INVESTIGATION OF MECHANISMS THAT SUPPRESS NON-ALLELIC HOMOLOGOUS RECOMBINATION

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INVESTIGATION OF MECHANISMS THAT SUPPRESS NON-ALLELIC HOMOLOGOUS RECOMBINATION

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Homologous recombination (HR) is a conserved pathway for repair of DNA double strand breaks (DSBs) and stalled or collapsed replication forks, and depends upon recognition of a homologous template on a sister chromatid or alternate parental copy. Non-allelic homologous recombination (NAHR) results from erroneous recognition of a similar but non-homologous template and can lead to lethal chromosomal deletions or rearrangements. To avoid errors, NAHR is actively suppressed by cellular mechanisms that disrupt heteroduplex recombination intermediates. Using a specialized type of recombination pathway single-strand annealing (SSA) as a model in yeast, I found that rejection of heteroduplex HR intermediate induces a *RAD9*-dependent cell cycle delay in the G2 stage of the cell cycle. Strains lacking the *RAD9* gene, and consequently a damage-induced G2 delay, less frequently allowed SSA between divergent sequences than identical ones. However, non-allelic SSA could be restored to wild-type levels if a G2 delay was induced by nocodazole treatment. These results indicate that that cell cycle delay induced by the Rad9-dependent DNA damage response can passively promote recombination between non-allelic sequences despite the potential for creating deleterious genome rearrangements. Secondly, following identification of the Msh2-Msh6 heterodimer and Sgs1 helicase as essential factors for unwinding of a heteroduplex intermediate during SSA (Sugawara et al., 2004; Goldfarb and Alani, 2005), our lab determined that these proteins interact through a direct physical interaction, similarly to mammalian homologs (Pedrazzi et al., 2003; Yang et al., 2004; Saydam et al., 2007). Next I asked whether other proteins that interact with

Msh6 and Sgs1 contribute to heteroduplex rejection, including the topoisomerase Top3-Rmi1 which is known to stimulate Sgs1 activity (Cejka et al., 2010; Niu et al., 2010) and the replication clamp PCNA which enhances the activity of Msh6 in the mismatch repair pathway. I found that Top3-Rmi1 contributes to heteroduplex rejection, but appears to do so mainly by stabilizing Sgs1. Additionally, I show that PCNA is dispensible for heteroduplex rejection; three mutants of the catalytic subunit *pol30* and a *msh6* mutant lacking the Pol30 interaction domain rejected SSA heteroduplexes to the same extent as wild-type. Finally, two of the *pol30* mutants displayed a reduction in SSA efficiency, revealing an unexpected role for PCNA in SSA.

BIOGRAPHICAL SKETCH

Carolyn ("Carrie") Marie George was born on October 14, 1982 in Altoona, Pennsylvania to parents Dennis and Marie and joined a 2-year-old brother Matthew. Three years later, Carolyn welcomed a younger sister Rebecca. At the time of her birth, Carolyn's mother wished to call her "Carrie Anne" as in the song by the Hollies, but since her father was not crazy about that idea they compromised by naming her Carolyn but calling her "Carrie" as a shortened version of the name. And so Carolyn has been known as "Carrie" to all ever since.

Carrie grew up in Altoona, and was surrounded by a large extended family with whom she is still very close. She attended the Our Lady of Lourdes Catholic School from Kindergarten through 8th grade where she was known to be very shy but a good student. As a child she enjoyed reading and learning about animals (penguins were her favorite) and was also involved with girl scouts, cheerleading, and attended summer theater camp. Carrie also enjoyed arts and crafts, and especially music. She sang as part of the children's choir at her church and, despite never having the opportunity for lessons, taught herself how to play a number of songs on the piano. Upon entering high school and becoming a member of a nationally ranked cheerleading squad, Carrie put many of her other interests and talents on the back-burner so that she could prepare for multiple annual state and national competitions as well as attend basketball and football games at her school and maintain her GPA at the same time. Cheerleading was brought to an abrupt halt for Carrie in her senior year of high school when she injured her knee on a trampoline and required surgery to repair several torn ligaments. Though she was originally devastated, she later found the injury a blessing as it allowed her to return focus to her other interests including academics and music.

Carrie graduated from Bishop Guilfoyle Catholic High School in May of 2001, and for the first time left Altoona, albeit not far, for Juniata College in Huntingdon, Pennsylvania. Having an interest in animals and her favorite subject in high school being biology, she began with a major in biology with hopes of potentially going to veterinary school. However, upon taking chemistry classes and having the opportunity to do research in a biochemistry laboratory, her mind was changed. She added chemistry to her major and decided to enter on a track to her Ph.D. While at Juniata, and finally unburdened by a demanding cheerleading schedule, Carrie explored other interests. She was involved with community service groups Sigma Phi Alpha and the Catholic Council, but also searched for a recreational activity to suit her needs. After trying out several activities including brief stints on the dance and gymnastics teams, joining the intramural lacrosse team, and even playing the cowbell for the pep band, she settled nicely on singing. She was a member of the Juniata Choral Union for six semesters and also sang for weekly Catholic services. It was here where she was first encouraged to try singing solo and where she found confidence in her voice. By the time graduation came along, she was asked to sing for a multi-denominational religious service held for graduating students and their families. In addition, Carrie made some true and lasting friendships while at Juniata, and these friends helped to transform her from the shy, solitary creature she was into a more outgoing person.

In the meantime, Carrie continued to excel in science. She gained experience both in the laboratory and in the classroom. The former being fulfilled by carrying out research projects in the labs of Dr. Tom Fisher and Dr. Ruth Reed and also during a summer research experience at the University of Vermont. She gained teaching experience as a four-year part-time employee with Juniata College's Science In Motion program. She assisted a high school chemistry teacher in preparing and teaching laboratory exercises and helped to organize workshops for high school

science teachers. Carrie also was employed as a peer tutor and organizer of the annual Science Olympiad competition. Carrie enjoyed these experiences so much that she had a difficult time deciding on whether to continue towards a Ph.D. or seek out a teaching degree. Ultimately, she decided to spend some time working in a research lab after college to help her with this decision.

