INVESTIGATION OF CHROMOSOME SIZE EFFECT ON THE RATE OF
CROSSOVERS IN THE MEIOTIC YEAST *Saccharomyces cerevisiae*

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ABSTRACT

Investigation of chromosome size effect on the rate of crossovers in the meiotic yeast *Saccharomyces cerevisiae*

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Meiosis is a specialized type of cell division characterized by a single round of DNA replication and two rounds of chromosome segregation, ultimately resulting in four haploid cells. During meiosis I, chromosomes align and reciprocal recombination results in the formation of a crossover, creating the tension required to properly segregate homologs during the first round of meiosis.

Two mechanisms involved in regulating the occurrence of crossing over are assurance and interference. Crossover assurance describes the phenomenon that at least one crossover will form between each pair of homologous chromosomes during prophase I. Crossover interference, on the other hand, describes the nonrandom placement of crossovers between homologs, increasing the probability that a second crossover will occur at a discrete distance away from the first one.

In addition to assurance and interference, chromosome size may play a role in the rate of meiotic recombination during prophase I. As a result of crossover assurance, small chromosomes receive a minimum of one crossover, the obligate crossover. Assuming chromosome size does not influence the rate of recombination, pairs of large chromosomes should experience the same number of crossovers per base pair as small chromosomes. Previous studies have been inconsistent: Kaback et al. (1999) saw decreased rates of crossing over between large chromosomes relative to small ones, suggesting that crossover interference acts across a larger distance on large chromosomes. Turney et al. (2004), however, saw no such effect, suggesting that these findings may be site- or sequence-specific.

The current study used the *Cre-loxP* system to create translocated chromosomes, decreasing the size of chromosome VIII from 562 kb to 125 kb. The rate of crossing over was evaluated using nutrient marker genes that were inserted on the left arm of chromosome VIII to facilitate phenotypic detection of crossing over between homologous translocated chromosomes in comparison to crossing over between homologous nontranslocated chromosomes.

Translocated strains were attempted, though further testing suggests that the translocation itself may be lethal. In the future, we plan to further investigate the potential lethal nature of the translocation.

We also experienced difficulty in curing yeast cells of the Cre expression plasmid: as pSH47 was removed, translocated chromosomes reverted to nontranslocated chromosomes. In addition, crossing over in nontranslocated yeast, along with subsequent molecular analysis, revealed that one of the marker genes presumed to be on the left arm of chromosome VIII is, in fact, located on a different chromosome, preventing analysis of crossing over in this region. As a result, we were unable to proceed with current experimentation.

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INTRODUCTION

Mitotic cycle

The cell cycle is divided into phases consisting of G1, S, G2, and M. The majority of a cell's life is spent in G1 phase, during which the cell grows and matures while its constituent proteins and organelles both replicate and carry out programmed biosynthetic functions. Upon reaching maturity, the cell enters S phase, characterized by the beginning of DNA replication. Chromosomes are duplicated and connected at the centromere, thus maintaining the cell's ploidy, or the number of sets of chromosomes. The cell then enters a second growth phase (G2), followed by mitosis (M). During mitosis, cell growth ceases as energy is directed towards the proper segregation of sister chromatids into two identical daughter cells (Figure 1). First, prophase marks the condensation of DNA, where sister chromatids remain attached at the centromere (Hartwell 1974). During metaphase, homologs align with one another, with spindle fibers attached at the kinetochore. Next is anaphase, marking the separation of identical sister chromatids by spindle fibers, pulling them towards opposite poles of the developing cells. Finally, cytokinesis describes the physical division of the parent cell into the two daughter cells. Two identical daughter cells are formed from a single round of mitosis, and ploidy is maintained.

Figure 1: Mitosis and meiosis. During mitosis, a single round of cell division results in two genetically identical daughter cells ($2n=2$). Sister chromatids are separated at the centromere, pulling chromatids into two identical daughter cells. Meiosis I is characterized by genetic recombination and subsequent separation of homologs. During meiosis II, sister chromatids are pulled to opposite spindle poles, ultimately resulting in four haploid daughter cells. (Cnudde and Gerats 2005)

Meiosis

Meiosis describes the process by which four haploid cells are formed from a single parental diploid cell, resulting from a single round of DNA replication followed by two rounds of chromosome segregation (Figure 1). This process is essential in sexually reproducing eukaryotes and results in genetically distinct haploid daughter cells, each having the opportunity to join with another unique haploid cell, forming diploid progeny. Furthermore, meiosis is highly conserved among eukaryotes, and it ensures genetic variation within a population.

Following pre-meiotic S-phase, cells begin two rounds of chromosome segregation: meiosis I and meiosis II (Figure 1). Each round is further divided into stages, including prophase, metaphase, anaphase, and telophase. During prophase I, homologous chromosomes pair together, making physical contact and forming crossovers as spindle fibers begin to form. Metaphase I is characterized by the aligning of homologous chromosomes. Next, anaphase I pulls apart homologous chromosomes, each consisting of two potentially nonidentical sister chromatids, to each of the spindle poles. Telophase I and cytokinesis result in two haploid daughter cells.

Meiosis II is the mitosis-like round of segregation of sister chromatids. Just as in mitosis, chromosomes condense and align, followed by the separation of sister chromatids. The completion of meiosis I and II results in four unique haploid cells.

Crossing over

Genetic variation among daughter cells is achieved as homologous chromosomes pair during prophase I. Leptotene, or the first of the five stages of prophase I, is marked

by the condensation of chromosomes in the nucleus (Figure 2). Homologous chromosomes, each consisting of two identical sister chromatids, pair together. The zygotene phase is characterized by the partial synapsis of homologous chromosomes; chromatin condenses around proteinaceous axial elements, which subsequently align corresponding homologs. Transverse elements connect the two axial elements, which are then referred to as lateral elements. This proteinaceous structure, called the synaptonemal complex, separates homologous chromosomes by approximately 200 nm (Westergaard and von Wettstein 1968); while it aligns homologs, it does not bring them into physical contact (Figure 3).

Figure 2: Prophase I. Crossing over occurs during prophase I ($2n=2$). See text for a detailed description of each stage. (Page and Hawley 2003)

A.)

B.)

Figure 3: Synaptonemal complex. Lateral elements (LE, green and blue) are pulled together by transverse filaments (orange and yellow) spanning the central region (CE), bringing homologs into close contact (Part A, Page and Hawley 2004). Part B shows an image of the synaptonemal complex (Westergaard and von Wettstein 1968).

Pachytene is characterized by the full synapsis of homologous chromosomes.

Following the formation of the synaptonemal complex, a sister chromatid experiences a site-targeted double-strand break (DSB) induced by the enzyme Spo11, a topoisomerase-

like enzyme (Keeney et al. 1997). DSBs occur nonrandomly and are often seen at “hotspots,” or site-specific regions of high levels of recombination (Wu and Lichten 1995; Blitzblau et al. 2007). Next, proteins Mre11, Rad50, and Xrs2 bind together to form the appropriately named MRX complex (Ajimura et al. 1992; Alani et al. 1990; Ivanov et al. 1994). The MRX complex, along with Sae2, degrades the 5’ ends of the broken strands, while the 3’ ends are protected by Replication Protein A (Wold 1997) and subsequently Dmc1 and Rad51 recombinase. Next, strand invasion occurs, describing the invasion of a broken 3’ overhang into the homologous region of the intact double strand DNA on the intact nonsister chromatid (Figure 4). The single invading 3’ end creates an intermediate displacement loop (D-loop), eventually resulting in either a crossover or a noncrossover.

Repair of a DSB without crossover formation is referred to as synthesis-dependent strand annealing (SDSA). In this event, the invading 3’ end uses the homologous region in the intact, invaded nonsister chromatid as a template for repair; the noninvading 3’ strand uses its complementary strand as a template for repair. This results in gene conversion, yielding only one altered chromatid, and three unaltered chromatids. Approximately two thirds of all DSBs in yeast are repaired without crossover.

At some DSB sites, an intermediate known as a double Holliday junction (dHJ) forms, often resulting in the formation of a crossover, or the physical exchange of a region of one homolog for the same region of the other homolog. As in SDSA, the 3’ end invades the homologous chromosome. The noninvading 3’ end, however, uses the intact, invaded homologous chromatid as a template for repair. Furthermore, the strands involved in the crossover physically exchange chromosome arms as endonucleases cut at

a Holliday junction. Should homologs contain opposing allelic variations, this swap will eventually result in four new, genetically distinguishable chromatids.

