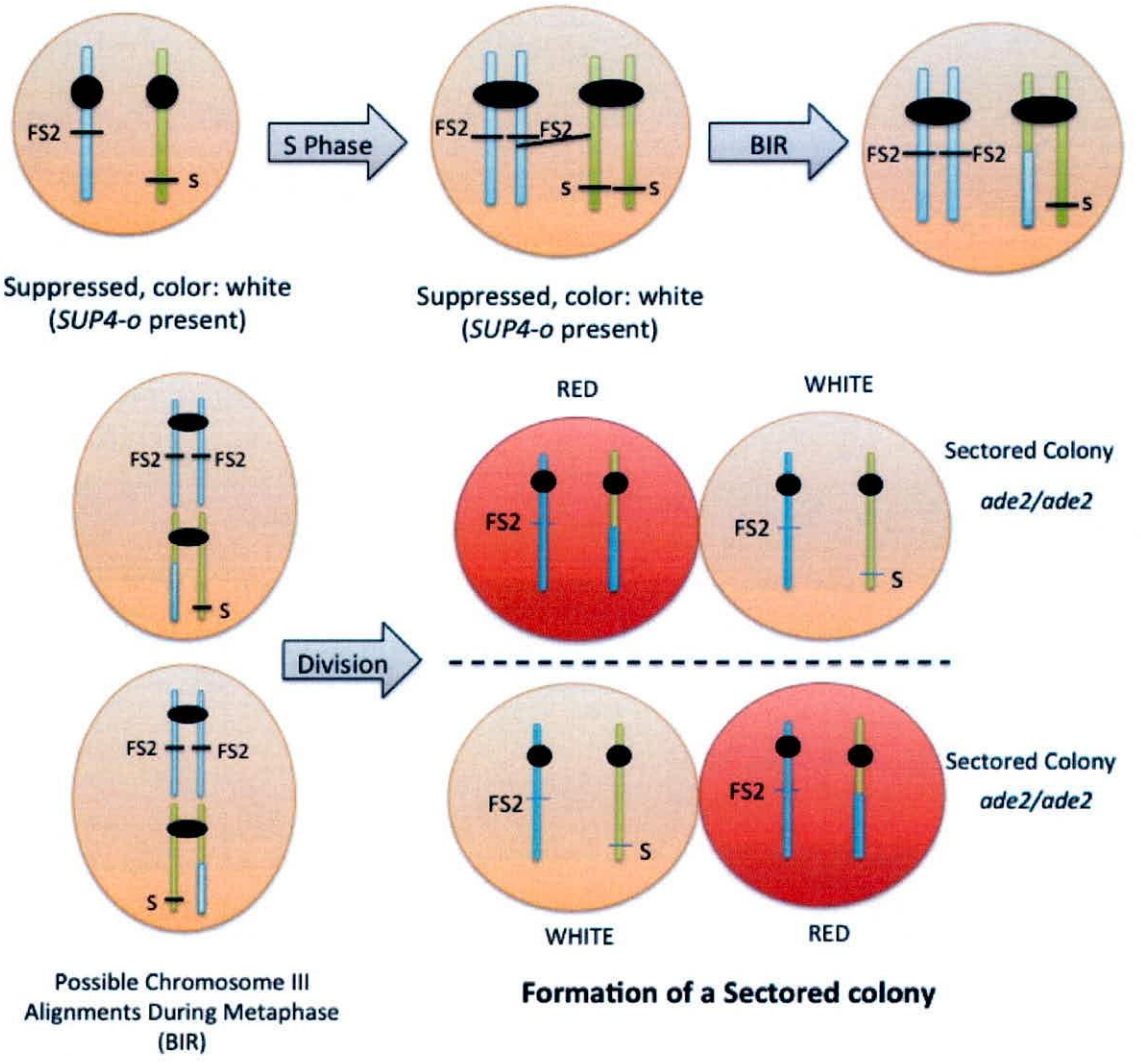


Figure 9: Formation of a Sectored Colony as a result of break-induced replication (BIR). *The above figure shows BIR used to repair a DNA break following S-phase. The sectored colony examples shown are the two possible results for the division of chromosome III along the metaphase plate. Unlike the RCO diagram above (Figure 8) both BIR possibilities can be detected through the absence of the SUP4-o gene, as both yield a half-colony color change; this is due to the presence of the ade2/ade2 genes on another (un-pictured) chromosome.*

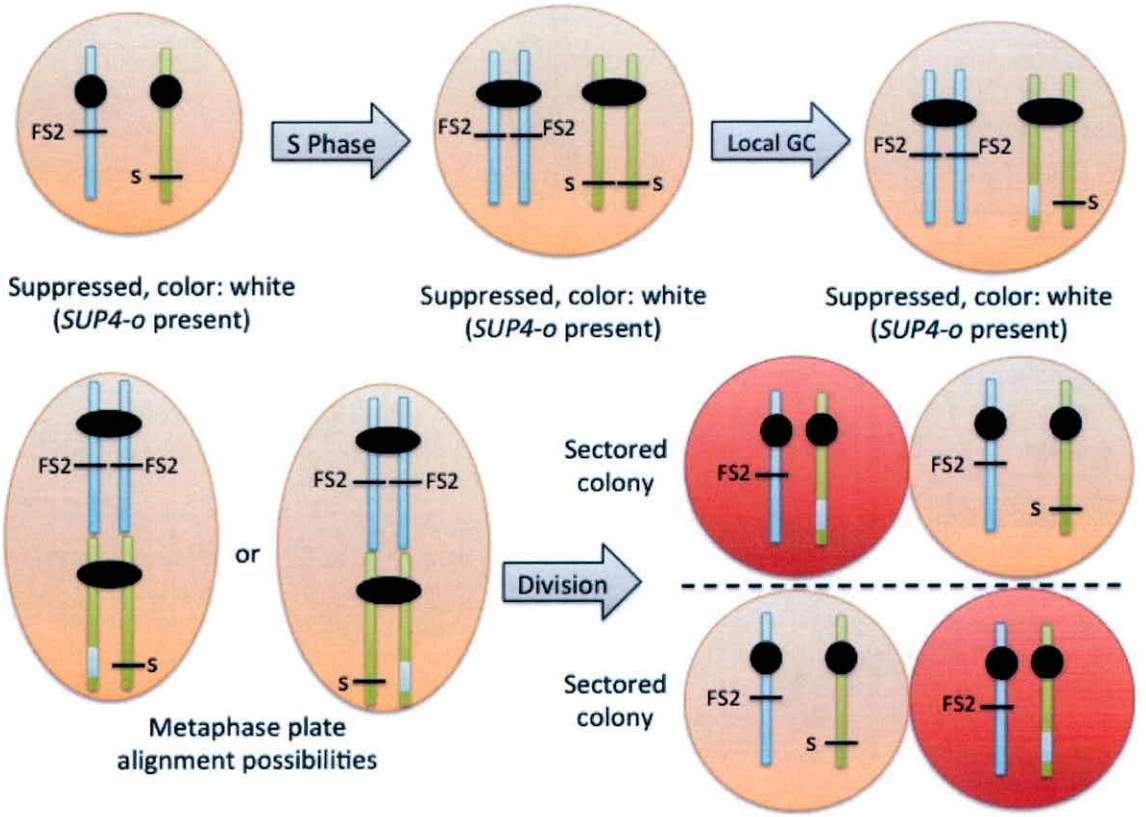


Figure 10: Formation of a Sectored Colony as a result of Gene Conversion (GC). *As with the other DNA break examples listed, the black circles represent centromeres, the blue bars are chromosomes that contain FS2, and the green bars are chromosomes that contain SUP4-o; our experimental strain contains this particular arrangement. Y383, however, has both FS2 and SUP4-o on the same chromosome arm. For gene conversion to be identified by the experimental system implemented, the GC must have knocked out SUP4-o to have the red color, and secored colony formation develop.*