Carrie graduated with distinction in biochemistry from Juniata College in May 2005, she worked for 2 years as a lab technician in the lab of Dr. Susan Michaelis at the Johns Hopkins School of Medicine, and then continued to graduate school at Cornell. In the meantime, she continued to be involved on the side with singing, first with the Johns Hopkins Medical Institute's Choral Society, then as a member of St. Catherine of Siena choir in Ithaca NY, where her talents have blossomed under the direction of choir director Sherry Scanza. Also during this time, Carrie developed an interest in crafting, especially in sewing and crochet, and likes to design pieces that combine both mediums. Carrie currently is planning for a move to New Hampshire with her dog and faithful companion, Dillon. She will continue to do research, this time focusing on mechanisms of aging using *Drosophila melanogaster* as a model, and will continue to sing and craft in her spare time.

DEDICATION

This thesis is dedicated to my family, my parents Denny and Marie and siblings Matt and Becky. I am who I am because of you and could never have achieved this degree without your love and support.

I also want to dedicate this to my good friends from Juniata College who brought me out of my shell and gave me the courage to face the world: Emily Meyers, Valerie Capotosto, Jenn Mikula, Nikki Bressler, Nathan Thompson, Levi Blazer, and Tina Ausherman.

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LIST OF ABBREVIATIONS AND SYMBOLS

µg = microgram

µl = microliter

1n = haploid

2n = diploid

ADP = adenosine diphophate

ATM = ataxia telangiectasia mutated

ATP = adenosine triphosphate

ATR = ataxia telangiectasia related

BIR = break induced replication

bp = base pair

CDK = cyclin dependent kinase

ChIP = chromatin immunoprecipitation

DAPI = 4',6-diamidino-2-phenylindole

DDR = DNA damage response

dHJ = double Holliday junction

DMSO = dimethyl sulfoxide

DNA = deoxyribonucleic acid

DSB = double-strand break

DSBR = double-strand break repair

dsDNA double-stranded DNA

EDTA = ethylenediaminetetraacetic acid

FACS = fluorescence activated cell sorting

G0 = growth 0, quiescent stage

G1 = growth 1, pre-replication stage

G2 = growth 2, post-replication stage

G418 = Geneticin

GCR = gross chromosomal rearrangement

HA = hemagglutinin

HCl = hydrochloric acid

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HO = homothallic switching endonuclease

HPHMX = Hygromycin B resistance cassette

HR = homologous recombination

HRP = horseradish peroxidase

HU = hydroxyurea

IgG = immunoglobulin G

IP = immunoprecipitate

KANMX = Geneticin (G418) resistance cassette

kb = kilobase

kDa = kilodalton

LiCl = lithium chloride

M = mitosis stage

MBC = methyl benzimidazol

MEPS = minimal efficient processing segment

mg = milligram

ml = milliliter

MMR = mismatch repair

MMS = methylmethane sulfonate

NaCl = sodium chloride

NAHR = non-allelic homologous recombination

NATMX = nourseothricin resistance cassette

NHEJ = non-homologous end-joining

nt = nucleotide

PCNA = proliferating cell nuclear antigen

PCR = polymerase chain reaction

PMSF = phenylmethylsulfonyl fluoride

RPA = replication protein A

S = synthesis, replication phase

S/TQ = serine or threonine followed by glutamine

SDS = sodium dodecyl sulfate

SDSA = synthesis dependent strand annealing

SSA = single-strand annelaing

ssDNA = single-stranded DNA

TBS = Tris-base SDS

TCA = trichloroacetic acid

TE = Tris-EDTA

TLS = translesion synthesis

wt = wild-type

YP = yeast peptone

YPD = yeast peptone dextrose

α-HA = antibody to hemagglutinin

Δ = deletion

CHAPTER I: INTRODUCTION

Multiple cellular mechanisms prevent chromosomal rearrangements involving repetitive DNA.[1]

a review, by

Carolyn M. George and Eric Alani

Abstract

Repetitive DNA is present in the eukaryotic genome in the form of segmental duplications, tandem and interspersed repeats, and satellites. Repetitive sequences can be beneficial by serving specific cellular functions (e.g. centromeric and telomeric DNA) and by providing a rapid means for adaptive evolution. However, such elements are also substrates for deleterious chromosomal rearrangements that affect fitness and promote human disease. Recent studies analyzing the role of nuclear organization in DNA repair and factors that suppress non-allelic homologous recombination have provided insights into how genome stability is maintained in eukaryotes. In this review we outline the types of repetitive sequences seen in eukaryotic genomes and how recombination mechanisms are regulated at the DNA sequence, cell organization, chromatin structure, and cell cycle control levels to prevent chromosomal rearrangements involving these sequences.

[1] Published in *Critical Reviews in Biochemistry and Molecular Biology*, 2012, 47(3):297-313

Introduction

Repetitive DNA is present throughout the eukaryotic genome; for example, centromeres and telomeres are composed of repeated elements, ribosomal DNA consists of tandem arrays, and different classes of transposable elements are present in multiple copies. Repetitive DNA provides a means for co-evolving multiple forms of a gene and for rapidly reorganizing the genome (Marques-Bonet and Eichler, 2009; Ohno et al., 1968). The amounts and types of repetitive DNA varies between organisms and may reflect how rapidly an organism evolves to changes in its environment. Such benefits, however, come with risks. For example, repetitive DNAs serve as substrates for chromosomal rearrangements that include disease-causing deletions, inversions, and translocations (collectively defined as gross chromosomal rearrangements, GCRs; reviewed in Chen J-M et al., 2010).

The consequences of GCRs will depend largely on when and where they occur. An aberrant recombination event has a greater likelihood of contributing to disease if it occurs during meiosis or in the germ-line of a multicellular organism, rather than in a somatic cell. GCRs in the germ-line, if they do not confer lethality during meiosis or embryogenesis, will be passed on to all cells of the body. Thus a genetic disease can result if the GCR greatly affects the normal function of any organ or tissue. If the same rearrangement occurs in a single somatic cell of a multicellular organism, that cell will most likely be eliminated and not affect the rest of the organism. An exception is if a somatic GCR affects a tumor suppressor gene or a cell cycle control pathway and allows uncontrolled cell proliferation of the affected cell, leading to the growth of a potentially cancerous tumor.

