Proposal for Undergraduate Thesis (Capstone Project)

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The Rem1 gene converter: Controlling Homologous Recombination in *S. pombe*.

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Possible co-advisor: Donald A. Martyn, Research Professor, Bioengineering, University of Washington

Homologous Recombination | Gene Conversion | DNA Repair | Rem1 | Double-strand break | Conversion Tract | Tetrad Analysis | Loss of Heterozygosity

I have read my advisee's senior capstone research proposal and approve it for review by the Student Affairs Committee of the Department of Bioengineering.

Introduction and Significance

If DNA is to remain stable, chromosomal breaks must be repaired *with perfection*. Among the various lesions that can occur, double-strand breaks (DSBs) are the most deadly. Fortunately, DSBs can be repaired by homologous recombination (HR, see next section), during which broken ends are annealed to homologous chromatids in G_1, and in G_2, between sisters as well. In addition to somatic cells, recombination is also *required* for proper (non-random) segregation of homologs at the first metaphase in meiosis, and thus, for formation of the germline (1). If this *meiotic* HR is faulty, gametes will be inviable, or mutations will be propagated onwards, causing congenital pathology. In humans, Rad51, a widely conserved recombinase, is mediated by BRCA1/BRCA2. Deficient BRCA2 causes susceptibility to breast and ovarian cancers (2-6). Mutations in both BRCA proteins have also been shown to cause symptomatic equivalence to Fanconi Anemia, which is characterized by extreme chromosome fragility and high occurrence of carcinoma (7). Additionally, HR seems to revive replication forks that have been arrested due to DSBs in the template strands (8, 9). In light of these recent discoveries, HR is becoming recognized as a major pathway in mammalian DNA repair (10, 11).

While HR is essential for both repair and resistance to large-scale chromosomal damage, it also has effects on the smaller scale of individual genes or DNA segments; these latter effects are arguably as significant as those on the karyotipic level. The successful completion of a recombination event does not guarantee chromosomal stability. In fact, inferior haplotypes can be produced if diploid strand exchange occurs between wild-type and mutated sequences, thereby swapping out the normal locus completely, resulting in a loss of heterozygosity (LOH). If this occurs in an essential gene, the resulting phenotype might be lethal or oncogenic. Therefore, it is of high priority to understand how HR is modulated in instances where multiple daughter genotypes are possible. In my project, I will examine how the creation of recombinants by HR can be regulated by the catalytic properties of a certain meiotic cyclin, Rem1. By determining the mechanism, we will find insight into how Rem1 biases the formation of one type of daughter DNA, as opposed to another, via its interaction with other proteins at various stages in the HR pathway. In order to study Rem1, mutant strains will need to be engineered, experimental protocols will need to be created, and a high-throughput genetic assay will need to be developed. These are real design element. This basic science will (and should) be applied to understanding, for instance, how LOH is a derivative of HR, how gene therapy might be feasible for the correction of defects in homologous recombination, and how HR can be used as a tool in embryonic stem-cell genetics.

Background and Clinical Need

There are several models of DSB-induced recombination that have been proposed. Nearly universal to these models is the formation of a hybrid joint between the damaged chromosome and its intact homolog (Figure 1). Each of these joints, or Holliday Junctions (HJs), can be resolved to give two kinds of molecules (Figure 2): a cross-over (XO) or non-crossover (NXO). In both cases, the resulting DNAs contain a vestige of the joint, which is still in a hybrid state. Since the two recombining chromosomes are *homologous*, and not identical, it is possible that non-perfect nucleotidic pairing occurs at the joint. Mismatch repair enzymes will then correct these anomalous pairings, resulting in a stable recombinant molecule. If the HJ spans a coding region (exon), the mismatch

repair can potentially convert one allele of a resident gene on one chromosome, to its homologous allele. This process is called *gene conversion*, and is the predominant mechanism by which *intragenic* recombination (within a gene) occurs (12). Gene conversion can either restore the nascent DNA to its parental form (restoration), or convert it entirely into the recombinant (conversion). Both XOs and NXOs can give rise to this scenario, but XOs always result in recombination *between* genes as well (intergenic). If two alleles in a heterozygote are sufficiently close together to be coincident on the HJ, then both alleles can convert in a process called *co-conversion.*