Pachytene concludes as crossing over is completed. Diplotene is marked by the dissolution of the synaptonemal complex; chromosomes appear to repel one another, though all four chromatids remain physically connected by crossovers (now termed chiasmata) and sister chromatid cohesion. Meiotic spindle fibers form, and tension created at the chiasmata in conjunction with the dissolution of cohesins connecting chromosome arms facilitates the pulling of homologous chromosomes to opposite poles of the developing daughter cells in order for meiosis II to proceed.

Figure 4: DSB repair. Following a DSB, 5'-3' resection leads to single strand invasion of the broken strand into the intact homologous chromosome strand. DSBs are repaired by synthesis-dependent strand annealing (SDSA), resulting in a noncrossover, or by the formation of a double Holliday junction, resulting in a crossover (double-strand break recombination, or DSBR). (Bugreev et al. 2011)

Nondisjunction and aneuploidy

If crossing over does not occur at least once along the length of homologous chromosomes, implying an error during recombination, no tension is created between spindle poles. As a result, the homologs frequently do not segregate properly during prophase I, a process called nondisjunction (Figure 5). Errors in chromosome segregation result in aneuploidy, the genetic defect in which a cell contains too many or too few copies of a chromosome, resulting in severe detrimental effects including cell death.

Figure 5: Nondisjunction and aneuploidy. The top shows proper disjunction of homologous chromosomes following the formation of a crossover during meiosis I, resulting in four viable, haploid cells ($2n=2$). When crossing over fails to occur, the tension required to separate homologs is absent, resulting in improper segregation and aneuploidy (bottom).

In humans, approximately 25% of aneuploid zygotes do not implant within the female, and of those that do, most are miscarried before pregnancy is detected (Hassold and Hunt 2001). Of the aneuploid zygotes that survive to birth, offspring cells contain either three copies of a chromosome, trisomy, such as in Down syndrome (three copies of

chromosome 21), or a single copy of a chromosome, monosomy, as seen in Turner syndrome (a single sex chromosome resulting in the genotype X,O). In these survivors, severe detrimental physiological effects manifest.

To ensure survival, cells employ multiple mechanisms to regulate the segregation of homologous chromosomes during meiosis I. The common baker's yeast *Saccharomyces cerevisiae* provides the best model to study the process of meiotic recombination as the species proceeds through meiosis in a manner similar to human cells. Past research has uncovered several mechanisms by which the crossover event is regulated in yeast.

Crossover assurance

Crossover assurance refers to the phenomenon ensuring that at least one genetic exchange event will occur between homologous chromosomes (Figure 6). This provides a guarantee that small pairs of chromosomes will form at least one crossover. This obligate crossover facilitates the creation of the tension at chiasmata required for proper homolog segregation.

Figure 6: Crossover assurance. Crossover assurance ensures the formation of at least one crossover per homologous pair of chromosomes. Part A shows a lack of crossover assurance, resulting in all three crossovers placed randomly between the larger chromosomes while the smaller chromosomes lack a crossover. Part B, on the other hand, shows that even small chromosomes will experience at least one crossover termed the obligate crossover.

Interestingly, yeast with non-null mutations of *spo11* (resulting in reduced, non-zero levels of expression) do not suffer from decreased crossing over, even though the overall number of DSBs is decreased (Martini et al. 2006). Instead, cells experience crossover homeostasis in which the number of crossovers is maintained, while the number of noncrossovers is decreased. Furthermore, using *Caenorhabditis elegans*, Rosu et al. (2011) showed that crossover formation is the heavily favored result of a single DSB along the length of homologs.

Crossover interference

While not fully understood, crossover assurance appears to be closely associated with another regulatory mechanism called crossover interference, describing the non-random distribution of crossovers along the length of homologous chromosomes (Figure 7) (Muller 1916; Hillers and Villeneuve 2003). For example, if these genetic exchanges occurred randomly along the arm of the chromosome, they might be seen spatially near to one another; crossover interference results in a low probability that a second crossover will form close to the first one. Although multiple DSBs are formed, only a subset results in crossover formation. Furthermore, multiple crossovers tend to be both evenly distributed and spatially distant from one another.

Figure 7: Crossover interference. If crossovers occurred randomly between homologous chromosomes, they might form close to one another (Part A). Crossover interference describes the probability that a second crossover will occur far down the length of the chromosomes from the first one (Part B).

The effect of chromosome size on crossing over

In addition to crossover assurance and interference, chromosome size may also influence the rate of crossing over between homologs (Figure 8), but results are inconsistent. Assuming equal rates of crossing over, homologous chromosomes of 100 kb will experience only a single crossover, for example, while homologous chromosomes of

1,000 kb will experience ten crossovers. This result would indicate that crossing over occurs at a rate of one per 100 kb, supporting that the rate is independent of chromosome size. This is not observed in natural chromosomes, though studies have been inconsistent.

Figure 8: Chromosome size effect. If size has little or no effect on the rate of crossing over, we would expect chromosomes to have the same number of crossovers per base pair regardless of length, suggesting that interference is consistent regardless of chromosome size (Part A). If chromosome size does, in fact, influence the rate of crossing over, we would expect smaller chromosomes to exhibit an increased number of crossovers per base pair than larger ones, suggesting that interference acts across greater lengths between larger chromosomes than smaller ones (Part B).

Several studies by Kaback et al. have shown an inverse relationship between chromosome size and rate of crossing over (Kaback et al. 1989; Kaback et al 1992; Kaback et al. 1999). After increasing chromosome size, a decrease in the rate of crossing over was observed, while decreasing chromosome size resulted in the increase in the rate

of crossing over. The decrease in crossing over between larger homologs was observed after translocating the right arm from chromosome II onto the right arm of chromosome I (creating a I-IIR translocant); approximately 550 kb from the right arm of chromosome II was translocated onto the right end of chromosome I, increasing its size from 230 kb to 780 kb. The rate of crossing over in diploid yeast homozygous for the I-IIR translocation was compared to that in nontranslocated yeast. Results showed a significant decrease in the rate of crossing over when comparing I-IIR homozygotes to nontranslocated homozygotes, supporting the hypothesis that crossover interference increases as chromosome size increases.

In an attempt to recreate a similar study, Turney et al. (2004) induced a reciprocal translocation between chromosomes III and VII. Chromosome III increased in size from 340 kb to 917 kb, and chromosome VII decreased in size from 1,085 kb to 508 kb. In analyzing the rate of crossing over, Turney et al. (2004) recorded no such effect of chromosome size, observing the same rate of crossing over regardless of length. Instead, researchers suggest that the rate of crossing over may be interval- or chromosome-specific. Furthermore, Turney et al. (2004) suggests that the rate of crossing over may be more heavily influenced by the sequence-specific frequency of DSBs.

The Cre-loxP system as a mechanism for recombination

Translocated chromosomes such as the ones described above can be created using the *cre-loxP* system. The *loxP* site is a sequence derived from the bacteriophage P1, a virus infecting *Escherichia coli* with a natural phagemid genome (Sternberg and Hamilton 1981). In *E. coli*, phage-derived Cre recombinase helps facilitate the integration

of P1 DNA into the genome. In the current study, *loxP* sequences are inserted at specific locations; Cre recombinase subsequently brings *loxP* sites together, facilitating reciprocal translocation between nonhomologous chromosomes (Sauer and Henderson 1988).

Cre recombinase, encoded in engineered plasmids, can be regulated by the *GALI* promoter. In the absence of galactose, the *GALI* promoter is turned off, and subsequently, Cre recombinase is not expressed. If shifted to a medium containing galactose, the *GALI* promoter is turned on, resulting in Cre-induced recombination at *loxP* sites. This mechanism can be used to create translocants, or chromosomes comprised of genetic information from two nonhomologous chromosomes.

Yeast life cycle

Yeast are eukaryotes possessing the ability to reproduce both sexually and asexually. They can be kept and cultured as either haploid or diploid cells, both reproducing by means of budding. As a single-celled organism, *S. cerevisiae* reproduces asexually following the process of mitosis. The parent cell creates a bud, actin and myosin pull the poles apart such that the new nucleus is placed in the bud (Hartwell 1974; Sobel 1997). Following the completion of mitosis, the bud breaks off, yielding two cells. Parent and daughter cells are genetically identical and haploid, and both can continue reproducing in this fashion.