An initial screening was done by colony PCR and restriction enzyme digest to differentiate the sectored colonies that resulted from LOH events near the *SUP4-o* locus from those associated with a point mutation at the *SUP4-o* locus; the reason being that only the sectored colonies from LOH events were pertinent to the topic of this study. The primers used for the initial PCR screening depended on the strain (Y383 or AMC310). For AMC 310 three primers were used: the forward primer, AMC396, and the two

reverse primers, AMC275 and AMC397 (Table 2 and Figure 11). The presence of the YJM chromosome and SGD chromosome were differentiated through the band patterns that resulted from the three primers (AMC275, AMC 396, and AMC397) had an approximately ~350bp product and a ~300bp product, respectively. If there is only a point mutation that inactivates *SUP4-o,* the initial screening will reveal a heterozygous product. However, if LOH has occurred, then there would be a homozygous result, i.e., two of the same bands, which would appear as one band on the agarose gel as the base pair length is the same. The initial genotyping PCR for the Y383 strain was done with primers AMC353 and AMC354 (Table 2), which amplify a SNP located near *SUP4-o.* The YJM and SGD chromosome had different cut sites to ensure accurate and interpretable results that differed on the chromosome and chromosome site that was cut by a given restriction enzyme.

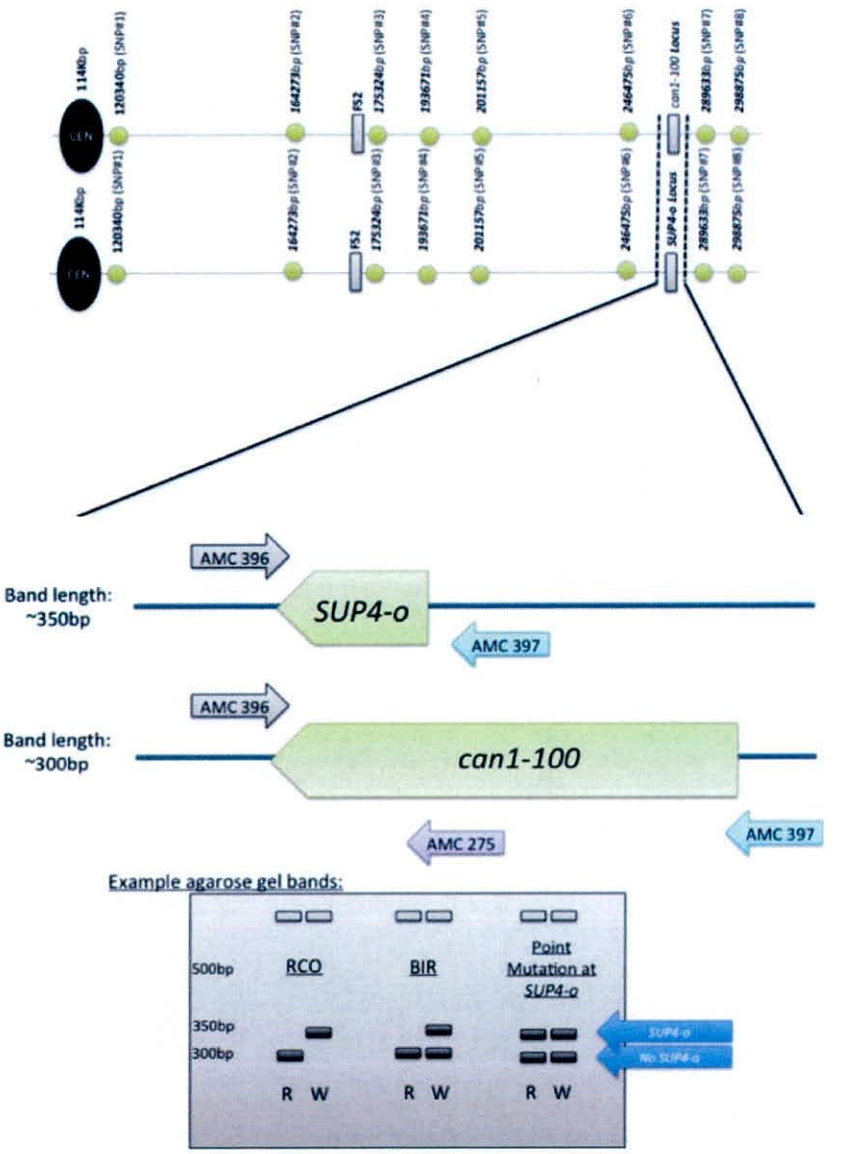


Figure 11: Initial screening for LOH induced sectored colonies.
The chromosome map in the upper portion provides orientation for the location of this PCR reaction. In the presence of SUP4-o the band size is ~350bp as shown above from forward primer AMC396 and reverse primer AMC 397. However, in the absence of the SUP4-o gene there is a ~300bp product that results from the forward AMC396 primer and the reverse AMC 275 primer; this amplified region contains the can1-100 gene which is homologous to SUP4-o, but is not relevant to this study. The reverse primer AMC 275 then produces the smaller product with the AMC 396. The lower half of the figure shows an example agarose gel layout for the PCR product that would result. The colors of the sectored colony halves purified for DNA are indicated as red (R) or white (W) below the example agarose gel band patterns.

After the initial screening, all sectored colonies with loss of heterozygosity at the *SUP4-o* locus were further analyzed. Eight SNPs along the right arm of yeast chromosome III were amplified and digested with the appropriate diagnostic restriction enzyme (Table 3). The 0.5% sequence divergence present between the two homologs allowed the use of SNP analysis. Specific restriction endonucleases (Table 3) were used in reactions with their corresponding PCR products to isolate the cut site present for any of the eight SNPs tested. The amplified PCR products contained the enzyme cut site on only one of the two homologs. The PCR product size would then vary based on the homologs present. Recall that the cut and or uncut DNA segments were separated through the use of agarose gel electrophoresis. The relative band sizes could then be visualized in the agarose gel. Band patterns observed through agarose gel electrophoresis indicated if genetic information from the YJM homolog, SGD homolog, or both, were present at each SNP. See Figure 12 for an example of how this SNP analysis can be used to construct chromosome maps of the events observed.