Gene conversion is responsible (but not the only reason) for LOH, as abovementioned, which can cause, if LOH occurs on a tumor suppressor gene, recessive-type inheritance of cancer susceptibility (13, 14, 15, 16). For instance, LOH on a region in human chromosome 17 is prevalent in ovarian and mammary neoplasms (17). Similarly, allelic loss of Rb1 is the genetic culprit of occlusive retinoblastoma (18). Thus, control of intragenic recombination (conversion), and the daughter DNAs formed thereof, is vital for genomic stability in germ cells. In the above instances, it is not clear whether gene conversion is at fault. However, LOH by conversion has been documented in a variety of cancers. In Bloom's syndrome, the gene product (a helicase, which upon mutation, causes ectopic recombination and LOH) evidently guides recombination in a non-destructive manner (19, 20). In mice, LOH was observed following recombination (21), and LOH was localized to the vicinity of DSBs following homologous repair (18, 22). At present, the means by which recombination is biased towards conversions or restoration is unknown. It is speculated that the condition of the parental strands, or the nature of the mismatch itself, could create the bias (23 – 27). We suppose that Rem1 has an epigenic effect (by interaction with protein substrates) on the control of this bias.

Design of Project

The focus of my project is precisely the control of gene-conversion by Rem1. The protein Rem1 is a meiotic cyclin in the fission yeast *S. pombe*. It was identified in Barcelona, and appears to be necessary for normal levels of intragenic recombination. Cells that are *rem1* mutant show a three-fold reduction in recombination in two tested genes (28), without showing decreased intergenic recombination. This behavior, which is postulated to exist at all loci across the genome, suggests that Rem1 acts on gene conversion to decrease the probability of *phenotypic* recombination through HR. The objective and *deliverable* of my project is to determine the molecular mechanism for this decrease in intragenic recombination among mutants, and, if possible, to investigate the interaction of Rem1 with other proteins whose functions in recombination are better understood. We have formulated three hypotheses to explain the observations above.

1. Recombination might be reduced due to a decrease in the frequency of intragenic DSBs. This is not expected to effect recombination between distant markers because the genome of *S. Pombe* has very little interference.
2. A reduction may occur by an increase in conversion tract length. Such an increase most likely proceeds via extended branch migration, or a change in the morphology of the JM.
3. The decrease may also occur if there is a low ratio of conversions to recoveries, or by preferential conversion to the double-mutant (increased conversion of wild-type alleles).

I shall study these hypotheses in the context of meiosis, using *S. Pombe* as my model organism. This is a good model because of its rapid growth, strength of phenotypes, and significant homology in DNA repair to *S. Cerevisiae* and *H. Sapiens*. Like asexual prokaryotes, *S. Pombe* normally divides by mitosis. However, certain heterothallic strains have one of two mating types, allowing entry into the meiotic cycle to be controlled by crossing strains of opposite mating type under certain conditions. We use a meiotic system for several reasons. Firstly, Rem1 is only expressed during meiosis, causing arrest if it prematurely spliced. Secondly, it is possible to screen for linkage and inheritance of a multitude of alleles. Thirdly, if proper markers are chosen, we can use the segregant phenotypes in the daughter cells as a genetic assay for gene conversion in the zygote. The first hypothesis is easily testable by standard DNA quantification methods (Southern blotting). The last two hypotheses require more subtle genotypic tests, which are greatly aided by this meiotic system, because it is not possible to visualize the fine details of recombination *in situ.*

In engineering the strains to be used, several design considerations are presented. We ideally want strains with genes that are dispersed across one chromosome (so that the linkage between them can only be broken by cross-over). In the case of intergenic recombination, these markers will allow me to analyze reciprocity, association with conversion, and so forth. Thus, four genes were chosen that are located on the third chromosome of *S. Pombe*: *ura4*, *ade6*, *tps16*, and *arg1*. Three of these markers cause auxitrophy for uracil, adenine, and arginine. The *tps16* maker creates temperature sensitivity; cells are unable to grow above 32 °C. The order of these markers is such that *ade6* is flanked by *ura4* and the *tps16/arg1* region (Figure 3). Of course, to measure linkage, the markers must be heterozygous in the diploid. For instance, if one strain is polymorphic in *ura4*, the meiotic partner will have the wild type allele ura^+.