Each haploid cell is one of two opposite mating types determined by the expression of different alleles of the *MAT* locus on the right arm of chromosome III: α and a (Herskowitz 1988). Cells secrete pheromones attracting the opposite mating type. If a cell detects the pheromone of the opposite mating type, a projection called a shmoo

will form, extending up the concentration gradient. Under favorable environmental conditions, conjugation occurs, and the two haploid cells and nuclei fuse together to form diploids. This process, known as karyogamy, results in diploid cells containing a genome from mating type a and from mating type α (*MATa* and *MAT α* , respectively). Diploids also reproduce asexually via budding, and all cells are genetically identical. Culturing of diploids on media lacking nitrogen induces sexual reproduction through meiosis.

Genetically unique haploid cells are produced in which two cells are *MATa* and two are *MAT α* . The cells, known as spores, are bunched together in a tough coat called an ascus.

As environmental conditions improve, *S. cerevisiae* begins to grow again, mating and consequently resulting in diploid yeast reproducing via budding. Should the ascus break open and separate spores prematurely, however, environmental improvement will result in haploid, asexually reproducing yeast as growth restarts.

Analysis of spores

Researchers can take advantage of predictable mating and survival techniques, spatially separating spores following sexual reproduction and in turn analyzing resulting offspring. The four spores resulting from meiosis and contained in an ascus are referred to as a tetrad. Using light microscopy and micromanipulation techniques, asci can be disrupted, and spores can be separated for subsequent genetic analysis, a process known as tetrad dissection. Asci can be partially digested using Zymolyase (Zymo Research, Irvine, CA). Using β -1,3-glucan laminaripentaohydrolase to hydrolyze glucose polymers at β -1,3- linkages within ascus walls, Zymolyase results in the weakening of the ascus coat. Following digestion, a fiber optic needle can pick up and move spores from a single

tetrad to distinct locations on a nutrient-rich medium. All four haploid cells can be grown up as colonies and subsequently tested for growth on selective nutrient media, providing a simple yet highly accurate assay of the products of meiosis.

Strains with inserted nutrient markers allow for the evaluation of rates of crossing over between homologous chromosomes. Growth on selective media allows researchers to quantify the number of crossovers among several tetrads. Researchers can then gauge the rate of crossing over on a per base pair basis, facilitating the analysis of a chromosome size effect.

Strains used for translocation

Previously designed yeast strains KHY 185 (*MAT* α) and KHY 214 (*MAT**a*) were chosen for the present study. A nutrient marker gene was previously inserted into the left arm of chromosome VIII in KHY 214; the *his5* gene, derived from *Schizosaccharomyces pombe*, is inserted into the open reading frame (ORF) of *pau13*, complementing the *his3* mutation (Table 1, Figure 9). As a result, KHY 214 can grow on media lacking the amino acid. Likewise, the ORF of *YHL010c* was presumably disrupted by the insertion of *TRP1*, resulting in the ability to grow on media lacking tryptophan.

Table 1: Strain genotypes.

Strain	<i>MAT</i>	Genotype
KHY 185	α	<i>arg4-1691-lop</i> , <i>HpaI-lopC</i> , <i>his3</i> , <i>lys2</i> , <i>leu2</i> , <i>ura3</i> , <i>ade2</i> , <i>trp1</i> , <i>CEN8-HPHMX4/pGDP/loxp</i> , <i>YCR101c-NATMX4/loxp/ADE2</i>
KHY 214	<i>a</i>	<i>pau13::his5 YHL010C::TRP1 arg4-1691-lop</i> , <i>HpaI-lopC his3 lys2 leu2 ura3 ade2 trp1 CEN8-HPHMX4/pGDP/loxp YCR101c-NATMX4/loxp/ade2</i>
KHY 352	<i>a</i>	<i>pau13::his5 YHL026c::LEU2 YHL010C::TRP1 arg4-1691-lop</i> , <i>HpaI-lopC his3 lys2 leu2 ura3 ade2 trp1 CEN8-HPHMX4/pGDP/loxp YCR101c-NATMX4/loxp/ade2</i>

Figure 9: Marker insertions. In KHY 214 (*MATa*), *his5* and *TRP1* were presumably inserted on the left arm of chromosome VIII within *pau13* and *YHL010c*, respectively (numbers 1 and 2). KHY 185 (*MATα*), however, does not contain the nutrient marker insertions. Number 3 corresponds to the site of the *loxP* construct.

KHY 185, however, does not contain gene insertions for *his5* and *TRP1*, indicating that growth is dependent on the presence of both amino acids. Diploids resulting from the mating of KHY 185 and KHY 214 can biosynthesize their own histidine and tryptophan. Upon starvation, diploids undergo crossing over during meiosis, resulting in four spores. Haploid progeny can then be spatially separated, allowing for the analysis of crossing over between loci. For example, a spore that synthesizes tryptophan but requires supplemental histidine indicates a crossover between the two loci.

Additionally, each of the strains contains the *loxP* construct on the right arm of chromosomes III and VIII, flanked by known sequences of marker genes. As seen in Figure 10, the strains house similar inserts, each containing the *loxP* sequence. Chromosome III is characterized by the gene conferring resistance to the antifungal nourseothricin sulfate (cloNAT), followed by the *loxP* site and the *ade2* ORF. Chromosome VIII, however, houses the gene conferring resistance to the antifungal hygromycin B, followed by the *ade2* promoter and a *loxP* site. The separation of the *ade2* promoter on chromosome VIII and the ORF on chromosome III makes cells *ade2*-. Blocking adenine biosynthesis at this step catalyzed by *ADE2* results in the buildup of a red intermediate when grown on YPD (Yugolini and Bruschi 1989). Translocation of chromosome arms at the *loxP* site, however, yields haploid cells with functional *ADE2*,

yielding white colonies. This marker for recombination serves as a simple assay for successful translocations.

Molecular analysis of the translocation

The success of translocation products can be evaluated using PCR with primers on either side of the *loxP* sites on chromosomes III and VIII (Figure 10). For example, in nontranslocated yeast, the forward primer (3) left of the gene conferring resistance to CloNAT of chromosome VIII would not yield a PCR product when paired with the reverse primer (2) located in the *ade2* ORF on chromosome III (Figure 10). Following translocation at the *loxP* site, however, the two primers would be located on the same chromosome, thus yielding a PCR product.

Figure 10: *LoxP* construct at *YCR101c*. Chromosomes III and VIII contain insertions at *YCR101c*. Chromosome III houses the gene conferring resistance to hygromycin B (Part A, yellow), the *loxP* site (red), and the *ade2* ORF (dark purple). Chromosome VIII houses the gene conferring resistance to CloNAT (green), the *ade2* promoter (light purple), and the *loxP* site (red). Translocation of chromosome arms at *loxP* sites results in an altered gene arrangement, resulting in the physical linkage of the *ade2* promoter to the *ade2* ORF (Part B). Numbers 1, 2, 3, and 4 represent primers nat in R, ade2 R lox, cen 8 test F, and cen 8 test R, respectively (Table 3).

Finally, chromosome size can be determined using pulsed-field gel electrophoresis (PFGE). In regular gel electrophoresis, segments of DNA larger than 15 kilobases tend to comigrate, yielding a large, smeared band lacking resolution. Instead of running voltage through the gel in a single direction, as in typical gel electrophoresis, PFGE switches the direction of the voltage throughout electrophoresis (Chu et al. 1988). The idea behind PFGE involves the orientation of chromosomes during electrophoresis: DNA molecules run straight along the central axis, and as the direction of voltage is altered, the molecules must adjust in orientation. Smaller DNA molecules can adjust more rapidly to the altered current, while larger molecules reorient more slowly. This technique is capable of resolving very large pieces of DNA, including whole, intact chromosomes.

While there are multiple types of PFGE, one of the most common techniques is referred to as clamped homogenous electrical field (CHEF) technology. CHEF periodically changes the direction of voltage through a gel, switching from the central axis to 60-degree perturbations to either side of the axis. Gels are run for 24-48 hours, and the resulting image differentiates large DNA molecules ranging in size from 200 kb up to 6 Mb, easily resolving yeast chromosomes, which range in size from 230 kb to 1.5 Mb.

Furthermore, PFGE can be used as physical evidence of altered chromosome size following the reciprocal translocation previously described. *S. cerevisiae* is commonly used as a ladder for PFGE as the species' genome is entirely sequenced and precise chromosome sizes are known. In the current study, PFGE can be used to visualize physical differences between nontranslocated and translocated *S. cerevisiae*

chromosomes: bands from nontranslocated strains will be missing or altered following chromosome manipulation at *loxP* sites, and new bands will be created.