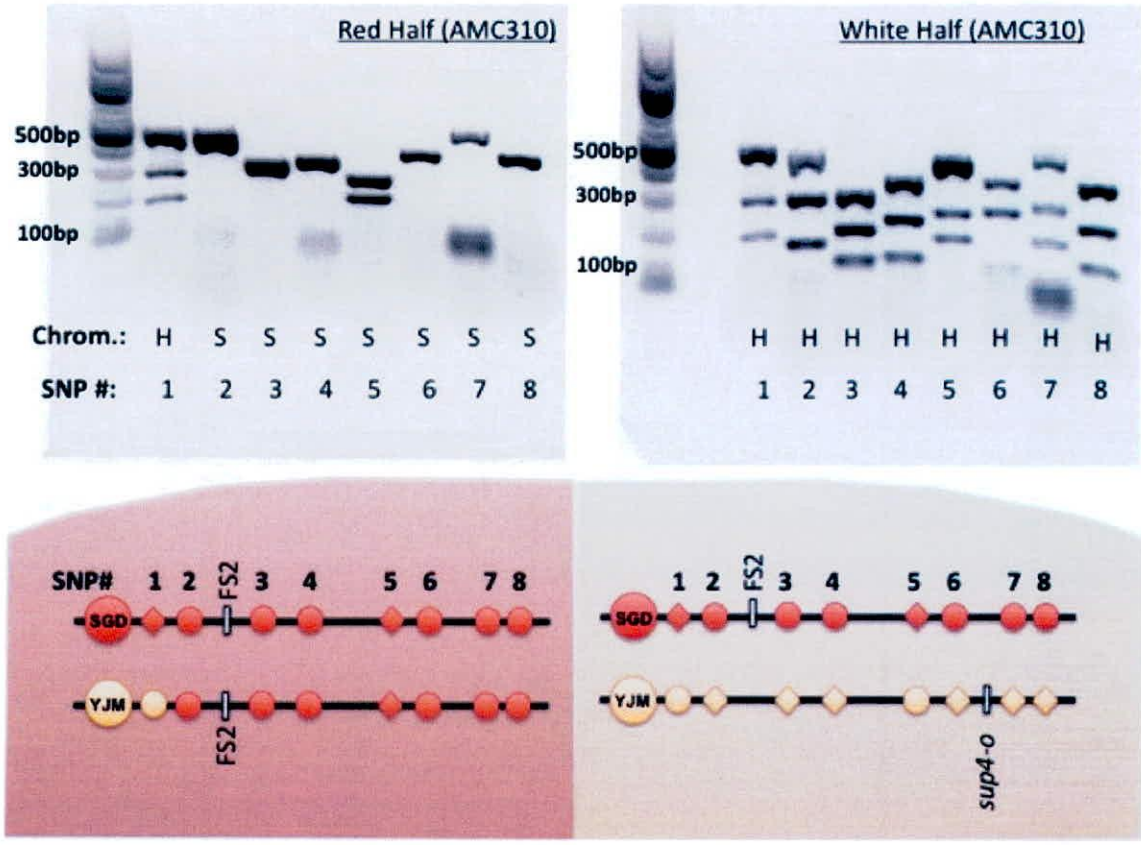


Figure 12: Agarose Gels for SNP Analysis Example
Two agarose gel images are pictured above with corresponding chromosome maps below of a break induced replication (BIR) event. In the example BIR event shown here, repair was initiated between SNP1 and SNP2. Note that the gene, SUP4-o is only present in the cells from the white side of the sectored colony. The letter "H" indicates the banding patterns were that of a heterozygote; "S" and "Y" indicate that the SGD and YJM chromosomes were present, respectively. The diamond shaped markers along the SGD and YJM chromosomes represent the chromosome with the cut site of the restriction enzyme for that SNP. The circles represent the homologous sequence of the SNP (with a polymorphism that eliminates the restriction enzyme cut site). Present on each intact SGD chromosome was FS2. The exact location of each SNP, PCR primers for each SNP, and diagnostic restriction enzyme for each SNP are reported in Table 3.

Chapter 3: Results

3.1 Replication stress leads to a higher frequency of mitotic recombination on yeast chromosome III

There were 34,546 total colonies screened during this study, of those, the number of LOH events that caused sectoring for Y383 HG treatments was highest in YPD treatments with19 events; of the AMC 310 treatments the YPD treatments also produced the most LOH events (Table 4).

The frequency of sectored colonies in cells under replication stress (low galactose) was compared to the frequency of sectored colonies in un-stressed cells (high galactose) for both the experimental and the control strains (Table 5). Recall that the experimental strain has an intact fragile region and the control strain has an interrupted fragile region. A region of homology for homologous recombination is present on the other chromosome that does not contain FS2. Both strains have the galactose inducible system but only AMC310 has an active fragile site (FS2).

We observed that both the control and the experimental strains had a greater frequency of sectored colonies resulting from loss of heterozygosity (LOH) events when grown in low galactose concentrations ($p = 0.0201$ and $p < 0.001$ for Y383 and AMC310, respectively, by chi-square contingency table). Statistical analyses were done using Vassar Stats (http://vassarstats.net). Chromosome loss was included in this comparison, despite not being a type of mitotic recombination, because it was an event that led to LOH. The frequency of sectored colonies resulting from LOH events was calculated by dividing the respective LOH event number by the total number of colonies screened for the given treatment.

Strain	Treatment	Total # Colonies Plated	No Change	Sectored colonies resulting from LOH events	RCO	BIR	Chrom. Loss (CL)
Control (Y383)	HG	9121	9117	4	0	2	2
	NG	7573	7557	19	0	9	10
Experimental (AMC 310)	HG	10664	10656	8	0	5	3
	NG	7188	7157	31	2	21	8

Table 4: Event Numbers per Treatment for AMC310 and Y383.
The above table shows the total number of colonies per media treatment as well as the numerical breakdown of events per event type. The numbers of colonies without an observable event, by our screening mechanism, were listed in the "No Change" category. HG represents the high galactose concentration (YPR+HG) and NG represents the low galactose (YPD) medium. The media composition can be found in Table 1.

We next compared the respective event ratios among the three main event types studied (RCO, BIR, and CL) in both Y383 and AMC 310 cells. Another chi-square analysis was performed to test the statistical significance of the null hypothesis, which stated; cells exposed to replication stress (no galactose) experience a similar ratio of RCO, BIR, and chromosome loss events as cells not exposed to stress (high galactose). The null hypothesis could not be rejected, as $p = 0.9003$ and $p = 0.6538$, respectively for Y383 and AMC310, by chi-square contingency table). Therefore, although a state of replication stress increases the total frequency of LOH events resulting in red-white sectoring in both strains Y383 and AMC310, there was no change in the relative ratio of LOH event types between stressed and non-stressed conditions.

3.2 The presence of FS2 leads to an increase in recombination events under replication stress conditions.

The experimental strain, AMC310, contains fragile site FS2, while the control strain, Y383, is not fragile at this site. Under non-stress conditions (high galactose), we

observed a 1.71-fold increase in the frequency of LOH events causing sectoring in the experimental strain (AMC 310) relative to the control strain (Y383) (Table 5). We evaluated this difference statistically by a chi-square contingency table, with this null hypothesis: Under high galactose conditions, Y383 and AMC310 have the same frequency of LOH events on chromosome III. The null hypothesis could not be rejected, as the p value was 0.8158; this indicates that AMC 310 and Y383 did not have a different frequency of breaks in HG conditions. Low galactose induced a state of replication stress, which was responded to by the active fragile site in AMC310 (FS2). There was an event fold increase of 9.83 when compared to the HG treatment of the control strain, as opposed to Y383, which had a 5.72-fold increase in event frequency. To evaluate this difference, we tested this null hypothesis in our chi-squared analysis: Y383 exposed to LG have the same frequency of events as AMC310 exposed to low gal. This null hypothesis was rejected ($p = 0.0039$, by chi-square contingency table), indicating that the greater frequency of LOH events in AMC310 under replication stress is statistically significant. Both the YPR+HG and YPD media appeared to have roughly doubled their event frequencies when the AMC310 and Y383 fold changes were compared for a given treatment (Table 5).

Strain	Treatment	Total frequency of LOH events causing sectoring	Fold change in total event frequency
Control (Y383)	HG	4.385E-04	1.00
	LG	2.509E-03	5.72
Experimental (AMC 310)	HG	7.502E-04	1.71
	LG	4.313E-03	9.83

Table 5: Total Event Numbers with Event Fold Changes.
The total frequency of sectored colonies in each strain for each condition is shown along with the fold changes, using high galactose (HG) treatment of Y383 as a reference. YPR with a high galactose concentration is abbreviated "HG" and YPD is shown as low galactose (LG).