In this meiotic system, I would like to optimize the output signal. To do this, I am using a certain adenine allele, *ade6-3049*, a known hotspot for DSBs during meiosis. In a bi-allelic system consisting of *ade6-3049* and *ade6-52*, we can expect DBSs to occur with high frequency, independently amplifying the effects of *rem1* on HR. This system gives a net signal-to-noise processing gain of approximately 80 dB. The *ade6-52* allele forms an aborted gene product that creates pink colonies on an adenine-poor medium. The *ade6-3049* haploids form dark red colonies. Thus I can use the *color* of haploid as a direct phenotypic assay for intragenic recombination in *ade6*. Both mutations are lethal if there is no adenine. Thus, wild-type recombinants are white, and can be selected against parentals on an adenine-free media, greatly assisting analysis of HR in this gene.

Gene conversion within *ade6* will form different sets of daughter recombinants. By assembling daughter cells into their quartets, all arising from the same meiosis, it is possible to determine the nature of the conversion involved. This process is made facile by the physiology of *S. Pombe*; spores are transiently enfolded in an ascus (Figure 4). Thus, I can reconstitute the recombination event based on the ratios of observed phenotypes within each ascus. This technique is called tetrad analysis, and involves the dissection and matrix colonization of spores from an ascus with a micromanipulator. I will perform a homozygous mating (to generate recombinant DNA) of two rem1 strains with the DSB hotspots *ade6-3049* and *ade6-52*, as mentioned above. The mitotic phenotypes of these two markers, and their recombinant wild-type ade^+, will be used in our tetrad analysis. I use the frequency of co-conversion between these two adjacent

intragenic markers (revealed as a 3:1 ratio of parental phenotypes in a tetrad) as a measure of the conversion tract length. I can use the frequency of occurrence of conversion tetrads (containing a $1:2:1^{+}$ motif), as a measure of conversion-to-restoration ratio.

Design and Phases: Experiments

- **Phase 1: Construct Parental DNA (strains)**

Transformation of Cells This phase is complete. Original strains were provided by the laboratory in Barcelona. These strains were mutagenized by PCR eclipse of the *rem1* gene with *kan*^{r}, which allows for selection of the transgenic cells on the antibiotic G418. The oligonucleotides used were **5'**-GCGAAAATCGATCAAAATTAGAAG-**3'** and **5'**-GTCACATCATGCCCCTGAGC-**3'** at *kan* (Ayté, communication). In order to create two strains suitable for the tetrad analysis, the *rem1*::*kan*^{r} construct had to be cloned into our DNA library. This was done by choosing two strains with different markers dispersed throughout the genome to cross with the original strains. In *ade6*, as mentioned above, we chose two different alleles: *ade6-52* and *ade6-3049*, which are single nucleotide polymorphisms (SNPs) with distinct phenotypes in an adenine-minimal medium, and hotspots for DSBs. The first parental strain was constructed by crossing GP 5674: h^{+} *ura4-D18 ade6-3049* ● JA 456: h^{-} *rem1*::*kan ura4-D18 arg1-230*. The resulting strain was VT 001: h^{+} *rem1*::*kan ura4-D18 ura4-aim ade6-52 arg1-230*. The strain was screened by replica plating random segregants from rich media to media with minimal adenine, uracil, arginine, as well as onto G418. The same process was repeated for the second parental strain, using GP 4917: h^{+} *ura4-D18 ura4-aim ade6-52 tps16* ● JA 456: *h-rem1 ura4-D18 arg1-230*. The resulting strain was VT 002: h^{-} *rem1*::*kan ura4-D18 ade6-3049 tps16*. Control strains VT 003 and VT 004 were isogenic to VT 001 and VT 002 except for *rem1*. The parents were repurified and will be tested for genotype.