MATERIALS AND METHODS

Primer design

Using the Serial Cloner software, approximately two dozen primers were designed, flanking the *loxP* insert to facilitate molecular testing of both wild-type and translocant strains. All primers were designed between 18-22 base pairs and consisted of approximately 50% GC content, allowing for similar annealing temperatures for subsequent PCR. Primer sequences can be found in Appendix A.

Insertion of YHL026c :: LEU2

Plasmid pUG73 was isolated from *E. coli* cells using a standardized miniprep protocol (Appendix B). The *LEU2* insert within pUG73 was amplified in 45 µL reactions using *leu2* non *lox* F and *leu2* non *lox* R, annealing at 59 C and extending for 3 minutes (Appendix C). Additional primers complementary to targeted insertion site at *YHL026c* in wild-type KHY 214 were designed, and additional PCR was conducted: one PCR used *YHL026c* small F and *YHL026c* test R2, annealing at 55 C and extending for 1.25 minutes, while a second PCR used *YHL026c* small R and *YHL026c* test F2, annealing at 55 C and extending for 1.25 minutes. A summary of PCR conditions and potential products is listed in Table 2. Gel electrophoresis was used to assess the amplification of the *LEU2* PCR product as well as the two small DNA segments complementary to the target insertion site in *YHL026c*. PCR products were purified to eliminate residual contaminants from PCR (Appendix D).

Table 2: PCR conditions and expected products involved in the strain construction of *YHL026c* :: *LEU2*.

Primers	Annealing temperature	Extension time	Expected product
leu2 non lox F leu2 non lox R	59 C	3 minutes	2,373 bp
YHL026c small F YHL026c test R2	55 C	1.25 minutes	518 bp
YHL026c test F2 YHL026c small R	55 C	1.25 minutes	535 bp
YHL026c test F YHL026c test R	55 C	3 minutes	2,700 bp, if transformed
YHL026c test F YHL026c test R	55 C	3 minutes	780 bp, if untransformed

The three PCR products were cotransformed into KHY 214 by electroporation (Appendix E). Potential transformants were first evaluated based on growth on selective media. First, colonies were streaked for singles on SD-leu. Plates were allowed to grow for 3 days at 30 C. Colonies were then patched to YPAD plates and grown overnight at 30 C. The next day, patches were replica printed to selective media based on the expected phenotype (Table 1). Plates were incubated at 30 C overnight, and growth was evaluated. Patches corresponding to an incorrect phenotype were discarded, while those corresponding to the correct phenotype were patched to a new YPAD plate and grown overnight at 30 C.

Genomic DNA was isolated (Appendix F), and PCR was used to assess possible transformants using primers YHL026c test F and YHL026c test R, annealing at 55 C and extending for 3 minutes. Gel electrophoresis was used to visualize DNA size at *YHL026c* in KHY 214. The new strain was named KHY 352.

Creating the translocation

To create the translocation, pSH47 was isolated from *E. coli* cells using the plasmid miniprep protocol. Diagnostic digests were completed using EcoRV, ClaI, and PvuII. Samples were allowed to digest for 1.5 hours at 37 C, and products were assessed using gel electrophoresis.

The plasmid was subsequently transformed into both KHY 185 and KHY 214 by electroporation and selected for on SD-ura plates. Transformants were selected and streaked for single colonies on SD-ura, and plates were grown for 4 days at 30 C. Colonies were then patched to YPAD and allowed to grow overnight at 30 C. Patches were replica printed to SD-dropout plates lacking various nutrients to assess the phenotype of each isolate. Correct strains were patched to YPAD and allowed to grow overnight at 30 C. Plates were stored at 4 C. Using primers pSH47 Cre F and pSH47 Cre R, transformation was assessed by PCR, annealing at 52 C and extended for 2 minutes. Products of PCR were evaluated using gel electrophoresis.

Yeast strains containing pSH47 were grown in 3 mL of liquid YPAD overnight at 30 C with shaking at 200 rpm. A volume of 300 µL of overnight culture was transferred to two separate tubes containing 3 mL of YPGalactose broth. Cultures were grown at 30 C with shaking at 200 rpm; one culture was allowed to grow for 1 hour, and the other was allowed to grow for 2 hours. Following incubation, 1.5 mL of culture was pelleted in a microcentrifuge tube at 7,000 rpm for 2 minutes, and the supernatant was discarded. The remaining 1.5 mL of culture was added to the tube and pelleted at 7,000 rpm for 2 minutes. Once again, the supernatant was discarded. Cells were resuspended in 1 mL of sterile diH₂O, pelleted at 7,000 rpm for 2 minutes, and the supernatant was discarded; this

step was repeated once. The pellet was resuspended in 1 mL of sterile diH₂O. Cells were diluted to 10⁻⁴ and 10⁻⁵ using diH₂O such that each dilution contained a total of 600 µL of cell mixture. A volume of 150 µL was added to each of 4 YPAD plates, and cells were spread using sterile glass beads. Each strain was spread to a total of 16 YPAD plates; for example, the KHY 185 overnight culture was added to YPGal liquid media, and one culture was allowed to grow for 1 hour while the other was allowed to grow for 2 hours (KHY 185-1hr and KHY 185-2hr, respectively). Cells were repeatedly washed with diH₂O, and each of KHY 185-1hr and KHY 185-2hr was diluted to 10⁻⁴ and 10⁻⁵. Finally, diluted cells were plated to each of 4 YPD plates, yielding a total of 16 plates per strain. Plates were incubated at 30 C for 5 days. White colonies of each strain were chosen and patched to YPAD plates.

Analyzing possible translocants

Genomic DNA was isolated from each of the potential translocant strains of KHY 185 and KHY 352. PCR was subsequently used to assess the presence or absence of the translocation using various sets of primers. Primers ade2R lox and cen 8 test F were used to assess the shortened, VIII-IIIIR translocation, annealing at 50 C and extending for 3 minutes; primers nat int R and cen 8 test R were used to lengthened, III-VIIIR translocation, annealing at 50 C and extending for 1.5 minutes; primers nat int R and ade2 R lox were used to assess strains lacking the translocation, annealing at 50 C and extending for 1 minute; and primers nat int R and YCR101c test F were also used to assess strains lacking the translocation, annealing at 50 C and extending for 3 minutes. Potential results are listed in Table 3. Gel electrophoresis visualized the success or failure

of inducing the translocation. Successfully translocated strains were patched to YPAD and grown overnight at 30 C. Plates were stored at 4 C. Translocated strains containing pSH47 were then evaluated by pulsed-field gel electrophoresis using Bio-Rad's CHEF-DR III variable angle system (Appendix G).

Curing translocated strains of pSH47

Translocated yeast were replica printed to YPAD and grown at 30 C overnight. The following day, the new plate was replica printed to 5-FOA plates to assess the presence or absence of pSH47. Patches yielding successful growth on 5-FOA were patched to YPAD and grown at 30 C overnight. DNA was isolated, and strains were tested for the presence of the plasmid. PCR was conducted using pSH47 CreF and pSH47 CreR, annealing at 52 C and extending for 2 minutes. PCR products were analyzed by gel electrophoresis.

Simultaneously, KHY 185 and KHY 352 with translocated chromosomes III and VIII were incubated overnight in 5 mL of liquid YPAD media at 30 C, shaking at 200 rpm. A volume of 5 μ L of overnight culture was used to inoculate 5 mL of fresh YPAD broth, and the culture was incubated overnight at 30 C, shaking at 200 rpm; this procedure was repeated four times. Each day, overnight culture was diluted with diH₂O to 10^{-4} , 10^{-5} and 10^{-6} . 50 μ L of each dilution was spread to 5-FOA, and plates were incubated at 30 C for 4 days.

Controls

KHY 185 (*MAT α*) was asymmetrically mated to KHY 352 (*MATa*) (Appendix H). Diploids were sporulated, and tetrads were dissected (Appendix I). Tetrads were scored by replica printing to SD-his, SD-leu, and SD-trp. Additionally, mating type of spores was assessed by replica printing to SD plates spread with KHY 148 (*MAT α*) and KHY 149 (*MATa*). Growth on selective media was compiled in tables, and spore arrangement was recorded.