Both germline and somatic GCRs are frequently seen in human cancers, and as many as hundreds to thousands of GCRs can exist within a single tumor (Chen J-M et al., 2010; Stratton

et al., 2009; Velculescu, 2008). In some cases, recurrent GCRs are found in tumor suppressors or oncogenes. Two well-known GCRs are chromosomal translocations that create *BCR-ABL* fusions seen in chronic myeloid leukemias (Chen et al., 2010) and the intrachromosomal rearrangements within the *BRCA1* and *BRCA2* genes that are found in some breast and ovarian cancers (Sluiter and van Rensburg, 2011). Also, many inherited neurological, muscular, and blood disorders are caused by germ-line rearrangements between sequences present in non-allelic chromosomal positions (Stankiewicz and Lupski, 2002), and smaller scale rearrangements, such as trinucleotide repeat (TNR) expansions and contractions, appear to be the primary cause of neurodegenerative diseases including Parkinsons, Huntingtons and Fragile X Syndrome (Kovtun and McMurray, 2008).

In this review we will briefly introduce the common types of repetitive elements that are present in eukaryotic cells. We will then describe the most common rearrangement events involving these sequences. Finally we will describe recent studies, that describe regulatory mechanisms that prevent such events from occurring, and outline the benefits and consequences of chromosomal rearrangements for uni- vs. multi-cellular organisms.

Types of repetitive DNA and why they exist

A. Segmental duplications. Segmental duplications (Figure 1.1A), also referred to as low-copy repeats, are among the most deleterious of repetitive sequences because rearrangements in some of these sequences are associated with disease and occur more frequently than predicted (Shaw and Lupski, 2004; Lupski and Stankiewicz, 2005). Segmental duplications, which can involve chromosomal regions of one to several hundred kilobases (KB), have arisen recently during evolution, most likely as the result of unequal sister chromatid recombination between smaller

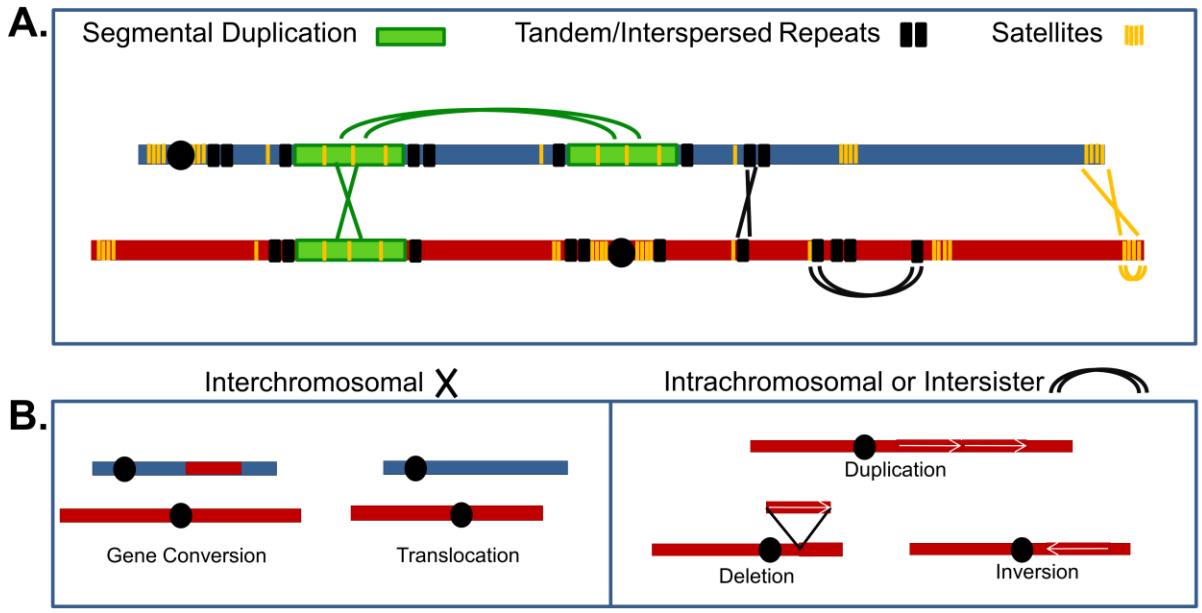

Figure 1.1 **(A)** Types of repetitive DNA sequences are illustrated on two hypothetical chromosomes (blue and red): segmental duplications (green boxes), interspersed repeats (black boxes), satellites (yellow lines) present in eukaryotic genomes and NAHR events that involve repetitive sequences. These include interchromosomal (X), intrachromosomal and intersister rearrangements (curved X). **(B)** Types of GCRs resulting from NAHR in repetitive sequences. Interchromosomal rearrangements can result in gene conversions (non-crossovers), translocations (crossovers), or unstable acentric or dicentric chromosomes (crossovers, not shown). Intrachromosomal or intersister rearrangements surrounding a chromosomal locus (white arrow) can result in duplications, deletions, or inversions.

repetitive elements and replication errors (see below). They appear unique to higher order primates and compose 5 to 10% of their genomes (Marques-Bonet and Eichler, 2009; Stankiewicz and Lupski, 2006; Bailey et al., 2001). However, some lower order organisms show evidence of whole or partial genome duplications which may have served a similar evolutionary role as segmental duplications (Timusk et al., 2011; Wolfe and Shields, 1997; Zhang et al., 2010; Zhou et al., 2011; Gu et al., 2004). The short time for divergence of the duplicated sequences has resulted in large genomic regions that share high (88 to 99%) sequence identity. The duplicated sequences arranged adjacently or on separate chromosomes can contain single or multiple genes. Segmental duplications are thought to contribute to evolution by providing the means for multiple copies of important genes to diverge and give rise to paralogs with specialized functions that can act in different environments and/or cell types (e.g. Ohno et al., 1968; Gu et al., 2004).