The frequency of each type of event leading to sectoring (RCO, BIR, and CL) is reported in Table 6. Frequencies for BIR and chromosome loss were calculated by dividing the total number of sectored colonies for a given event type by the total number of events screened. The frequency of RCO was calculated by dividing the total number of sectored colonies for that event type by the total number of events screened, then multiplying by two to account for the RCO events that are undetected due to segregation (refer to Figure 8). Chromosome loss occurred at a frequency of 2.19×10^{-4} events per colony in the control strain under non-stress conditions; the greatest frequency of chromosome loss was found in the treatment under replication stress, the experimental strain, with a frequency of 2.92×10^{-3} events per colony. For the frequencies of BIR, the lowest was in the control strain grown under conditions of non-replication stress with a frequency of 2.19×10^{-4} and the highest was from the experimental strain grown under replication stress with a frequency of 2.92×10^{-3} events per colony. The only RCOs observed were found in the replication stress treatments of the experimental strain with an event frequency of 2.78×10^{-4}.

Strain	Treatment	Total frequency of RCO events causing sectoring	Total frequency of BIR events causing sectoring	Total frequency of CL events causing sectoring
Control (Y383)	HG	0	2.19E-04	2.19E-04
	LG	0	1.19E-03	1.32E-03
Experimental (AMC 310)	HG	0	4.69E-04	2.81E-04
	LG	2.78E-04	2.92E-03	1.11E-03
Strain	Treatment	Fold change in RCO event frequency	Fold change in BIR event frequency	Fold change in CL event frequency
Control (Y383)	HG	1	1	1
	LG	1	5.4	6.0
Experimental (AMC 310)	HG	1	2.1	1.3
	LG	1	13.3	5.1

Table 6: Event Frequencies for AMC310 and Y383.
The frequencies for reciprocal cross over (RCO), break induced replication (BIR), and chromosome loss (CL) events are shown above. As explained in Figure 8, the RCO event frequency was multiplied by two, to account for the 50% of events that went undetected. All others were calculated by dividing the events of the respective type observed by the total colony count for the strain and treatment in question.

3.3 Location of mitotic recombination events on chromosome III

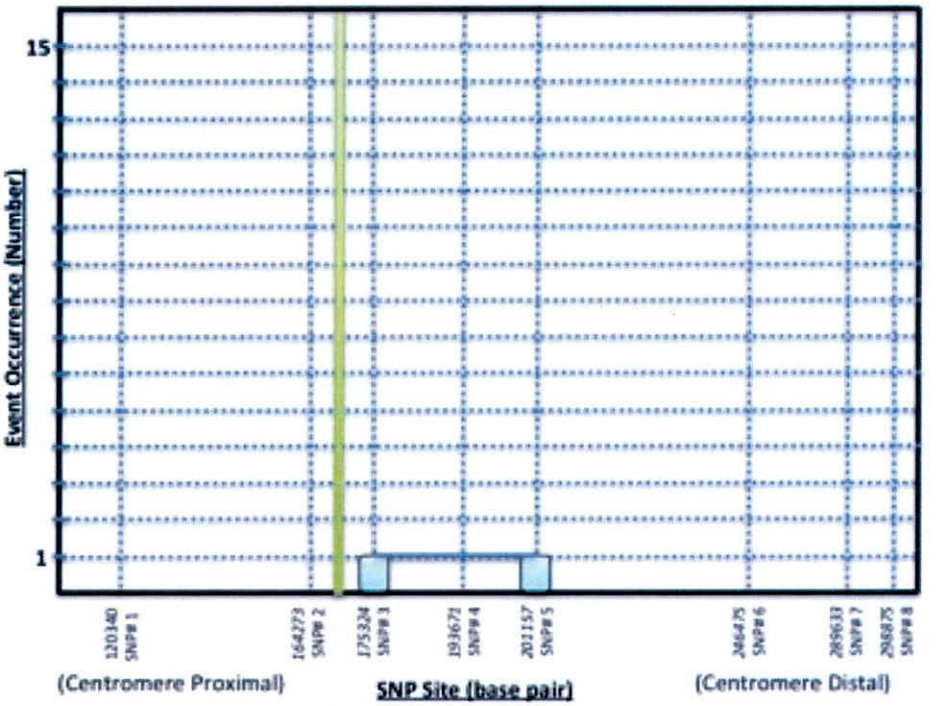
Eight points along chromosome III of *S. cerevisiae* were examined through SNP testing, as described in Figure 12. Testing this panel of SNPs allows us to determine what type of event has occurred (BIR, RCO, or chromosome loss), and in the case of BIR or RCO, it allows us to determine where the recombination event was initiated. In Figure 13 and Table 7, the results of the SNP testing are summarized. The experimental strain under replication stress (Low Galactose concentration) presented the highest concentration of recombination events initiated near the fragile region (represented as the

vertical green bar in Figure 13) SNPs #s 2 and 3 flank FS2 and they are at 164273bp and 175324bp, respectively; FS2 spans a distance of ~12.28 kb and is located in-between SNPs #2 and #3 (Rosen et al., 2013). Also present in the experimental strain under stressed conditions (Low Galactose concentration) was an accumulation of events 5' of SNP#6. Six total recombination events appeared to occur for AMC 310 in the LG treatment at SNP #6, which represented 26% of the total recombination events in the AMC 310 LG treatment. SNP 6 is located on chromosome III at base 246475. The buildup of events at this location is discussed in more detail in the Discussion section. Y383 LG had nine recombination events near the fragile region and Y383 HG had two events total, however, only one was close in proximity to the fragile site.

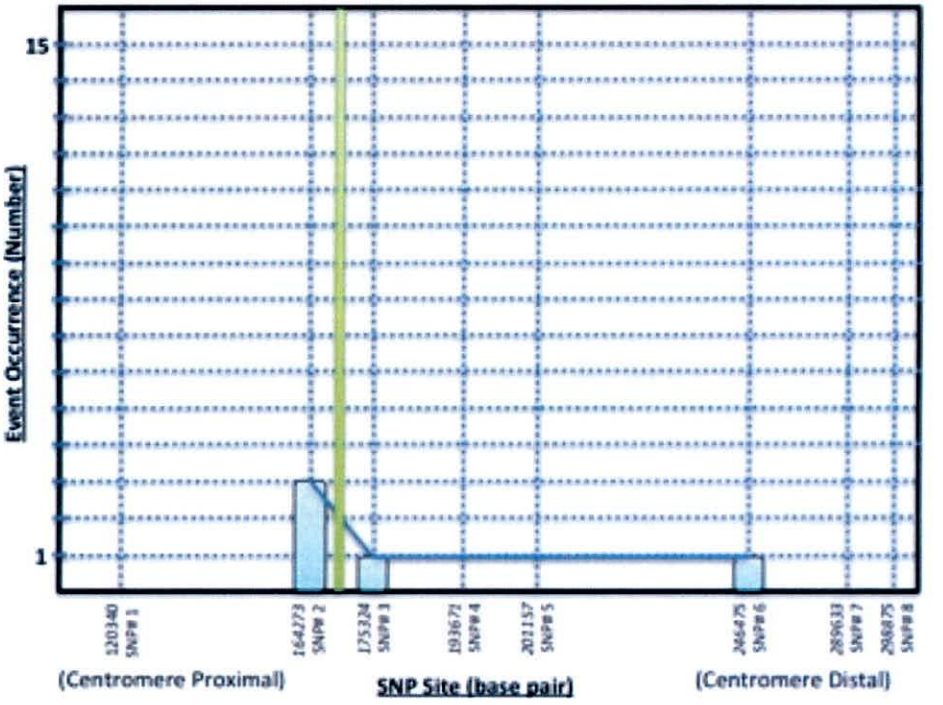
SNP Interval	Distance between SNPs (kb)	Events per kb, Y383 HG	Events per kb, Y383 LG	Events per kb, AMC310 HG	Events per kb, AMC310 LG
1 to 2	43933	0	9.10E-05	6.83E-05	0.137
2 to 3	11051	9.05E-05	4.52E-04	9.05E-05	0.909
3 to 4	18347	0	0	0	0
4 to 5	7486	1.34E-04	0	0	0.133
5 to 6	45318	0	0	2.21E-05	0.132
6 to 7	43158	0	0	0	0
7 to 8	9242	0	0	0	0
Events per kb averaged across entire arm of chr III:		3.20E-05	7.76E-05	2.58E-05	0.129

Table 7: Initiation of BIR and RCO Events per Interval on chromosome III
The number of events per kilobase pairs were examined to add clarity to the accumulation of events at the 6th SNP of AMC 310 low galactose (LG). The number of events in the area was divided by the inter-SNP base pair length. Note, the "2 to 3" SNP interval is the one that houses FS2.