RESULTS

Strain construction: insertion of *LEU2* into *YHL026c*

Before creating the translocation, KHY 214 needed to be altered such that an additional nutrient marker was inserted into the left arm of chromosome VIII. After consideration, the distance between *paul3 :: his5*, at coordinates 11,923-12,285 base pairs, and *YHL010c :: TRP1*, at coordinates 83,721-84,221 base pairs, in chromosome VIII was large enough to allow potentially undetectable double-crossover events, confounding analyses. Thus, *LEU2* was chosen for transformation into the left arm of chromosome VIII in KHY 214, within *YHL026c*, a nonessential gene spanning coordinates 53,219-54,166. *LEU2* was amplified from pUG73 using primers constructed with 3' ends complementing the plasmid sequence and 5' ends complementing a small region within *YHL026c* in KHY 214 (Figure 11A). By this design, *LEU2* was successfully amplified from pUG73, and the 5' ends containing 60 bp of sequence complementary to the targeted insertion site within *YHL026c* should have allowed for proper integration of the *LEU2* PCR product.

After several unsuccessful transformations, it became apparent that the 5' ends did not provide enough overlap with the targeted insertion site within *YHL026c*. To create larger regions of overlap, an additional pair of primers was designed (YHL026c small F and YHL026c small R). By pairing primer YHL026c small F with YHL026c test R2, a sequence of DNA could be used to create increased overlap between the *LEU2* PCR product and the target region within *YHL026c*, increasing the region of overlap from 60 bp to 578 bp on the right (3') side of the *LEU2* PCR product (Figure 11B). Likewise,

primer YHL026c small R was paired with YHL026c test F2, increasing the overlap on the opposite (5') end of the *LEU2* PCR product from 60 bp to 595 bp (Figure 11C).

Figure 11: Construction of *YHL026c :: LEU2*. *LEU2* was inserted into *YHL026c* using a multistep process. First, *LEU2* was amplified from *pUG73* (Part A) using primers containing homology to both the plasmid (green) and the insertion site at *YHL026c* (red). Next, small flanking segments of DNA complementary to *YHL026c* were amplified (Part B). In Part C, *LEU2* and the two accessory segments were cotransformed into KHY 214 by electroporation, resulting in the integration of *LEU2* at *YHL026c* (Part C).

Potential transformants were first evaluated for growth on selective media based on the expected phenotype (Table 1). Strains potentially containing *YHL026c :: LEU2* grew on SD-leu, SD-his, SD-trp, YPAD+CloNAT, and YPAD+HYG, and did not grow on other selective media. These results were consistent with successful insertion, and presumed transformants were subsequently used for PCR using primers to the left and right of the *YHL026c* ORF. Using primers YHL026c test F and YHL026c test R, untransformed yeast would yield a PCR product of approximately 780 bp while successfully transformed yeast would yield a product of approximately 2700 bp. Gel electrophoresis confirmed successful *LEU2* insertion at *YHL026c*, and the new strain was named KHY 352 (Figure 12).

A.

B.

Figure 12: *LEU2* transformation. *LEU2* was successfully inserted at *YHL026c*. Part A depicts the transformation. In part B, lane 1 shows the 1 kb ladder, lane 2 contains untransformed 214 (negative control) yielding a band at approximately 780 bp, and lane 3 contains transformed 214, yielding a band at approximately 2,700 bp.

Creating the translocation

To facilitate the creation of translocant strains, plasmid pSH47 was transformed into KHY 185 (*MAT α*) and KHY 352 (*MATa*). This plasmid houses the sequence for Cre recombinase under control of the *GALI* promoter. It also houses *URA3*, allowing potential transformants to be selected for on SD-ura. Successful transformation was confirmed by PCR and subsequent gel electrophoresis.

The 10^{-4} dilution resulted in the growth of approximately 150 colonies on YPD plates following induction of the translocation. The 10^{-5} dilution yielded approximately 30 colonies. The incomplete biosynthetic pathway for the production of adenine leads to a build up of a pink intermediate; in successfully translocated yeast, the *ade2* promoter and *ade2* ORF are physically connected, resulting in the completed biosynthetic pathway and thus white colonies. Approximately 20% of colonies were white, corresponding with successful translocation. All 16 plates from each strain were evaluated, and 15 of the largest white colonies were patched to YPAD for molecular analysis.

Molecular analysis of the translocation

Several sets of primers were used as molecular confirmation of potential translocation. Primer pairs (Table 3) were chosen such that translocated chromosomes III and VIII could be analyzed (Figure 13A). For example, primer nat int R (1), located on the left side of the *loxP* site in chromosome III, and primer cen 8 test R (4), located to the right of the *loxP* site on chromosome VIII, would yield a PCR product in chromosome III-VIII, but not in nontranslocated yeast (see Figure 10 for primer locations). In another analysis, primers that would only yield a product in nontranslocated yeast were

used, and nontranslocated yeast were used as a positive control. Interestingly, translocated and nontranslocated yeast yielded the same results. Both positive and negative control strains yielded identical products (Figure 13B).

Table 3: PCR of potential translocants.

Primers	Annealing temperature	Extension time	Expected product	
			nontranslocated	translocated
nat int R cen 8 test R	50 C	1.5 minutes	none	900 bp
ade2 R lox cen 8 test F	50 C	3 minutes	none	2,700 bp
nat int R ade2 R lox	50 C	1.5 minutes	600 bp	none
nat int R YCR101c test F	50 C	3 minutes	2,400 bp	none

A.

B.

Figure 13: Analyzing the translocation. In both Parts A and B, lanes 1, 6, and 11 contain 1 kb ladder; lanes 2 and 7 contain the nontranslocated KHY 352 control; lanes 3 and 8 contain potentially-translocated KHY 185 (*MATα*); lanes 4 and 9 contain potentially-translocated KHY 352 (*MATα*); and lanes 5 and 10 contain a translocated KHY 214 control. In part A, bands 2-5 correspond to PCR using primers nat in R and cen 8 test R (Table 3), and bands 7-10 correspond to PCR using primers ade2R lox and cen 8 test R. In part B, lanes 2-5 correspond to PCR using primers nat int R and ade2 R lox, and lanes 7-10 correspond to PCR using primers nat in R and YCR 101c test F. See Table 3 for a summary of expected results.

Pulsed-field gel electrophoresis was used to provide physical evidence of translocation by evaluating intact chromosomes within each yeast strain. Results for

nontranslocated and translocated strains were similar, though chromosome dosage could not be accurately determined. Nontranslocated chromosomes III and VIII were expected to yield products at 315 kb and 563 kb, respectively; translocated yeast were expected to lack bands corresponding to these sizes, and should have gained bands at approximately 757 kb and 125 kb (chromosomes III-VIIIR and VIII-IIIR, respectively). Both translocated and nontranslocated strains showed identical bands at approximately 750 kb (Figure 14). Band intensity, however, could not be determined; translocated chromosome III, at 757 kb, would likely overlap with chromosome X, at 746 kb, while translocated chromosome VIII band at 125 kb should be visible.

Figure 14: PFGE of potential translocants. Lanes 1-8 contain either nontranslocated or potentially translocated yeast evaluated by CHEF PFGE. All strains show bands at identical locations.

Curing yeast cells of pSH47

To analyze the effect of chromosome size on the rate of crossing over, translocant strains had to be cured of pSH47. *URA3*, located on pSH47, encodes orotidine-5'-phosphate decarboxylase, or ODCase. When grown on media containing 5-fluoroacetic acid, ODCase converts the acid into toxic 5-fluorouracil, causing cell death (Boeke et al. 1984). This was used as a selection for cells that have lost pSH47.

Multiple methods were used in an attempt to cure yeast cells of pSH47. Evidence for translocated chromosomes was only found in yeast still containing pSH47. Results suggest that the translocation cannot exist in the absence of the plasmid.

Controls: tetrad dissection and spore analysis

Spore viability was assessed in two ways. First, overall spore viability was calculated. Additionally, the number of 4-, 3-, 2-, 1-, and 0-spore viable tetrads was recorded. Results are provided in Table 4.

Table 4: Nontranslocated yeast: spore viability.

	4 s.v.	3 s.v.	2 s.v.	1 s.v.	0 s.v
Number of tetrads	480	174	91	30	9
%	61.22	22.19	11.61	3.83	1.15
Overall Spore Viability:	89%				

Tetrads were evaluated based on spore phenotype. Parental ditypes (PD) describe a tetrad in which two phenotypes are observed, both of which correspond to parental genotypes, indicating a lack of crossing over. In nonparental ditypes (NPD), two phenotypes are observed, but neither corresponds to the parental genotypes, thus indicating recombination in all four spores. Finally, tetratypes (TT) result from four unique genotypes in which two spores correspond to the two parental genotypes, and two

spores correspond to crossing over between loci. Table 5 shows examples of scoring between loci. Growth was marked by a plus (+) sign, and lack of growth was marked by a minus (-) sign. Growth was evaluated on SD-his, SD-leu, and SD-trp plates, in addition to SD+70 α and SD+227a. SD+70 α and SD+227a were SD plates spread with KHY 148 (*MAT α*) and KHY 149 (*MATa*), respectfully, and were used to determine the mating type of each spore. With the compiled data, the genetic map distance was calculated using the Perkins equation, a formula using the relative frequencies of the spore arrangements to determine the map distance between two loci assuming 0, 1, or 2 crossovers within an interval (Ma and Mortimer 1983).