Segmental duplications pose threats to genome stability because they can serve as substrates for non-allelic homologous recombination (NAHR) using repair mechanisms that the cell normally uses to maintain genome stability (Shaw and Lupski, 2004; Figure 1.1B; Figure 1.2). Crossing over and non-conservative recombination events between segmental duplications can result in GCRs such as deletions, duplications, inversions, and translocations, which can in turn subject the cells to gene dosage effects, perturbations in chromosome structure, and defects in chromosome segregation (Stankiewicz and Lupski, 2006). Similar types of rearrangements occur with significant frequency between gene paralogs and ectopic sequences in budding yeast (Jinks-Robertson and Petes, 1985, 1986; Lichten et al., 1987; Bailis et al., 1992; Putnam et al., 2009; Kolodner et al., 2002), making this organism a model for studying instability of segmental duplications.

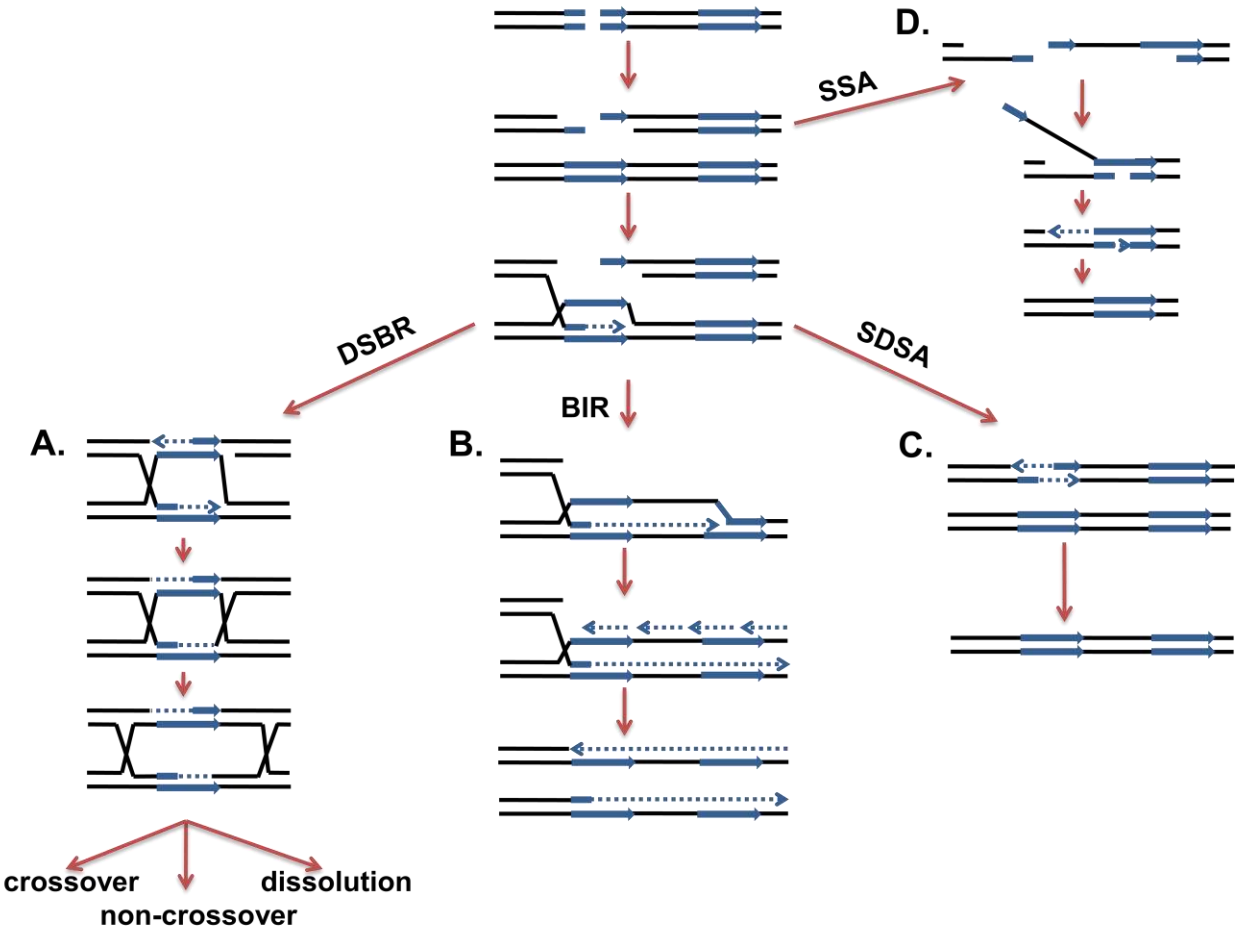

Figure 1.2. Recombination mechanisms (DSBR, SDSA, BIR and SSA) that can use repetitive DNA sequences as substrates. **(A)** DSBR and **(B)** BIR can result in crossovers and non-crossovers, SDSA **(C)** creates only non-crossovers, and SSA **(D)** creates only deletions or chromosome fusions (not shown). See the text for further details.

B. Tandem and interspersed repeats. Tandem repeats (Figure 1.1A) are multiple iterations of a few hundred to a few thousand base pairs that are often arranged in arrays of a few or many repeats. These repeats can also be interspersed throughout the genome, and can be arranged in direct or inverted orientations. However, inverted repeats are extremely unstable because they can form secondary structures that disrupt DNA replication, and consequently, are rarely seen (Cook et al., 2011; Kurahashi et al., 2009; Paek et al., 2009; Lobachev et al., 2002). Many tandem and interspersed repeats are active transposons such as Ty elements in yeast, or have their origins in transposable elements, the most common of which are *Alu* and *LINE* elements in humans. Approximately 50% of the human genome is derived from transposable elements (International Human Genome Sequencing Consortium, 2001). In addition, ribosomal DNA exists in highly repetitive arrays that are condensed into highly packed chromatin (Németh and Längst, 2011).