- **Phase 2: Duplicate Published Result (Barcelona experiment)**

Inability to Duplicate the Result Since the previous revision of this draft, this phase has presented considerable difficulty. In the second attempt, I have not yet been able to reproduce the putative decrease in intragenic recombination (V. Tseng and G. Smith, unpublished results). A described previously, I investigated the effects of nitrogen (N) and supplements (S) by performing homozygous crosses on the extreme media SPA – N – S and EMM2 – N – S, as well as the intermediate media: EMM2 + N – S, EMM2 – N + S, and EMM2 + N + S. On the extreme media, I performed crosses with 500 % cell volume from our standard protocol, as well as the method used in Barcelona (J. Ayté, communication), in which confluent layers of cells grown on EMM2 + N + S are mixed onto mating media without inoculation in culture. The results from these experiments are inconclusive. We observed similar recombinant frequencies in the mutant and wild-type cells. However, the wild-type frequency was over five times higher than those reported in the original paper. Since the absolute frequencies of recombination are different, and consistently so, we believe that disparities in methods are still suspect, rather than an actual absence of effect. Thus, further experimentation is necessary. I have designed two definitive experiments to determine whether *rem1* has the advertised phenotype.

Independent Crosses As before, I will cross the strains JA 427: h^{-} *rem1::kn ura4-D18 ade6-M26 arg1-230* ● JA 493: h^{-} *rem1::kn ura4-294 ade6-52 leu1-32*. We will also cross the wild-type isogenics GP 488: h^{-} *ura4-D18 ade6-M26 arg1-230* ● GP 720: h^{-} *ura4-294*

ade6-52 leu1-32. These crosses will be performed in quadruplicate; two isolates of each strain will crossed with each other on EMM2 – N – S. We will desist from using SPA since we would like to adhere to the conditions used in Barcelona as exactly as possible. Spores will be harvested and serially diluted onto selective media for recombinants. In this step, we will diverge from the protocol used in Barcelona, and use the guanine-negative method: if *rem1* has an interesting effect, it should not be contingent on the selection processes.

Collaboration with Barcelona In addition to a dependency on media, *rem1* might only be observable in strains with certain genotypes. Since we do not know the genotypes of the strains used in Barcelona, we have contacted the laboratory, and they will send us the strains used in the published experiment. We will also resend them the strains used in our unsuccessful crosses (JA 427, JA 493, GP 488, GP 720), so that they may repeat the experiment. If these experiments do not prevail, then I will submit a revised proposal on a new project, which will have equally important design components.

- **Phase 3: Random Spore Analysis**

Meiosis between VT 001 ● VT 002 and the isogenic VT 003 ● VT 004 will be performed in the appropriate manner as determine by Phase 2. Asci will be harvested from sporulation media and digested with glusulase to free the spores. As is standard, I will eliminate all unmated cells by treatment with ethanol. Recombinant frequency is determined by diluting spores onto a permissive and differentiating medium for *ade-* (to count parentals) and onto an inhibitory medium for *ade-* (to count recombinants). Of course, the major limitation of this method is the treatment of all parental-type DNAs as non-recombinant, whereas most of them will arise by gene conversion.

- **Phase 4: Tetrad Analysis**

Dissection of Asci I will perform the mating between VT 001 ● VT 002 and VT 003 ● VT 004. The asci will not be dissolved for random spore analysis, but instead, dissected by arraying them in a grid and incubating until the outer membrane has lysed. The four spores will then be individually placed across the grid and grown on minimal adenine so that phenotypes will be expressed. There are eight possible recombination events that may occur within *ade6*. A reciprocal XO between the alleles may occur (rare). There may be no recombination. Two modes of co-conversion can occur, and there are four modes of single conversion.

Classification of Recombinant Genotypes For tetratypes that contain the same phenotypes, but are generated by different HR events, a series of back-crosses will be necessary to disambiguate them. The *ade6-52 ade6-3049* double point-mutation will have the same pink phenotype as *ade6-52*. By crossing the double-mutant with a strain with only the *ade6-3059* SNP, will be unable to produce $ade^{+/+}$, whereas the *ade6-52* strain could produce recombinants. This cross will be necessary in cases where asci arise from conversion of $ade6\text{-}52^{+}$ to *ade6-52*, conversion of *ade6 –*3049^{+} *ade6-3049*, or if no recombination has occurred (parental di-type). All three of these tetrads will contain a 2:2 ratio of phenotypes. The one tetrad arising from a reciprocal XO will contain the double-mutant, and will have the same phenotype as the single conversion of *ade6*-3049 to $ade6\text{-}3049^{+}$. Thus, the back-cross must be performed in these two cases as well. The two tetrads arising from co-conversion are unique and can be identified from each other and other recombination events. The final tetrad will show the conversion *ade6-52* to $ade6\text{-}52^{+}$, and is phenotypically different than all other tetrads.