Table 5: Example of data collection.

Tetrad	Spore	SD-his		SD-leu		SD-trp	SD+227a	<i>MAT</i>	SD+70 α
2T	a	+	PD	+	NPD	-	+	α	-
	b	-		-		+	-	a	+
	c	-		-		+	+	α	-
	d	+		+		-	-	a	+
2B	f	+	TT	+	PD	+	-	a	+
	g	-		+		+	-	a	+
	h	+		-		-	+	α	-
	i	-		-		-	+	α	-

The calculated map distances seen in Table 6 implied an error at the presumed *YHL010c* :: *TRP1* insertion. Though KHY 214 and KHY 352 could successfully grow on media lacking tryptophan, 23.13% of tetrads showed nonparental ditypes at the *YHL026c* :: *LEU2* to *YHL010c* :: *TRP1* interval, corresponding to recombination within this interval in 91% of spores. The known distance of approximately 30 kb between the *YHL026c* and *YHL010c* target insertion sites and a map distance of 91 cM provides evidence that *TRP1* is located on a different chromosome. To further evaluate the issue, PCR was conducted using primers YHL010c test F and YHL010c test R (binding to the

left and right of the *YHL010c* ORF, respectively), annealing at 55 C and extending for 2.5 minutes. Results confirmed the absence of *TRP1* at *YHL010c*.

Table 6. Nontranslocated yeast tetrad data.

Interval	Tetrad Type	Number of Tetrads	Percentage of Tetrads	Map Distance
<i>pau13::his5</i> to <i>YHL026c::LEU2</i>	parental ditype	249	51.88%	25.1 cM
	nonparental ditype	2	0.42%	
	tetratype	229	47.71%	
<i>YHL026c::LEU2</i> to <i>YHL010c::TRP1</i>	parental ditype	164	34.17%	90.73 cM
	nonparental ditype	111	23.13%	
	tetratype	205	42.71%	

Map distance was calculated using the Perkins equation such that map distance in cM = $(100 (6N + T)) / (2(P + N + T))$ where N, T, and P represent the number of nonparental ditypes, tetratypes, and parental ditypes, respectively. (Ma and Mortimer 1983)

Finally, gene conversion at each of the confirmed nutrient marker intervals was analyzed for future use. These results, compiled in Table 7, represent DSBs that were repaired by SDSA rather than crossing over.

Table 7. Gene conversion in nontranslocants.

Locus	Number of tetrads	Percentage of tetrads
<i>pau13 :: his5</i>	6	1.25
<i>YHL026c :: LEU2</i>	18	3.75

n = 480

DISCUSSION

Previous studies have provided evidence to support the hypotheses of crossover assurance, interference, and homeostasis. The effect of chromosome size on crossing over, however, is perhaps least explored with studies by Kaback et al. (1999) and Turney et al. (2004) drawing different conclusions.

This investigation set out to evaluate the effect of chromosome size on the rate of crossing over along the left arm of chromosome VIII. First, nutrient marker genes were inserted along the left arm of chromosome VIII. Using the *Cre-loxP* system, reciprocal translocation between chromosomes III and VIII was conducted, changing VIII in structure from the nontranslocated size of 562 kb to the VIII-IIIIR size of 125 kb. In analyzing control data, however, it was revealed that *TRP1* was inserted at a locus on a chromosome other than VIII.

Translocation

The Cre-induced reciprocal translocation between chromosomes III and VIII initially appeared to be successful, as molecular analysis confirmed successful translocation. PCR using primers flanking the *loxP* sites yielded expected products in translocated and nontranslocated yeast, providing evidence of both III-VIIIIR and VIII-IIIIR. Additionally, positive and negative controls yielded expected results.

However, when using primers expected to yield a product only in nontranslocated yeast (primers 1 and 2 in Figure 11), the positive control, negative control, and presumed translocants showed identical bands. Furthermore, results from pulsed-field gel

electrophoresis showed an identical band pattern between translocated and nontranslocated yeast. There is a lack of evidence to rule out altered chromosome dosage resulting from overlapping bands (III-VIIIR, at 757 kb, overlapping with the band for chromosome X, at 746 kb). Alternatively, this could be due to differences in cell density during PFGE plug preparation. In evaluating PCR results, however, it is possible that the translocation is a lethal one, and as such, diploids must retain copies of both translocated and nontranslocated chromosomes.

Curing yeast cells of pSH47

Several failed attempts at curing translocated yeast of pSH47 have led to the reevaluation of using this particular plasmid. Multiple techniques resulted in the same reversion of chromosomes, and we have been unable to identify a cause for the chromosome rearrangement. Future work will include the use of a different plasmid, also containing the *GALI-Cre* system. The alternate plasmid, pNH3, will be used to induce the translocation to evaluate the possibility of plasmid-specific reversion of translocated chromosomes to nontranslocated ones.

Spore analysis of nontranslocated diploids

Following the collection of control data, it was clear that *TRP1* was inserted into a chromosome other than chromosome VIII. As a result, only two nutrient marker genes remain on the left arm of chromosome VIII in KHY 352, spanning 12-84 kb: *pau13* :: *his5* and *YHL026c* :: *LEU2*. Without the third marker, experimentation regarding the effect of chromosome size could not be continued. We will progress with strain

construction to insert additional genes on the left arm of chromosome VIII to analyze crossing over in multiple intervals, and we will repeat dissection of nontranslocated yeast tetrads.

CONCLUSION

The aim of this investigation was to evaluate the effect of chromosome size on the rate of crossing over during meiosis. Had experimentation progressed successfully, we would have compared the rate of DSB formation in nontranslocated yeast to those containing the VIII-IIIR translocation. This would have been achieved by tagging the far left end of chromosome VIII with a fluorescent tag and arresting yeast in various stages of meiosis, enabling us to take a “snapshot” of DSB formation and frequency (Baudat and Nicolas 1997). Blitzblau and Hochwagen (2011) previously used this method to identify recombination hotspots within the *S. cerevisiae* genome by analyzing a microarray of ssDNA that naturally accumulates at DSB hotspots. We would utilize a similar technique to identify the frequency and location of DSBs, allowing us to overlay the rate of crossing over with the rate of DSB formation between long and short chromosomes.

At this point in the project, we are unable to draw conclusions other than those gained in the evaluation of new techniques. Significant progress was made in exploring the methodology of creating translocated strains using the *Cre-loxP* system. Using these techniques, the experiment can be repeated and further investigated in an attempt to gain an understanding of the effect of chromosome size on the rate of crossing over.

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APPENDICES

Appendix A: Primer sequences.

Table 8: Primer sequences

Primer	Sequence
leu2 non lox F	5'-ACCTTTTCGTCATCCAAGCGCTAGGTTCTGC AATAATCTCGGGCGGCATCGAGTTTGCCAAtctaga gatccgcaggctaac-3'
leu2 non lox R	5'-GTCGTGGGACCCGACCTTATGGCCTATACCA GCAAGGATACCCATAGTGACTGGCCAAATtccca gcaaaggcttacaag-3'
YHL026c small F	5'-TGATCCTAGCCATTGCCATG-3'
YHL026c test R2	5'-AAATCACACATGCCCTAGCG-3'
YHL026c test F2	5'-TATGCCCTGCTTGTTCCAGT-3'
YHL026c small R	5'-CATATGCGATGGCAAACCTCG-3'
YHL026c test F	5'-CCGGGTTCAGAAATTCCATC-3'
YHL026c test R	5'-CCTCGTGAAACTGGTTTCTC-3'
pSH47 Cre F	5'-CGGCTGGCGACTGAATATTA-3'
pSH47 Cre R	5'-GGAGAAAAAACCCCGGATTC-3'
cen8 test R	5'-CACATCCCTTCTTTGTTGACG-3'
ade2 R lox	5'-CCAATTGTCCCCCTCCTAAT-3'
cen8 test F	5'-GTGGATGTGTAATTGTTGGAG-3'
nat int R	5'-GTGTCGTCAAGAGTGGTAC-3'
YHL0101c test F	5'-ACACATAGAGGCAATAACGG-3'
YHL0101c test R	5'-GAATGCAAGAATGGTATGGC-3'
YCR101c test F	5'-CCAAAAGTAACGGAGGCTCC-3'