Like segmental duplications, tandem and interspersed repeats are substrates for NAHR (Jeffreys et al., 2004; McVean, 2010). Packing of ribosomal DNA and some high copy sequences, such as Ty elements in yeast, into compact heterochromatin-like structures prevents the formation of DNA lesions (e.g. DSBs) that initiate recombination, thus providing a mechanism to prevent recombination between these sequences (Ben-Aroya et al., 2004). However, sequences such as *Alu* and *LINE* elements are frequently found at the breakpoints of disease-associated rearrangements, suggesting that they act as recombination hotspots, perhaps by forming structures that disrupt DNA replication (Argueso et al., 2008; Narayanan et al., 2006; Puget et al., 2002; Lobachev et al., 2002; Abeysinghe et al., 2003; Pentao et al., 1992; reviewed in Chan and Kolodner, 2011). Consistent with this idea, some repetitive arrays are thought to form DNA structures that are more sensitive to breakage during DNA replication (Chuzhanova

et al., 2009; Lobachev et al., 2002; Figure 1.3), whereas others carry signature hotspot sequences that are known to increase their sensitivity to recombination by up to 10-fold (McVean, 2010; Myers et al., 2005, 2006, 2008). For example, the *RFB* and *HOT1* sequences increase recombination frequency within the ribosomal DNA array in budding yeast (Ward et al., 2000). Interestingly, recombination between *Alu* elements in the germ line appears to be a major route for generating segmental duplications (Shaw and Lupski, 2004; Zhou and Mishra, 2005; Bailey et al., 2003). Why do cells maintain repetitive sites that are hotspots for recombination? One possibility is that such events contribute to the adaptation of a species in specific environments by altering genome organization while specifically avoiding recombination within essential genes (McVean, 2010).

C. Satellites. Satellite sequences are also tandem repeats but differ from larger tandem repeats in their overall size (1 to ~ 100 nt), function, location and mode of instability (Figure 1.1A). Satellites are subdivided into minisatellites (14-100 nt) and simple sequence repeats, or microsatellites (1 to 13 nt), which tend to occur within non-coding DNA but may also occur in coding regions (Richard and Pâques, 2000). Simple sequence repeats constitute roughly 3% of the human genome (International Human Genome Sequencing Consortium, 2001). Essential genomic features such as centromeres and telomeres are composed of satellite sequences that could thus protect them from the loss of unique functional sequences. Some satellite sequences are highly unstable, undergoing frequent expansions and contractions when reaching a threshold repeat size. Though satellites could undergo homologous recombination in the presence of double-strand breaks, their instability is thought to result primarily from strand slippage during replication (Richard and Pâques, 2000; Cleary and Pearson, 2005; Strand et al., 1993; Figure

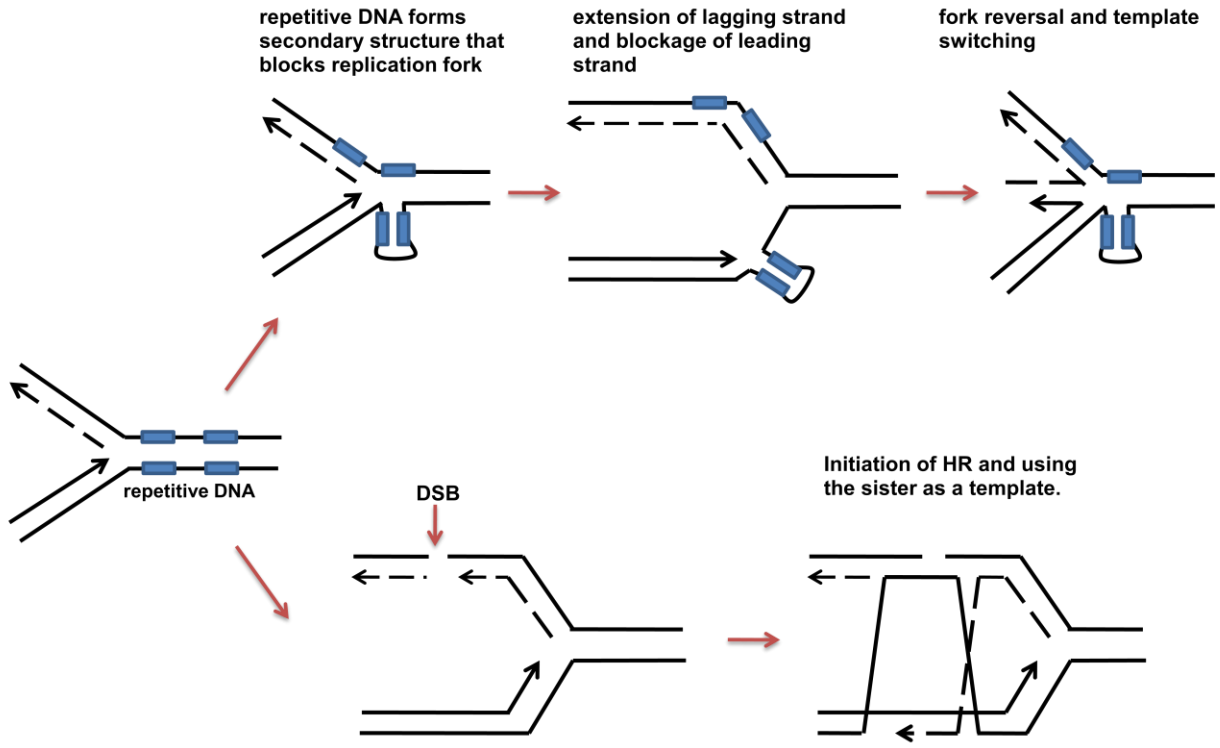

Figure 1.3. Model for how NAHR is initiated during replication. **(A)** Repetitive sequences form secondary structures that block progression of the replication fork and induce fork reversal which can result in sequence duplications. Physical stress on a stalled replication fork can also cause breakage of the fork (a DSB) and subsequent repair by homologous recombination (not shown). **(B)** Replication across single-strand gaps may also produce DSBs that may initiate homologous recombination by using the adjacent sister chromatid as a template.

1.3). Extensive expansions of satellite sequences, especially trinucleotide repeats, have been associated with neurological diseases and cancer (Claij and te Riele, 1999; Hannan, 2010). Literature regarding the dynamics of simple sequence repeats is vast; see Kovtun and McMurray (2008) for an excellent review of the evolutionary significance, mechanisms, and diseases associated with trinucleotide repeat expansion.

Mechanisms of recombination between repetitive elements

Homologous recombination (Figure 1.2) is a major cellular mechanism for repairing DNA lesions that appear due to DNA replication errors (Paques and Haber, 1999; Krogh and Symington, 2004). Homologous recombination also repairs DNA lesions resulting from environmental insults that occur during and outside of DNA replication. A single-stranded DNA gap or a stalled replication fork can induce homologous recombination, primarily through processing steps that create double-strand breaks (DSBs; e.g. Lobachev et al., 2002; Figure 1.3). Much of what is known about DSB repair pathways in eukaryotes has been obtained from work in the budding yeast *Saccharomyces cerevisiae*. Almost all of the repair factors identified in yeast have homologs in other eukaryotes, suggesting that DSB repair mechanisms are functionally conserved (reviewed in Paques and Haber, 1999; Krogh and Symington, 2004).