Post Meiotic Segregation Detection of uncorrected heteroduplex joints (which occur at low frequency) can be achieved by observing split colonies that contain both phenotypes (pink and dark red) after the chimeric haploid has replicated through mitosis. Colonies that show this split are indicative of post-mitotic segregation (PMS). We hypothesize that an apparent reduction in recombination could be caused by PMS, but the effect will be minor.

Perpetuation of the Cell Line Since the analysis will need to be conducted for at least 250 asci to characterize gene conversion, crosses will need to be continuously performed since asci degrade automatically. I will keep a constant line of purified cells. A genotype check will be performed during the experiment.

- **Phase 4: Analysis of Intergenic Recombination**

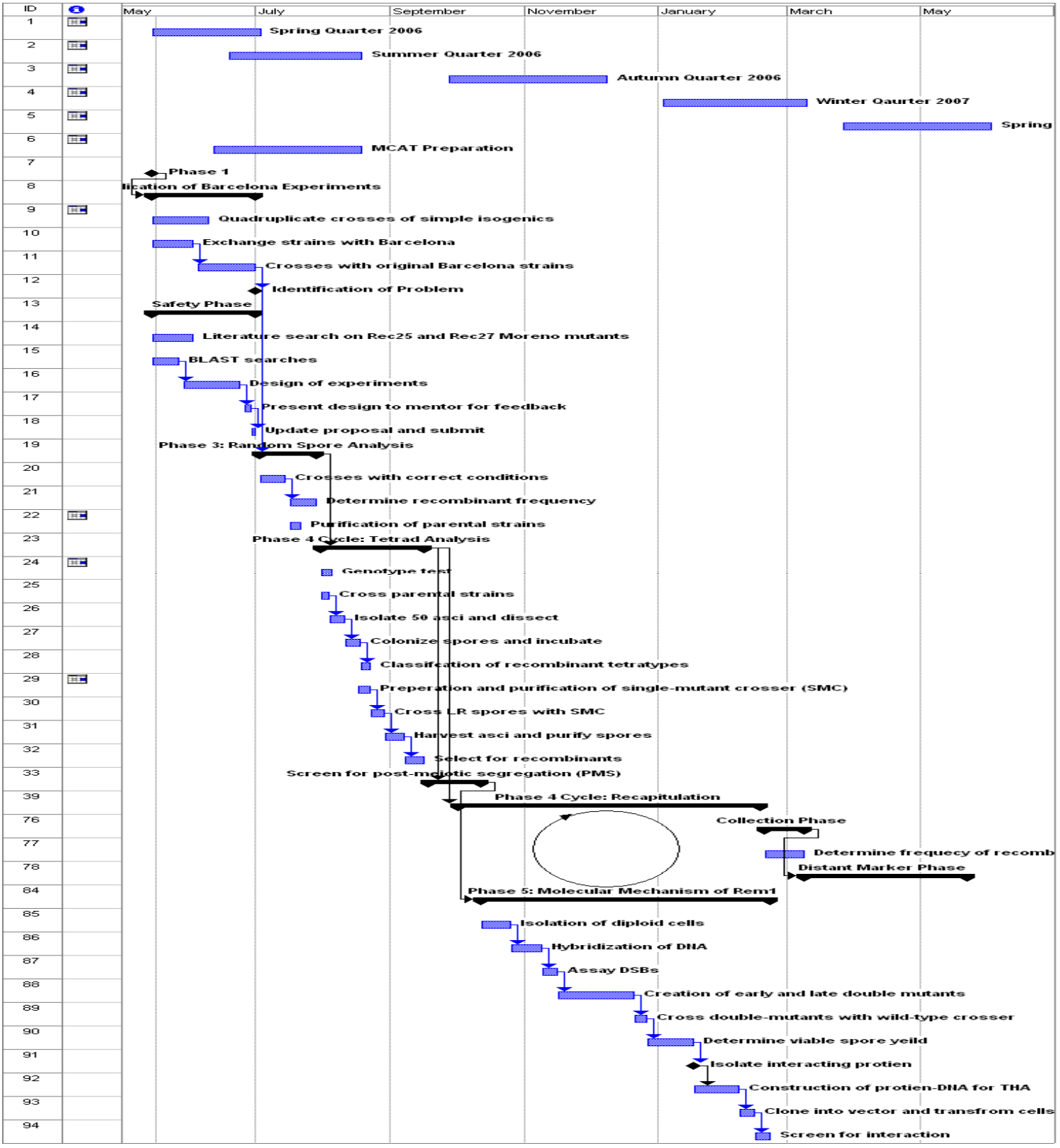
Intragenic recombination will be measured between three intervals: *ura4 – tps16*, *ura4 – arg1*, and *tps16 – arg1*. This will be achieved by replicating ~ 100 segregants from the tetrads onto media without uracil and arginine, as well as onto a medium at 32 °C. Reciprocals will be observed by reference to the original genotypes of the parents. Crossovers are indicated by the following recombinants: *ura4-D18 tps16*$^{-}$: *ura4-D18 ura4-aim tps16*$^{+}$, *ura4-DI8 arg1-230* : *ura4-D18 ura4-aim arg1*$^{+}$, and *tps16*$^{+}$ *arg1*$^{+}$: *tps16*$^{-}$ *arg1-230*. If differences are seen in a *rem1* background, we can isolate the effects from intragenic recombination in *ade6* since we know where the cells came from (they were chosen from the tetrads).