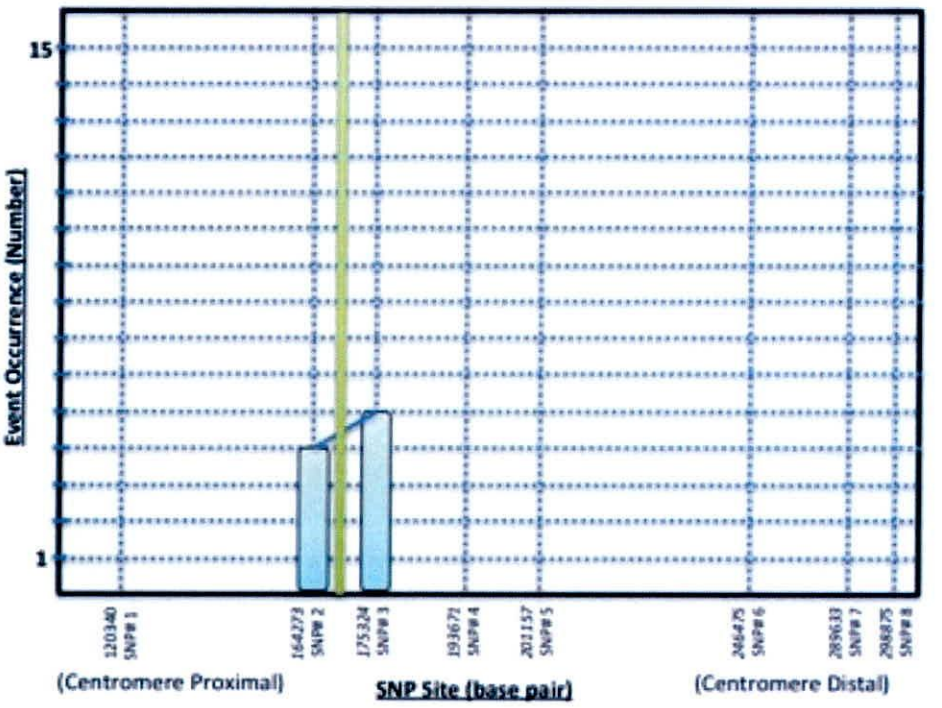
Y383 High [Galactose]



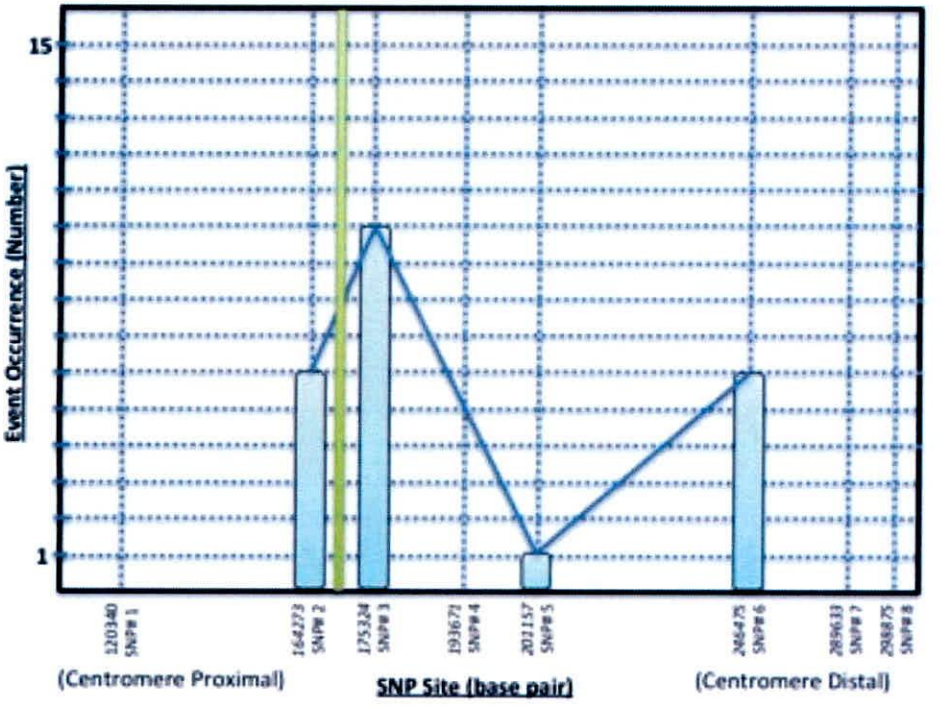
AMC310 High [Galactose]



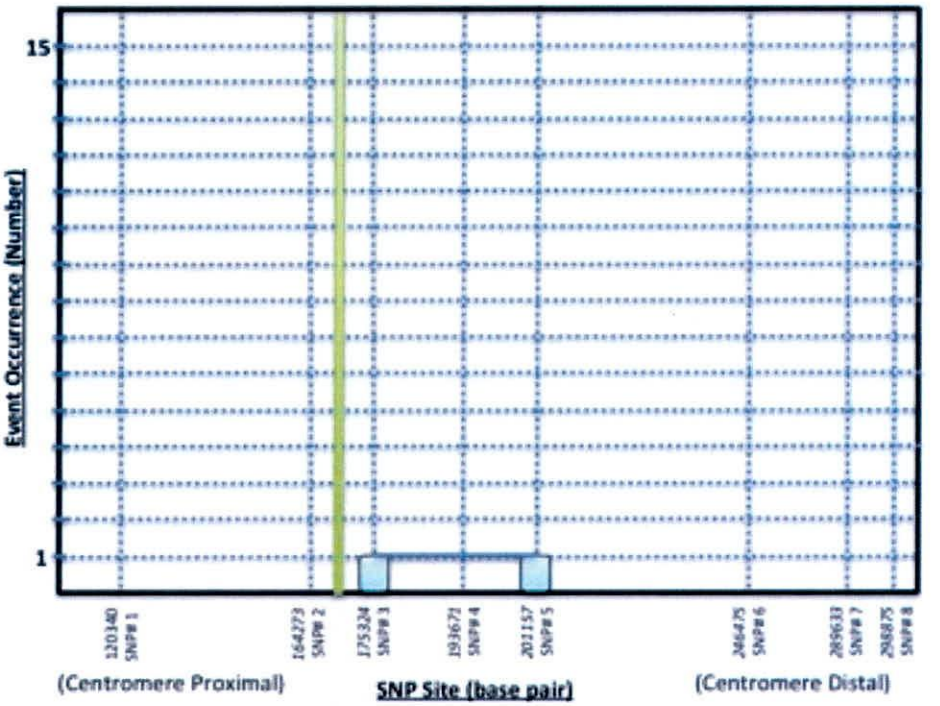
Y383 Low [Galactose]