Homologous recombination is regulated by the type of initiating lesion and the time at which it occurs in the cell cycle. For example, during meiosis in budding yeast, repair of programmed DSBs is biased towards an allelic template located on a homologous chromosome (Roeder, 1997). The distribution of DSBs and repair bias results in the formation of crossovers between all homologs and is critical for the proper alignment and segregation of homologous chromosomes in Meiosis I. In somatic growth, repair events that lead to crossing over between

homologs are rare; other types of recombination are promoted such as double-strand break repair (DSBR) involving sister chromatids, synthesis-dependent strand annealing (SDSA), break-induced recombination (BIR) and single-strand annealing (SSA; Krogh and Symington, 2004; Paques and Haber, 1999; see below). By restricting repair between repetitive DNA sequences in non-allelic positions during somatic growth, the cell can avoid crossover events that can lead to chromosomal translocations, inversions and deletions. Below we will briefly summarize DSB repair pathways using the budding yeast nomenclature. We will indicate differences in nomenclature for the higher eukaryotic organisms when appropriate. Extensive reviews have been written on homologous recombination mechanisms (Pâques and Haber, 1999; Krogh and Symington, 2004; Mimitou and Symington, 2009a; Mimitou and Symington, 2009b; Mimitou and Symington, 2011; Symington, 2002); we will briefly discuss the currently accepted models for homologous recombination as they pertain to events involving repetitive DNA sequences.

A. DNA repair that can lead to crossing over: the canonical DSBR pathway and BIR.

DSBs created by nucleases in somatic growth and meiosis are primarily shuttled into a DSBR pathway in which the 5' strands on each end of the DSB are subject to nucleolytic degradation, revealing 3' ended single-stranded DNA (ssDNA; Szostak et al., 1983; Figure 1.2A). This 5' to 3' resection is initiated by Mre11-Rad50-Xrs2 (MRX; MRN in mammals) and Sae2 and will utilize either Exo1 or Sgs1-Top3-Rmi1 (BLM-TopoIII-RMI1 in mammals) and Dna2 for further resection (Tsubouchi and Ogawa, 2000; Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008; Mimitou and Symington, 2009a). The naked ssDNA is rapidly coated by the ssDNA binding protein RPA, which is thought to protect the ssDNA from forming secondary structures that inhibit repair. In a series of steps involving multiple factors, RPA is replaced by Rad51,

resulting in a nucleoprotein complex capable of invading into a complementary double-stranded DNA (dsDNA) template (Sugiyama et al., 1997; Sung et al., 2003). When one of the ssDNA ends invades a homologous template it will form a stable strand invasion "D-loop" intermediate and serve as a primer for DNA synthesis. Rad52 assists Rad51 filament formation by directly interacting with RPA and making it amenable to displacement by Rad51 (Sung, 1997; Sugiyama and Kowalczykowski, 2002). In addition, it promotes strand invasion by stabilizing the displaced ssDNA of the D-loop. Rad52 is also thought to assist the displaced DNA within the D-loop to capture the second ssDNA end and initiate strand synthesis, leading to the formation of a double-Holliday junction (dHJ) structure (Nimonkar and Kowalczykowski, 2009; Lao et al., 2008).

Double Holliday junctions can undergo branch migration to extend the region of sequence that will be involved in the recombination event. Less is known about the factors that promote branch migration in eukaryotes, but it appears that Rad54 and the Mph1 and Sgs1 helicases can modulate branch migration (Bugreev et al., 2006; Lo et al., 2006; Rossi and Mazin, 2008; Tripathi et al., 2007; Zheng et al., 2011). Finally, the dHJ is resolved by one of three mechanisms: 1. Resolution by an endonuclease (e.g. Mus81-Mms4 or Yen1/human GEN1) to create either a crossover or non-crossover product; 2. Dissolution by a helicase-topoisomerase (Sgs1-Top3-Rmi1/human BLM-TopoIII-RMI1) to create a non-crossover; 3. Removal of the dHJ during normal replication (Svendsen and Harper, 2010; Ira et al., 2003; Ashton et al., 2011; Plank et al., 2006; Hickson and Mankouri, 2011; Dayani et al., 2011; Esposito, 1978).
It should be noted that dHJs are thought to be resolved by endonucleases during meiosis to promote crossover formation, whereas recombination intermediates involving homologs in

somatic growth are thought to be dissolved by Sgs1-Top3-Rmi1 to promote intersister or intrachromosomal recombination (Dayani et al., 2011; Matos et al., 2011).

In contrast to recombination that occurs in meiosis, random DSBs that appear during vegetative (or somatic) growth primarily use the sister chromatid as a template for repair (Kadyk and Hartwell, 1992; reviewed in Krogh and Symington, 2004). Such a repair bias is thought to prevent interactions between chromosomes (both homologous and non-homologous) that could increase the likelihood of a chromosomal rearrangement. The resulting sister chromatid repair will not result in mutations unless mistakes are made during repair or repair occurs through unequal sister chromatid exchange (Petes, 1980; Szostak and Wu, 1980). In cases where a sister chromatid is unavailable, for example, a haploid yeast cell in G1 phase, a DSB is most often repaired non-conservatively by the non-homologous end joining pathway (NHEJ; reviewed in Symington and Gautier, 2011).

A specialized type of DSBR, BIR (Figure 1.2B), has been described which appears important for rescuing degraded chromosome arms and for maintenance of telomeres (reviewed in Kraus et al., 2001), and can be viewed as creating non-reciprocal or "half" crossover products. In BIR, a resected DNA end has formed a D-loop with a homologous template and begins replication. However, when it is unable to identify the second broken end, either because it is trapped in another repair intermediate or has been degraded, the first end will continue to replicate (McEachern and Haber, 2006; Llorente et al., 2008). Replication will continue until the second break end is found or replication reaches the end of the chromosome. Consequently BIR can result in extensive gene conversion tracts (up to a few hundred KB) or copying of an entire chromosome arm that may or may not be from a homologous chromosome.