Appendix B: Plasmid miniprep

E. coli cells containing the plasmid of interest were grown overnight at 37 C in liquid LB medium. A 1.5 mL volume of overnight culture was added to a 1.5 mL microcentrifuge tube and pelleted for 2 minutes at 13,200 rpm. The supernatant was decanted. Another 1.5 mL volume of culture was added to the pellet, and the tube was pelleted for 2 minutes at 13,200 rpm. The pellet was resuspended in 100 µL of Solution 1 (MO BIO, Carlsbad, CA). Next, 200 µL of freshly prepared Solution 2 (MO BIO, Carlsbad, CA) was added to the mixture. The solution was inverted 5-10 times to mix and placed on ice for two minutes. A volume of 150 µL of Solution 3 (MO BIO, Carlsbad, CA) was added. The solution was inverted 5-10 times and incubated on ice for an additional 5 minutes. The tube was spun in the microcentrifuge for 10 minutes at 13,200 rpm. Approximately 400 µL of supernatant was removed and placed into a sterile 1.5 mL microcentrifuge tube. The pellet and original tube were discarded. To isolate RNA, lipids, and proteins from the DNA of interest, 350 µL of phenol/chloroform was added, and the solution was vortexed for 20 seconds. The solution was spun in a microcentrifuge for 4 minutes at 13,200 rpm. Approximately 350 µL of the top aqueous layer was removed and placed into a new, sterile 1.5 mL microcentrifuge tube. Care was taken to assure that fluid at the protein-containing interface and the RNA- and lipid-containing organic layer was avoided. Phenol waste was discarded in an appropriate waste container, and the tube was placed in the waste container found in the fume hood. A volume of 800 µL of ice-cold EtOH was added, and the tube was inverted 5-10 times for mixing. It was then placed on ice for 10 minutes. Following incubation, the solution was pelleted in a microcentrifuge for 10 minutes at 13,200 rpm, and the supernatant was decanted. The pellet was then

washed with 500 μL of 70% EtOH using a 1000 μL pipettor. The solution was pelleted for 2 minutes at 13,200 rpm, and the supernatant was discarded. The tube was pulse-spun, and remaining EtOH was removed using a 200 μL pipettor. The open-top tube was placed in a vacuum centrifuge and spun for 7 minutes at 30 C. The dried pellet was resuspended in 50 μL of TE pH 8.0 and 1 μL of 20 $\mu\text{g}/\text{mL}$ RNase. Resuspended DNA was stored at -20 C for further use.

Appendix C: General PCR conditions

At a total volume of 15 μL , each reaction consisted of the following: 7.35 μL of dH_2O , 0.3 μL of dNTPs, 3 μL of 5X GoTaq Buffer, 0.9 μL of MgCl_2 , 0.45 μL of GoTaq Polymerase, 1 μL of F primer, 1 μL of R primer, and 1 μL of template. PCRs were run with an initial denaturing step at 94 C for 10 minutes, followed by 30 cycles of the following: denaturation at 94 C for 30 seconds, annealing at a predetermined primer-specific temperature for 1 minute, and extension at 72 C for an amount of time corresponding to product length (approximately 1 minute per kb of expected product). After cycling, an additional extension at 72 C for 7 minutes was followed by a 4 C hold. PCRs were stored at -20 C.

Appendix D: PCR cleanup

5 volumes of chaotropic salts were added, and the solution was transferred to a spin column. The solution was centrifuged at 13,200 rpm for 1 minute, and the filtrate was removed from the collection tube. 300 μ L of Solution 4 (MO BIO, Carlsbad, CA) was added, and the spin column was centrifuged at 13,200 rpm for 1 minute. The filtrate was removed using a 1000 μ L pipettor. The spin column was centrifuged again at 13,200 rpm for 1 minute to dry the basket, which was subsequently moved to a new, sterile 2 mL microcentrifuge tube. 50 μ L of solution 5 (MO BIO, Carlsbad, CA) was added to the basket and allowed to incubate for 1 minute. The column was centrifuged at 13,200 rpm for 1 minute, and the purified PCR products were stored at -20 C.

Appendix E: Transformation by electroporation

Strains were grown overnight in 5 mL of liquid YPAD medium, incubating at 30 C with shaking at 200 rpm. Following incubation, 2 mL of overnight culture was added to a baffled flask containing 25 mL of liquid YPAD medium. The remaining 3 mL of overnight culture was added to a different baffled flask containing 25 mL of YPAD medium. Cultures were incubated at 30 C, shaking at 200 rpm, for 3 hours. The optical density of each was checked, and the culture reading an OD₆₀₀ between 0.4 and 0.6 was chosen for the remainder procedure. The other culture was discarded. Cells were pelleted in a 50 mL conical tube, spinning at 1500 rpm for 3 minutes, and the supernatant was discarded. The pellet was resuspended in 1.8 mL of TE pH7.5 and subsequently transferred to a 15 mL conical tube. A volume of 200 µL of 1 M LiOAc was added, and the cells were allowed to incubate for 45 minutes at 30 C, shaking at 55 rpm. Following incubation, 100 µL of 0.5 M DTT was added, and the cells were incubated at 30 C, shaking at 55 rpm, for an additional 15 minutes. (Concurrently, electroporation cuvettes were cleaned with 70% EtOH and placed on ice for use later in the procedure.) 8 mL of sterile diH₂O was added to each tube, and cells were pelleted at 1500 rpm for 3 minutes. The supernatant was discarded. Cells were resuspended in 10 mL of sterile diH₂O and pelleted at 1500 rpm for 3 minutes, and once again, the supernatant was discarded. The pellet was resuspended in 500 µL of cold 1 M sorbitol and then transferred to a 1.5 mL microcentrifuge tube. Tubes were spun at 13,200 rpm for 15 seconds. The supernatant was removed and discarded using a 200 µL pipettor. Cells were resuspended in the smallest volume of cold 1 M sorbitol that allowed for each transformation to receive 40 µL of cell slurry. Tubes were kept on ice. To 0.5 mL PCR tubes, 40 µL of cell slurry, 2.5

μL of 10 mg/mL boiled salmon sperm, and varying volumes of transforming DNA were added and mixed using a pipettor. The contents of each PCR tube was added to respective chilled electroporation cuvettes. Care was taken to assure that the volume reached and covered the bottom of the cuvette. All cuvettes and 1 M sorbitol were kept on ice, and the electroporator was set to the following parameters: 1.5 kV, 25 μF , and 200 ohms (setting 129 R5). Each cuvette, processed one at a time, was removed from ice and dried using a Kim wipe. The cuvette was placed into the chamber and electroporated. Immediately, 1 mL of cold 1 M sorbitol was added, and the cuvette was replaced on ice. Each cuvette was handled as described until all transformations were processed. The contents of the cuvette was transferred to a sterile 1.5 mL microcentrifuge tube and pelleted at 5,000 rpm for 1 minute, and the supernatant was decanted. Cells were resuspended in 300 μL of cold 1 M sorbitol. 150 μL of sample was plated onto media containing the appropriate selective marker. Plates were incubated at 30 C for 3-5 days, but not longer than 7 days.

Appendix F: Yeast sorbitol gDNA prep

Cultures were grown overnight at 30 C, shaking at 200 rpm. The next day, 1.5 mL of culture was added to a 1.5 mL microcentrifuge tube and pelleted at 3000 rpm for 2 minutes. The supernatant was discarded. Another 1.5 mL was added to the pellet, and the tube was respun. The supernatant was discarded, and a final 1.5 mL volume of overnight culture was added to the pellet. The tube was pelleted under the same conditions, and the supernatant was discarded. The pellet was resuspended using 1 mL of diH₂O, and the tube was pelleted for 2 minutes at 3000 rpm. The supernatant was discarded, and the pellet was resuspended in 500 µL of 1M sorbitol. 15 µL of 0.5 M DTT and 7.5 µL of 10 mg/mL Zymolyase were added, and the tubes were incubated at 37 C for 1 hour, shaking at 200 rpm. 200 µL of TE and 70 µL of 10% SDS were added. Tubes were incubated in a heat block at 65 C for 10 minutes. 320 µL of 5 M KOAc was added, and tubes were inverted 6-10 times to mix before placing on ice for 30 minutes. Following incubation, tubes were spun at 13,200 rpm for 6 minutes. During the spin, 1 mL of isopropanol and 200 µL of NH₄OAc was added to 2 mL microcentrifuge tubes (one per gDNA isolation). After spinning, 650 µL of the supernatant was removed and added to its respective, previously prepared 2 mL tube. The 2 mL tubes were spun at 4000 rpm for 1 minute, and the supernatant was discarded. Tubes were spun at 4000 rpm for 15 seconds, and the remaining supernatant was removed using a 200 µL pipettor. Open top tubes were then spun in a vacuum centrifuge at 30 C to dry the pellets. The pellets were then resuspended in 250 µL TE, and 1 µL of 10 mg/mL RNase was added. Tubes were incubated at 37 C for 30 minutes, and gDNA was stored at -20 C.