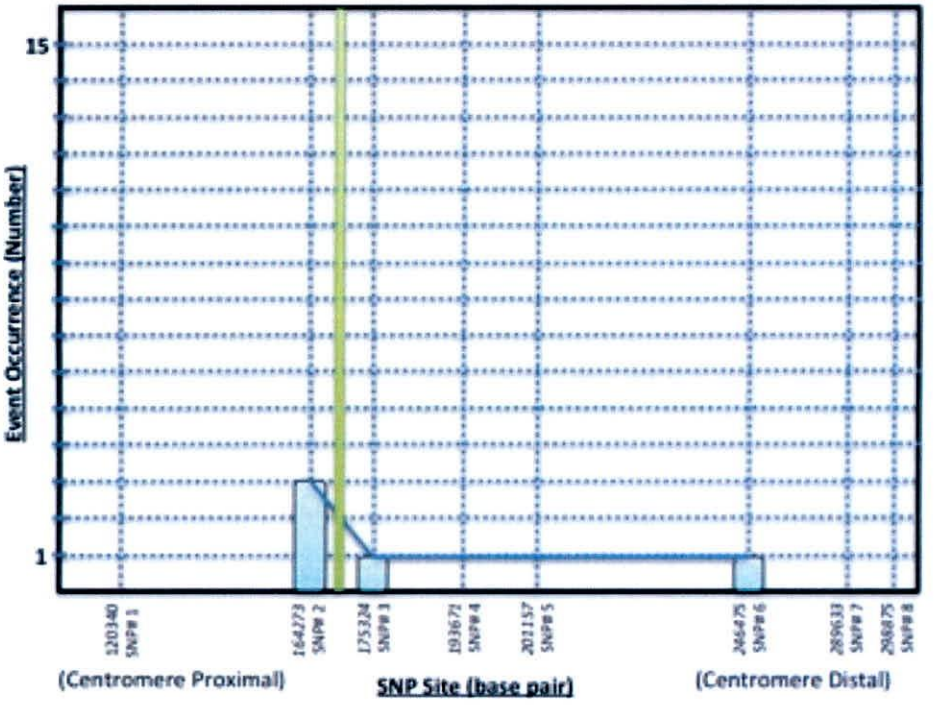
AMC310 Low [Galactose]



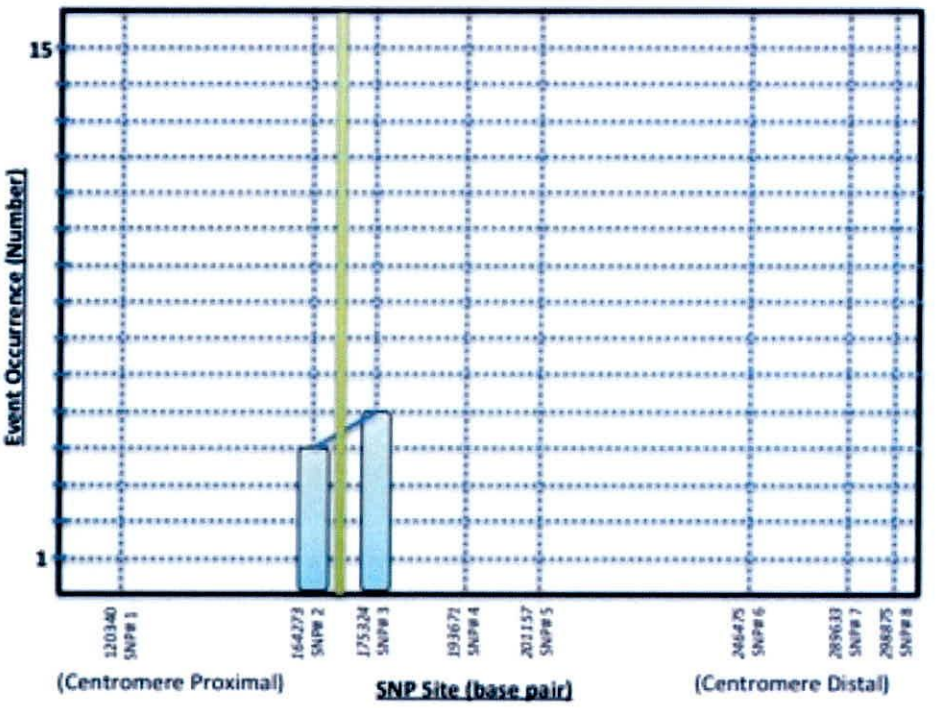
Y383 High [Galactose]



AMC310 High [Galactose]



Y383 Low [Galactose]



AMC310 Low [Galactose]

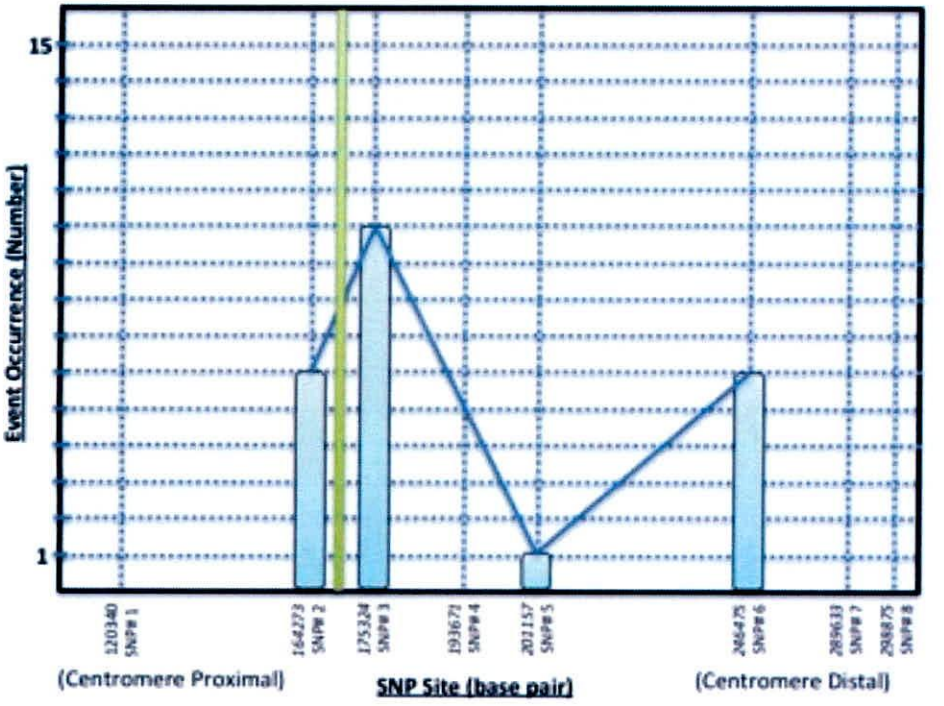


Figure 13: Analysis of the location of BIR and RCO events: Events per SNP Site. *The blue bars represent the number of events observed at each SNP site for Y383 or AMC310 in either low or high [galactose] conditions. The green line represents the location of FS on chromosome III. Also, note that the SUP4-o locus is located in between SNP #6 (246475) and SNP #7 (289633), approximately 159kb centromere distal (Rosen et al., 2013)*