B. DSBR that does not involve crossing over: SDSA and SSA. When a sister chromatid is available, in S or G2 phase for example, crossing over is suppressed and most of the breaks are shuttled into the SDSA pathway (Ira et al., 2003; Figure 1.2C). The resection step of SDSA is essentially the same as for DSBR. Like DSBR, one or both of the break ends will proceed to create a D-loop with a homologous chromosome, but instead of assembling both ends into a dHJ structure, the D-loop(s) will dissociate and the newly copied DNA ends will anneal to each other. At this point, further DNA synthesis can occur to fill in any gaps and DNA ligase is required to seal the nicks. Only non-crossover products are formed by SDSA.

SSA (Figure 1.2D) allows rapid repair of breaks within tandem repeat arrays, for example at the yeast and mammalian ribosomal DNA loci (Liang et al., 1998; Elliott et al., 2005; Fishman-Lobell et al., 1992; Liefshitz et al., 1995; Park et al., 1999). SSA initiates at resected DSB ends, but unlike other types of homologous recombination, it is intrachromosomal; it does not involve strand invasion and does not require a homologous chromosome or sister chromatid. Consistent with this, SSA can occur independently of Rad51 but is dependent on strand annealing factors such as Rad52 and Rad59 (Fishman-Lobell et al., 1992; Davis and Symington, 2001). In fact, Rad51 must be excluded so that Rad52 can instead catalyze strand annealing of the two complementary repetitive elements that have been revealed on opposite ssDNA ends (Wu et al., 2008; Sugiyama and Kantake, 2009). After annealing, intervening non-homologous DNA is displaced as 3' ssDNA tails and are clipped off by the Rad1-Rad10 endonuclease. DNA synthesis fills the gaps and DNA ligase seals the nicks. Since SSA results in deletions it is not conservative. However, the deletions are typically small; SSA does not occur between repeats spaced much more than 5 kb apart (Jain et al., 2009), and they may offset repeat expansions that occur frequently during replication (Kobayashi, 2011).

Suppression of NAHR

All of the homologous recombination pathways described above require a template to repair the DNA lesion; in diploids there are at most three templates located in allelic chromosomal positions; one on the sister chromatid and two on the homolog. What would happen if a resected DNA end first found a template in a non-homologous chromosome? Studies performed in budding yeast grown vegetatively or induced to enter meiosis have shown that gene conversion and crossing over between ectopic or dispersed homologous sequences can occur frequently (Jinks-Robertson and Petes, 1985, 1986; Lichten et al., 1987; Bailis, 1992). A screen in budding yeast (Putnam et al., 2009), using substrates that resemble segmental duplications in mammalian cells, showed that homologous recombination, DNA mismatch repair, and DNA damage checkpoint pathways played specific roles in suppressing chromosomal rearrangements between the segmental duplication substrate compared to rearrangements involving single copy sequences. The above studies have encouraged us to entertain the following: 1. How does the DSB repair machinery direct broken ends to the "correct" template? 2. How does it decide how much homology is sufficient to ensure that the template chosen is an allelic sequence? 3. Is the homology decision process the same across species or cell types, or even at different times during cell growth and division? As outlined below, the relatively high stability of eukaryotic genomes is accomplished by regulating early (A) and subsequent (B) steps in homologous recombination and by cell cycle control (C) and cellular organization (D) mechanisms. The combination of these regulatory mechanisms results in the avoidance of recombination between closely related non-allelic sequences yet permits recombination between slightly divergent allelic sequences.

A. Regulation of NAHR during the strand exchange step and strand annealing steps. As described above and in Figure 1.1, segmental duplications threaten genome stability because they can serve as substrates for NAHR. The initial strand invasion step (seen in DSBR, BIR and SDSA) in homologous recombination is sensitive to sequence heterology between the invading sequence and the template (reviewed in Surtees et al., 2004). For example, in baker's yeast, Rad51 is sufficient in promoting recombination between sequences with up to 10% sequence divergence (Datta et al., 1997). Using *in vitro* strand transfer reactions with the *S. cerevisiae* proteins, Holmes et al. (2001) demonstrated that Rad51-ssDNA is very efficient in promoting strand transfer between 3 KB substrates having a region of heterology up to 9-bp long, but was extremely inefficient in allowing transfer between substrates with 10 or more base pairs of heterology, indicating that there is a critical threshold for the amount of heterology that is tolerated during strand invasion. Similarly, Rad51 will allow branch migration across regions of heterology only up to 6 bp in length (Namsaraev and Berg, 2000). These studies, however, were limited by the use of insertion/deletion loop substrates, and the stringency of strand exchange was not tested with substrates containing single or multiple dispersed mismatched bases. For human Rad51, Gupta et al. (1999) demonstrated that as few as two mismatched bases within an 83-mer was enough to significantly impair strand exchange *in vitro* while 6 or 7 evenly spaced mismatches completely abolished strand exchange.

It is important to note that the activities of yeast and human Rad51 may be similar because both organisms share a similar minimal efficient processing segment (MEPS), which is defined as the smallest stretch of perfect homology that is needed for efficient recombination *in vivo*. This value is estimated to be approximately 200 bp for both mammals and yeast but only 23 to 90 bp for *E. coli* (Shen and Huang, 1986; reviewed in Waldman, 2008). The MEPS value,

however, is likely to depend on factors in addition to Rad51; for example DNA mismatch repair proteins that act in heteroduplex rejection (see below) are likely to contribute.

Holthausen et al. (2010) suggested that the length of a continuous Rad51-ssDNA filament affects the ability of the strand exchange protein to bypass DNA sequence heterology, and may correlate with amount of repetitive sequence present in the host genome. Rad51 filament nucleation *in vitro* is less efficient than the bacterial strand exchange protein RecA and consequently Rad51-ssDNA filaments are less continuous and more flexible compared to RecA-ssDNA filaments. While having more flexibility may increase the rate of homology search (allowing multiple contacts with dsDNA at once), the shorter stretches of Rad51-ssDNA filament may be incapable of stabilizing strand invasion intermediates with large regions of heterology. This could afford yeast and mammals a better chance at finding the proper homologous template within the densely packed structure of eukaryotic chromosomes while also giving them enough stringency to avoid NAHR within their highly repetitive genomes.