Appendix G: Pulsed-field gel electrophoresis

Preparation of 1% low-melting point (LMP) agarose

0.5 g of LMP agarose was added to 50 mL of 0.125 M EDTA pH7.0, in a 250 mL glass bottle. The solution was microwaved for a few seconds, and the agarose was swirled in the bottle. This process was repeated until the agarose was completely dissolved. The bottle was placed in a water bath at 55 C for immediate use.

Sample preparation

Yeast were streaked onto YPAD for single colonies and incubated at 30 C for 4 days. Single colonies were used to inoculate 4 mL of liquid YPAD media. Cultures were incubated overnight at 30 C, shaking at 200 rpm. The next morning, 1.5 mL of cells were pelleted in a microcentrifuge tube for 10 seconds at 13,200 rpm, and the supernatant was discarded. The pellet was gently resuspended in 1 mL of 50 mM EDTA pH 8.0, and the cells were pelleted for 30 seconds at 13,200 rpm. The supernatant was carefully removed using a pipettor. 100 µL of 50 mM EDTA pH 8.0 was added. Next, 50 µL of 10 mg/mL Zymolyase, and 250 µL of previously prepared 1% LMP agarose solution was immediately added. The solution was pipetted up and down until uniform in color, and the entire mixture was immediately added into 5 wells of the plug mold. Each mold was filled to the very top (approximately 90 µL per well). This step was repeated one strain at a time to prevent agarose from solidifying prematurely. Plugs were placed in a freezer at -5 C for exactly 5 minutes. Tape was removed from the molds, and the 5 identical plugs from each strain were extruded into the barrel of a 5 mL syringe.

Wearing gloves in a fume hood, EDTA-Tris- β -mercaptoethanol (ETB) solution was freshly prepared by combining 9 mL of 0.5 M EDTA pH 8.0, 1 mL of 1 M Tris-HCl pH 8.0, and 0.5 mL β -mercaptoethanol. (This volume of ETB solution was sufficient for 4 yeast strains.) To each syringe barrel containing 5 plugs, 2.5 mL of ETB solution was added using the following technique: the syringe nozzle was blocked using a gloved finger as ETB solution was added to the barrel. The plunger was gently inserted about 0.5cm into the barrel, and then the syringe was inverted; the nozzle was very carefully uncovered, aiming the syringe away from the face. The syringe nozzle was sealed with Parafilm, and the plugs were incubated at 37 C for 4 hours to overnight, gently rotating at 55 rpm.

Proteinase solution was mixed using 9 mL of 0.5 M EDTA, 1 mL of 10% N-lauroylsarcosine, and 100 μ L of 10 mg/mL RNase. In the fume hood, the Parafilm was carefully removed from the syringe nozzle, and then the plunger was removed, allowing the ETB solution to pour from the nozzle. The plugs were rinsed twice with 5 mL of 50 mM EDTA. To the syringe barrel, 2.5 mL of proteinase solution was added in the same manner as the ETB solution, blocking the nozzle with a gloved finger and inserting the plunger as the syringe was inverted. The syringes were placed horizontally in an incubator at 37 C, shaking at 55 rpm for 6 hours to overnight. The plunger was removed, and proteinase solution as allowed to drain from the nozzle. The plugs were rinsed with 5 mL of 50 mM EDTA. Approximately 3-4 mL of 1X TE was added, and the syringes were incubated horizontally at 37 C for 1 hour, shaking at 55 rpm.

(Following this step, the plugs were ready to use. Any unused plugs were stored inside syringe barrels with Parafilm-sealed nozzles at 4 C. If stored, 1 mL of fresh 1X TE

was added and incubated at room temperature for 30 minutes. The TE was poured off, and another 1 mL of 1X TE was added. After 30 minutes, the plugs were ready for use.)

Preparing and running the gel

The 14 cm x 13 cm gel platform was assembled. 100 mL of 1% agarose was boiled in 0.5X TBE, and the solution was cooled to 60 C. Approximately 1 mL of melted agarose was pipetted into a 1.5 mL microcentrifuge tube, and the tube was kept in a water bath at 60 C. The remaining agarose was poured into the gel platform, and the gel was allowed to set for 1 hour.

The Bio-Rad CHEF DRIII variable angle system was assembled, and 2 L of 0.95X TAE was poured into the electrophoresis chamber. The drive module, control module, and chiller were all turned on, and the system was allowed to cycle electrophoresis buffer as the gel set. The chiller was set to 12 C. Gel parameters were as follows: temperature at 12 C, block1 initial and final switch time of 60 seconds for 15 hours, block 2 initial and final switch time of 80 seconds for 7 hours, run time of 22 hours, angle at 120 degrees, and voltage gradient at 5.8 V/cm.

Once the gel was prepared, plugs were poured from the syringe barrel into a small petri dish. Using a small spatula, plugs were carefully inserted into the wells. The tops of plugs were cut and removed using a razor blade, and air bubbles were removed from wells by pressing gently with two fingers. The top of the wells was sealed with the remaining 1 mL of agarose. The casting platform was removed, and the gel was gently placed in the electrophoresis chamber. The system was started, and the gel ran overnight.

The completed gel was placed in a large, dark container containing 300 mL of 0.5X TBE and 0.5 mg/mL of ethidium bromide solution and allowed to stain for 45 minutes. The gel was removed and placed into a different container containing diH₂O and allowed to destain for 45 minutes. Finally, the gel was imaged using the GelDoc EQ system.

Appendix H: Asymmetrical mating

Haploid strains were mated asymmetrically in a 100:1 ratio of *MAT α* : *MATa*. Using a wooden stick, strains were thoroughly mixed, and haploid controls were left untouched as controls. Plates were incubated at 30 C for 48 hours. Patches were then replica printed to SD-his, and plates were incubated at 30 C for 48 hours. Following incubation, the mixed, potential diploids were streaked for single colonies on SD-his and incubated at 30 C for 4 days. In addition to haploid controls, 10 colonies per mating were patched to YPAD and incubated at 30 C overnight.

Haploid strains KHY 148 (70, *MAT α*) and KHY 149 (227, *MATa*) were incubated overnight in 5 mL of liquid YPAD medium at 30 C, shaking at 30 C. 100 μ L of overnight culture was spread to an SD plate and allowed to dry in the laminar flow hood for 30 minutes. The plate of potential diploids, including haploid controls, was replica printed to SD-227 and SD-70. Plates were incubated at 30 C for 48 hours, and ploidy was analyzed based on growth (or lack thereof).

Appendix I: Tetrad dissection

Yeast were spread across a YPAD plate and incubated overnight at 30 C. Cells were replica printed to SPO and allowed to sporulate for 4 days. A small amount of sporulated yeast was picked up off of a SPO plate using a 200 μL pipette tip by scraping approximately 3 cm across the plate. Yeast were then suspended in a 0.5 mL tube containing 5 μL of dH_2O and 5 μL of 10 mg/mL Zymolyase. The solution was incubated for exactly 5 minutes, and 0.2 μL of cells were streaked along the side of a YPAD plate (see image below). Using the Singer MSM series 400 dissecting microscope, spores within a single tetrad were spatially separated. The fiber optic needle tip micromanipulated spores, placing them in an arranged grid on the YPAD plate. The Singer setup was chosen, in which 19 columns and 9 rows comprise the grid. Only 18 columns and 8 rows were utilized; the first column was reserved for the initial streak (large streak on the left in the image), and the middle row served as a spacer between spores of different tetrads. The image below shows the arrangement of spores on a plate. A total of 18 tetrads were dissected per plate, and plates were incubated at 30 C for 3 days.

Figure 15: Tetrad dissection. See text for a detailed description.