Chapter 4: Discussion and Conclusion

4.1 Analysis of Homologous Recombination Data

A. Analysis of LOH event frequencies

We observed an increase in the frequency of homologous recombination events on yeast chromosome III as a result of replication stress. The galactose inducibility of the system served as a general model for a number of possible scenarios where the cell was experiencing less than ideal conditions for replication; as has been discussed previously, the state of replication stress is often a characteristic of early stage cancer cells (Arlt et al., 2006). The recombination event frequencies can be found in Table 6. The only full RCOs observed occurred in the experimental strain (AMC310) under LG growth conditions. The experimental strain contained the intact fragile site, FS2. Growth in LG media provided higher levels of replication stress than HG, per the *GAL-POL1* genetic system present in the yeast strains used. The control strain (Y383), grown in HG media, had the lowest frequency of events. Our hypothesis was supported, DNA breaks at FS2 during mitosis, under conditions of replication stress, appeared to be repaired by homologous recombination. The experimental strain from the LG and HG treatments always had a higher frequency of events than the control strain of the respective treatment; other than for RCOs, which were all absent except for two events in the AMC310 of the LG treatment that was described above (Table 4). Other studies have found higher frequencies of recombination events, particularly cross over events; Yim et

al. (2014) examined much larger portions of the yeast genome and found that roughly 40% of the studied events ended in a cross over. As previously mentioned, Rosen et al. (2013) also found higher frequencies of RCOs. The work of Yim et al. (2014) covered a significant portion of the genome with fragile inverted transposable elements present. Rosen et al. (2013) also used yeast but focused on the yeast chromosome III and FS2 the same fragile site of interest in this study. It seems probable that the sample size was a limiting factor in our study as well as the resolution of the SNP testing done; by increasing the number of points along the chromosome examined through SNP testing one increases the resolution of the data.

The fold changes calculated supported our hypothesis as well and can be found in Table 5. It is important to note that the event fold changes always increased when comparing Y383 to AMC310 or HG to LG (e.g. replication stress and the presence of the intact FS2 both increased the prevalence of homologous recombination during mitosis.) The most significant fold increase occurs when comparing the event numbers of Y383 from the YPR+HG treatment to those of the AMC310 YPD treatment (LG). The Y383 (HG) treatment was the control strain, while given unstressed replicative conditions. It was for this reason that all other strains-treatment combinations where compared to what should be the baseline level of homologous recombination events.

The underlying trends that our data supported were consistent with the work done by Rosen et al (2013). That is to say, replication stress induced mitotic recombination and strains with a fragile site present showed an increase in mitotic recombination activity as well. One difference present was the raw number of total colonies screened and selected for further analysis. Rosen et al., (2013) screened roughly 14,000-30,000

colonies per treatment; the treatments in this study were all in the colony numbers range of the low 7,000s to low 10,000s. It is possible that this study simply did not screen enough colonies per treatment to have a robust yield of RCOs. Rosen et al. (2013) found a frequency of 202 x 10^{-5} for RCOs compared to the 2.78 x 10^{-4} that we observed for the same strain (AMC 310) under stressed conditions. With that being said, given previous studies, it does follow that the RCO numbers would fall in a range below that of the BIR for mitotic recombination. Mitotic recombination is about DNA repair; it follows that mitotic recombination appears to favor RCOs over non-reciprocal crossovers that might leave unresolved DNA damage (Andersen and Sekelsky, 2010).

B. Analysis of recombination event locations

Recall that we hypothesized that DNA breaks at FS2 during mitosis, under conditions of replication stress, will be repaired by homologous recombination. The SNP analyses to determine the location of recombination event initiation further supported our hypothesis. We observed that more events accumulated in the regions flanking the intact FS2 in AMC310. Also present, was an accumulation of events in the LG treatments tested, showing a particularly dense compilation of recombination events in the LG treatment of the experimental strain, AMC310 (7.75 fold increase in events). The LG treatment of Y383 still had a higher frequency of events (4.75 fold increase), than the HG treatment of Y383; however, much less recombination was evident in Y383 (LG) than in AMC310 (LG). While SNP analysis does not show exactly where a break occurred, it can indicate which chromosomes segments are present in a particular region. The information yielded can provide essential information about any potential changes in

heterozygosity. An anomaly of note was found in the accumulation of recombination events present at the 6th SNP (246475bp) of the AMC310 (LG) treatment. As mentioned previously, 26% of the events for the AMC 310 LG treatment were found on SNP #6. With that being said, the probable cause for this apparent aberration in the data is low "resolution" of data points from only eight SNP sites along the chromosome. The raw base pair difference present between the 6th SNP and the preceding SNP was similar to the base pair difference between the 2nd SNP and the 1st SNP; it would be the difference of roughly 45,318 and 43,933, respectively. No such anomalies were found at the 6th SNP of the Rosen et al. (2013) study.

4.2 Implications of a Higher Frequency of Mitotic Recombination

Common fragile sites have been reported as drivers of early cancer development through oncogenic amplification and deletion of tumor suppressors (Tsantoulis et al., 2007). Homologous recombination during mitosis was examined in order to better understand possible mechanisms of fragile site breaks. LOH induced by mitotic recombination events from fragile site instability was the primary area of interest. Homologous recombination events, such as RCOs and BIRs, do lead to intact chromosomal arms. However, this genetic recombination, by copying off of the homologous chromosome, leaves the cell in a state of LOH (Casper et al., 2012). Chromosome loss can occur from a lack of homologous recombination, and as a result, can lead to DNA breaks and LOH as well. The results of our study support previous work that also found homologous recombination to result in cells exposed to a stressed replicative environment (Arlt et al., 2006; Casper et al., 2012; Rosen et al., 2013).

4.3 Conclusion

Studies done on the underlying mechanisms of illness progression are necessary for advancement of both research and our understanding of medical treatments. Cancer is a major cause of human mortality. For more clinical means of examination to progress, there must first be a thorough groundwork laid out that helps us to understand the innate properties of the problem itself. In this study, we examined the nature of homologous recombination and its probable role in the repair of DNA breaks at common fragile sites under replication stress. Our system used the naturally occurring fragile site, FS2, on the right arm of the yeast chromosome III in *S. cerevisiae*. The yeast cells were used as model organisms to examine what has the potential to occur in mammalian somatic cells. More work on the properties of fragile site breaks is warranted, as we do not yet understand the exact cause of DNA breaks in a number of different circumstances. Specific to this study, increasing the number SNP sites tested would help to add additional clarity and increased resolution to the results already obtained. As for the study of fragile sites in general, further investigation into the origins paucity hypothesis could be beneficial, especially to future work examining the overlap of the prevailing hypotheses, such as the origin paucity hypothesis. While epigenetic regulation, and thus, origin activation is not directly tied to the sequence itself, there could be converging characteristics. Perhaps a link between epigenetic tagging and secondary structure formation could be a potential issue in the fragility of DNA fragile regions. Although there are several avenues for future investigation, this study has provided additional support for the role of mitotic recombination, resulting from fragile site instability, as a potential mechanism for cancer development as a result of the LOH that follows.

Bibliography

Andersen, S.L., and Sekelsky, J. (2010). Meiotic versus mitotic recombination: Two different routes for double-strand break repair. BioEssays *32*, 1058–1066.

Arabshahi, L., Brown, N., Khan, N., and Wright, G. (1988). Inhibition of DNA polymerase alpha by aphidicolin derivatives. Nucleic Acids Res. *16*, 5107–5113.

Arlt, M.F., Casper, A.M., and Glover, T.W. (2003). Common fragile sites. Cytogenet. Genome Res. *100*, 92–100.

Arlt, M.F., Durkin, S.G., Ragland, R.L., and Glover, T.W. (2006). Common fragile sites as targets for chromosome rearrangements. DNA Repair *5*, 1126–1135.

Barlow, J., Faryabi, R.B., Callen, E., Wong, N., Malhowski, A., Chen, H.T., Gutierez-Cruz, G., Sun, H.-W., McKinnon, P., Wright, G., et al. (2013). A novel class of early replicating fragile sites that contribute to genome instability in B cell lymphomas. Cell *152*, 620–632.