- **Phase 5: Molecular Interactions of Rem1**

Analysis of DSBs If the project is complete, I will isolate the DNA at the pachytene stage to test for DSBs. This will be done by PCR amplifaction, and hybridization to probes. We know the expected DNA fragments that should result by a DSB at *ade6*, and we can thus compare the intensity of DSBs in *rem1* and in wild-type. I will then identify the part of the pathway that *rem1* acts upon. If DSBs are reduced, then *rem1* diminishes breaks at the hotpot by interaction with the break protein Rec12, or by preventing the action of the meiotic homolog cohesin Rec10. These two actions (cohesion and DSBs) are considered *early* parts of HR. If DSBs are not reduced, and we see that gene conversion has been changed to generate less recombinants, then we will then know whether Rem1 acts during, or after, the strand-exchange proteins Rad50S and Rad51. The two late parts of the HR pathway are HJ resolution (regulated by Mus81) and mismatch repair (regulated by ExoI). I will test interaction of Rem1 with all proteins, barring Exo1, with a double-mutant assay. If Rem1 acts early in HR, I will test if Rem1 suppresses the high rate of chromosomal missegregation in *rec10* and *rec12* mutants. Thus will be done by seeing if there is increased viable spore-yield in the *rem1*::*kan* Δ*rec10* and *rem1*::*kan* Δ*rec12* double mutant (constructed by a cross, as above). The same analysis can be applied to proteins in the late pathway. We can see if there is increased viability of spores in *rem1*::*kan* Δ*rad51* double mutant, for instance.

Two-hybrid Assay If there is a strong correlation in these assays, then we will test for direct interaction of Rem1 with the candidate substrate (for instance, Rec10) by using a two-hybrid assay. This will be done by fusing a DNA binding domain to Rem1 by a 600 bop flexible DNA sequence. The same thing will be done between Rec10 and a strong activating domain for *his4*. If there is an interaction, then the bound Rem1 – Rec10 will activate transcription at *his4*.

Timeline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May
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Spring Quarter 2006
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Summer Quarter 2006
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Winter Qaurter 2007
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Spring
6
MCAT Preparation
7
Phase 1
8
lication of Barcelona Experiments
9
Quadruplicate crosses of simple isogenics
10
Exchange strains with Barcelona
11
Crosses with original Barcelona strains
12
Identification of Problem
13
Safety Phase
14
Literature search on Rec25 and Rec27 Moreno mutants
15
BLAST searches
16
Design of experiments
17
Present design to mentor for feedback
18
Update proposal and submit
19
Phase 3: Random Spore Analysis
20
Crosses with correct conditions
21
Determine recombinant frequency
22
Purification of parental strains
23
Phase 4 Cycle: Tetrad Analysis
24
Genotype test
25
Cross parental strains
26
Isolate 50 asci and dissect
27
Colonize spores and incubate
28
Classification of recombinant tetratypes
29
Preperation and purification of single-mutant crosser (SMC)
30
Cross LR spores with SMC
31
Harvest asci and purify spores
32
Select for recombinants
33
Screen for post-meiotic segregation (PMS)
39
Phase 4 Cycle: Recapitulation
76
Collection Phase
77
Determine frequecy of recomb
78
Distant Marker Phase
84
Phase 5: Molecular Mechanism of Rem1
85
Isolation of diploid cells
86
Hybridization of DNA
87
Assay DSBs
88
Creation of early and late double mutants
89
Cross double-mutants with wild-type crosser
90
Determine viable spore yeild
91
Isolate interacting protien
92
Construction of protien-DNA for THA
93
Clone into vector and transfrom cells
94
Screen for interaction

Acknowledgments

This work is supported by the Mary Gates Endowment (Research Scholarship), and by NIH grant: XXXX.

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Figures

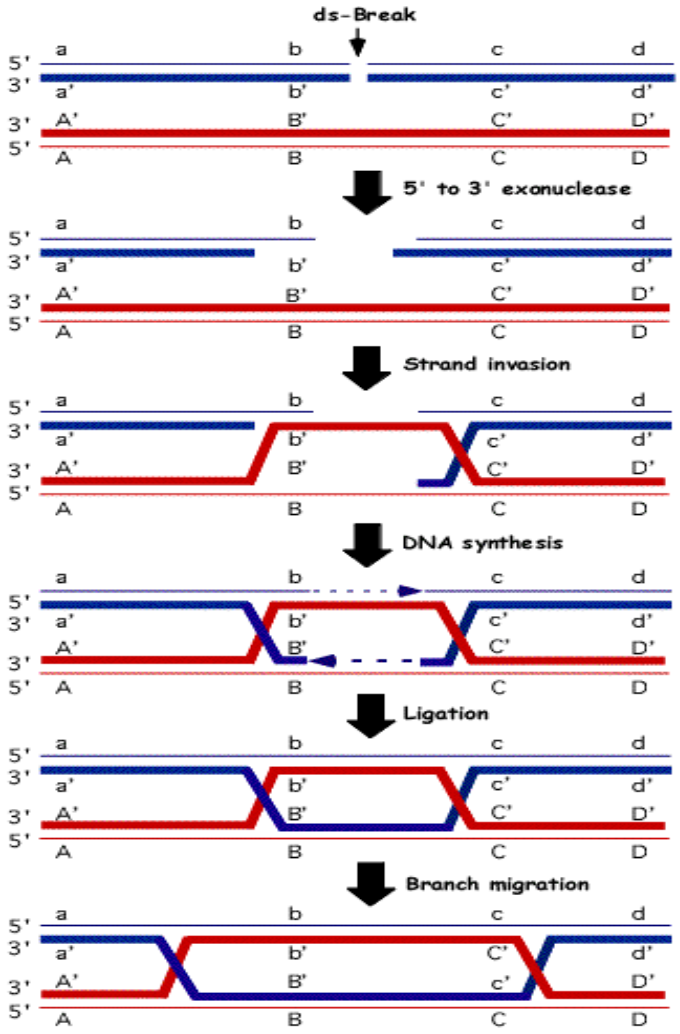

Figure 1 The DSB model of homologous recombination. Recombination is initiation by invasion of one DNA stand by the single-stranded (ss) homologous complement, displacing the original complement into a D-loop. This D-loop is then captured by the other DNA and synthesis reconnects the synapse. The above processes result in a joint molecule (JM) with joints in two positions, composed of hybrid DNA, and adopting a chiasmic shape. Each of these joints, or Holliday Junctions (HJ), can be resolved in one of two ways (Figure 2).