Bester, A.C., Roniger, M., Oren, Y.S., Im, M.M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D.S., and Kerem, B. (2011). Nucleotide Deficiency Promotes Genomic Instability in Early Stages of Cancer Development. Cell *145*, 435–446.

Bray, F., Ren, J.-S., Masuyer, E., and Ferlay, J. (2013). Global estimates of cancer prevalence for 27 sites in the adult population in 2008. Int. J. Cancer *132*, 1133–1145.

Casper, A.M., Mieczkowski, P.A., Gawel, M., and Petes, T.D. (2008). Low Levels of DNA Polymerase Alpha Induce Mitotic and Meiotic Instability in the Ribosomal DNA Gene Cluster of Saccharomyces cerevisiae. PLoS Genet *4*, e1000105.

Casper, A.M., Greenwell, P.W., Tang, W., and Petes, T.D. (2009). Chromosome Aberrations Resulting From Double-Strand DNA Breaks at a Naturally Occurring Yeast Fragile Site Composed of Inverted Ty Elements Are Independent of Mre11p and Sae2p. Genetics *183*, 423–439.

Casper, A.M., Rosen, D.M., and Rajula, K.D. (2012). Sites of genetic instability in mitosis and cancer. Ann. N. Y. Acad. Sci. *1267*, 24–30.

Durkin, S.G., and Glover, T.W. (2007). Chromosome Fragile Sites. Annu. Rev. Genet. *41*, 169–192.

Feng, W., Rienzi, S.C.D., Raghuraman, M.K., and Brewer, B.J. (2011). Replication Stress-Induced Chromosome Breakage Is Correlated with Replication Fork Progression and Is Preceded by Single-Stranded DNA Formation. G3 Genes Genomes Genet. *1*, 327–335.

Glover, T.W., Berger, C., Coyle, J., and Echo, B. (1984). DNA polymerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. Hum. Genet. *67*, 136–142.

Glover, T.W., Arlt, M.F., Casper, A.M., and Durkin, S.G. (2005). Mechanisms of common fragile site instability. Hum. Mol. Genet. *14*, R197–R205.

Goldstein, A.L., and McCusker, J.H. (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast Chichester Engl. *15*, 1541–1553.

Kobayashi, T., Wang, T., Qian, H., and Brachmann, R.K. (2003). Genetic Strategies in Saccharomyces cerevisiae to Study Human Tumor Suppressor Genes. In Tumor Suppressor Genes, W.S.E.-D. MD, ed. (Humana Press), pp. 73–86.

Lambert, S., Mizuno, K., Blaisonneau, J., Martineau, S., Chanet, R., Fréon, K., Murray, J.M., Carr, A.M., and Baldacci, G. (2010). Homologous Recombination Restarts Blocked Replication Forks at the Expense of Genome Rearrangements by Template Exchange. Mol. Cell *39*, 346–359.

Lemoine, F.J., Degtyareva, N.P., Lobachev, K., and Petes, T.D. (2005). Chromosomal Translocations in Yeast Induced by Low Levels of DNA Polymerase: A Model for Chromosome Fragile Sites. Cell *120*, 587–598.

Letessier, A., Millot, G.A., Koundrioukoff, S., Lachagès, A.-M., Vogt, N., Hansen, R.S., Malfoy, B., Brison, O., and Debatisse, M. (2011). Cell-type-specific replication initiation programs set fragility of the FRA3B fragile site. Nature *470*, 120–123.

Lukusa, T., and Fryns, J.P. (2008). Human chromosome fragility. Biochim. Biophys. Acta BBA - Gene Regul. Mech. *1779*, 3–16.

Mitchel, K., Zhang, H., Welz-Voegele, C., and Jinks-Robertson, S. (2010). Molecular Structures of Crossover and Noncrossover Intermediates during Gap Repair in Yeast: Implications for Recombination. Mol. Cell *38*, 211–222.

Ozeri-Galai, E., Lebofsky, R., Rahat, A., Bester, A.C., Bensimon, A., and Kerem, B. (2011). Failure of Origin Activation in Response to Fork Stalling Leads to Chromosomal Instability at Fragile Sites. Mol. Cell *43*, 122–131.

Ozeri-Galai, E., Bester, A.C., and Kerem, B. (2012). The complex basis underlying common fragile site instability in cancer. Trends Genet. *28*, 295–302.

Pearson, C.E., and Sinden, R.R. (1998). Trinucleotide repeat DNA structures: dynamic mutations from dynamic DNA. Curr. Opin. Struct. Biol. *8*, 321–330.

Roman, H. (1956). A system selective for mutations affecting the synthesis of adenine in yeast. Physiology 299–314.

Ronne, H., and Rothstein, R. (1988). Mitotic sectored colonies: evidence of heteroduplex DNA formation during direct repeat recombination. Proc. Natl. Acad. Sci. *85*, 2696–2700.

Rosen, D.M., Younkin, E.M., Miller, S.D., and Casper, A.M. (2013). Fragile Site Instability in Saccharomyces cerevisiae Causes Loss of Heterozygosity by Mitotic Crossovers and Break-Induced Replication. PLoS Genet *9*, e1003817.

Schwartz, M., Zlotorynski, E., and Kerem, B. (2006). The molecular basis of common and rare fragile sites. Cancer Lett. *232*, 13–26.

Tsantoulis, P.K., Kotsinas, A., Sfikakis, P.P., Evangelou, K., Sideridou, M., Levy, B., Mo, L., Kittas, C., Wu, X.-R., Papavassiliou, A.G., et al. (2007). Oncogene-induced replication stress preferentially targets common fragile sites in preneoplastic lesions. A genome-wide study. Oncogene *27*, 3256–3264.

Walsh, E., Wang, X., Lee, M.Y., and Eckert, K.A. (2013). Mechanism of replicative DNA polymerase delta pausing and a potential role for DNA polymerase kappa in common fragile site replication. J. Mol. Biol. *425*, 232–243.

Wang, Y.-H. (2006). Chromatin structure of human chromosomal fragile sites. Cancer Lett. *232*, 70–78.

Ward, A.C. (1992). Rapid analysis of yeast transformants using colony-PCR. BioTechniques *13*, 350.

Wei, W., McCusker, J.H., Hyman, R.W., Jones, T., Ning, Y., Cao, Z., Gu, Z., Bruno, D., Miranda, M., Nguyen, M., et al. (2007a). Genome sequencing and comparative analysis of Saccharomyces cerevisiae strain YJM789. Proc. Natl. Acad. Sci. U. S. A. *104*, 12825–12830.

Wei, W., McCusker, J.H., Hyman, R.W., Jones, T., Ning, Y., Cao, Z., Gu, Z., Bruno, D., Miranda, M., Nguyen, M., et al. (2007b). Genome sequencing and comparative analysis of Saccharomyces cerevisiae strain YJM789. Proc. Natl. Acad. Sci. U. S. A. *104*, 12825–12830.

Yim, E., O'Connell, K.E., Charles, J.S., and Petes, T.D. (2014). High-Resolution Mapping of Two Types of Spontaneous Mitotic Gene Conversion Events in Saccharomyces cerevisiae. Genetics *198*, 181–192.

Zhang, H., and Freudenreich, C.H. (2007). An AT-Rich Sequence in Human Common Fragile Site FRA16D Causes Fork Stalling and Chromosome Breakage in S. cerevisiae. Mol. Cell *27*, 367–379.

Zimmermann, F.K., Kern, R., and Rasenberger, H. (1975). A yeast strain for simultaneous detection of induced mitotic crossing over, mitotic gene conversion and reverse mutation. Mutat. Res. Mol. Mech. Mutagen. *28*, 381–388.