Image from http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/genetic-analysis/recombination/rec-molecular.html

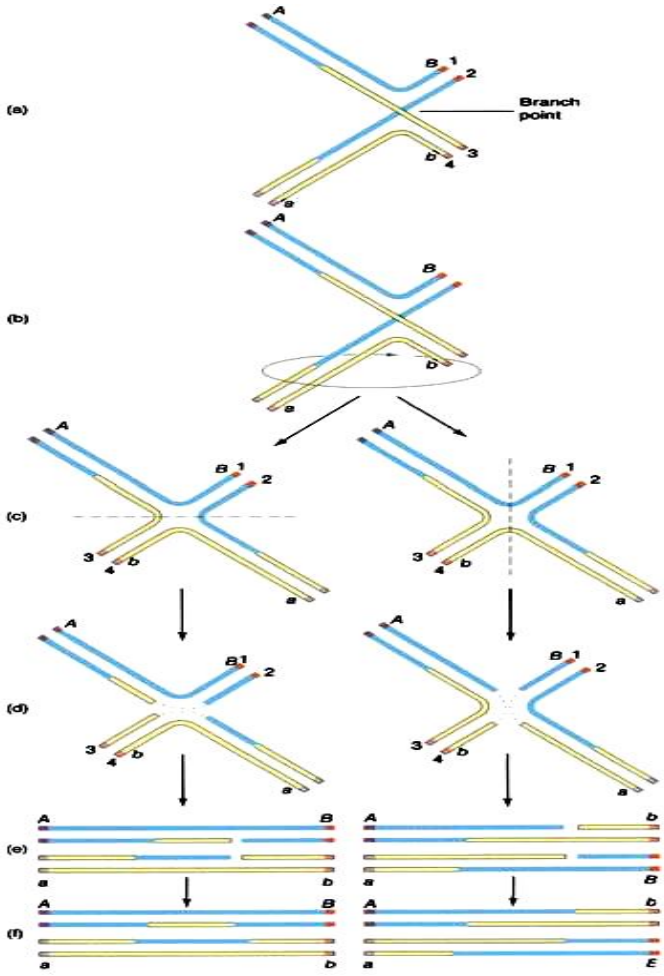

Figure 2 The Holliday junction can be resolved into two recombinant molecules. Each line represents a DNA single strand. On the left, the heteroduplex DNA, caused by branch migration, is sandwiched between regions of parental DNA. In this situation, (non-crossover), recombinants can only be formed by repair of the parental DNA to the homologous template in the heteroduplex region (gene conversion). On the right, a crossover has occurred and both molecules are recombinant.

From http://nitro.biosci.arizona.edu/courses/EEB320-2005/Lecture13/lecture13.html

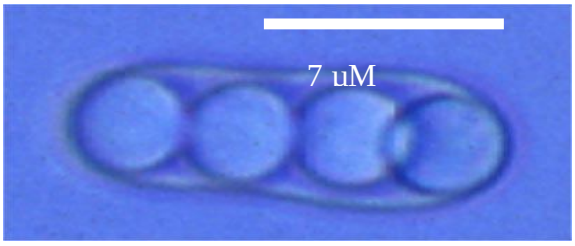

Figure 4 A yeast ascus containing four spores, after the second cytokenetic division of meiosis. In a random spore plating, the ascal membrane is removed by treatment with glusulase, allowing the spores from different tetrads to mix freely.

From
http://bugs.bio.usyd.edu.au/Mycology/IMAGES/Topics/Taxonomy/yeastAscus.jpg

Figure 3

The genotypes of the strains used in this study were screened so that the *ade6* locus contained two hotspots (*ade6-52* and *ade6-3049*) for recombination. The table below lists the intragenic genotypes and corresponding phenotypes. To illustrate the importance to tetrad analysis, if we see a light red spore in a random sample of spores, we do not know if it was produced by a co-conversion of the *52*$^{+}$ *3049* markers to *52* *3049*$^{+}$, or if the spore is simply parental.

ura4-D18: deletion, causing inability to synthesize uracil
ura4-aim: artificial inserted mutation, equivalent to ura^{+}

tps16: inability to grow above 32°C

arg1-230: inability to synthesize arginine