On the other hand, the structure of the filaments *in vivo* may not share the same amount of flexibility as suggested by *in vitro* nucleation reactions. The incorporation of Rad51 paralogs Rad55 and Rad57 into filaments (Liu et al., 2011) may alter their flexibility and bypass requirements (Ragone et al., 2008; Holthausen et al., 2010). Regardless, Rad51 and RecA require ATP hydrolysis for bypassing heterology, which in itself serves as a barrier to strand exchange between sequences of imperfect homology (Rosselli and Stasiak, 1991; Sung, 1994).

The mechanism by which the Rad51-ssDNA filament searches for homology and invades dsDNA is currently being investigated *in vitro* using single-molecule magnetic tweezer and total internal reflection fluorescence microscopy technologies. Studies using human Rad51 and *E. coli* RecA have been able to follow filament formation and strand invasion, respectively, in real

time (Miné et al., 2007; van der Heijden et al., 2008). Single molecule studies with a variety of heterologous substrates should prove to be revealing about how and to what extent Rad51 and related proteins bypass heterology.

The strand annealing activity of Rad52 is also capable of bypassing limited amounts of heterology. For example, mismatch recognition and helicase mutants in yeast will allow SSA between tandem repeats sharing only 97% homology, indicating that Rad52 must allow efficient annealing between at least modestly divergent sequences (Sugawara et al., 2004). *In vitro* single-strand annealing by human Rad52 observed by fluorescence resonance energy transfer generated a model for the initial homology search and subsequent extension of annealing (Rothenberg et al., 2008). Initial homology is first identified in patches of four nucleotides followed by sampling of several adjacent nucleotides for homology. In order for annealing to initiate, there must be sufficient homology between the adjacent nucleotides such that annealing is more energetically favorable than the hRad52-ssDNA interaction, since hRad52 cannot be bound to dsDNA. The model predicts that a stretch of approximately ten complementary bases are required to initiate annealing. Extension of the annealed region is then predicted to occur in segments of several nucleotides. The segments are initially brought into proximity by the interaction of two hRad52 oligomers, one bound to each ssDNA, and if the ssDNA segments are sufficiently homologous they will anneal and release the hRad52 oligomers. Considering this mechanism, hRad52 may allow annealing across occasional single nucleotide mismatches that do not significantly contribute to the energetic transaction required for annealing, but more extensive regions of heterology would not be able to overcome the energy barrier unless they could be "looped out." RPA could assist in annealing by removing secondary structure to open up the DNA for assembly of Rad52 oligomers and more efficient searching, and it may also

minimize "loop out" structures that could be bypassed by Rad52. Indeed, appropriate concentrations of RPA can enhance hRad52-mediated annealing (Grimme et al., 2010). Continued single molecule studies with more diverse DNA substrates will reveal more specifically the extent to which Rad52 can bypass heterology.

Another possible level of regulation is control of homologous recombination through the Rad54 motor protein. Rad54 has been called the "Swiss Army knife" of HR (Heyer et al., 2006) because of its multi-functional roles at almost every step of homologous recombination. Rad54 is involved in stimulating strand invasion and D-loop formation through contacts with the Rad51 filament (Ceballos and Heyer, 2011; Kiianitsa et al., 2006), but perhaps more importantly, Rad54 is essential for the transition from strand invasion to recombination-associated DNA synthesis (Li and Heyer, 2009). In yeast, Rad54 is required to displace Rad51 from the 3'OH end of the invading strand to allow assembly of DNA polymerase δ for extension of the invading strand. But what inhibits Rad54 from displacing Rad51 prematurely or from a template of insufficient homology? There is likely to be a minimum amount of homology, length, or level of stability of the D-loop that is required before DNA synthesis can be initiated. Research into the mechanism and homology requirements for this role of Rad54 is critical for determining whether it has a significant impact on NAHR. Furthermore, a role for mammalian Rad54 in initiating DNA synthesis from a D-loop has yet to be confirmed (Li and Heyer, 2009).

B. Regulation of NAHR: Disrupting heteroduplex intermediates (Figures 1.4 and 1.5).

Allelic sequences on homologous chromosomes can differ on the order of 1-2%; therefore regulation of NAHR must be finely balanced so that recombination with an extensively divergent non-allelic sequence is avoided but recombination with an allelic sequence is allowed.

Though strand annealing and exchange enzymes can limit NAHR between significantly divergent sequences (see above), they are not stringent enough to distinguish more closely related non-allelic sequences from minimally divergent allelic sequences. For this reason, organisms have evolved an additional level of regulation of NAHR that can disrupt heteroduplex recombination intermediates between modestly divergent sequences. These closely related sequences are often called homeologous sequences, and generally display sequence divergence of approximately 2-15%. Disruption of heteroduplex intermediates, termed heteroduplex rejection, involves the concerted action of mismatch repair (MMR) proteins, helicases, and topoisomerases (Sugawara et al., 2004; Surtees et al., 2004; Bailis et al., 1992; George and Alani, unpublished results).

DNA MMR proteins are commonly known for their role in repairing mismatched bases arising during replication. The MMR system in eukaryotes is composed of two mismatch recognition heterodimers: Msh2-Msh6 which indentifies single-base mismatches and insertion/deletion loops of 1-2 bases and Msh2-Msh3 which recognizes larger insertion/deletion loops. An additional heterodimeric complex Mlh1-Pms1 transmits the MMR signal to nucleases including Exo1, which excise the nascently replicated strand containing the DNA mismatch (reviewed in Kolodner and Marsischky, 1999; Li, 2008). Extensive evidence for MMR proteins suppressing homeologous recombination has been obtained in bacteria and lower and higher eukaryotes (reviewed in Surtees et al., 2004). Studies in yeast using recombination reporter cassettes revealed that MMR mutants show increased crossing over and more extensive gene conversion tracts (Datta et al., 1997; Datta et al., 1996; Chen and Jinks-Robertson, 1998; Selva et al., 1995), suggesting that MMR factors act at both early and late stages of homologous recombination, perhaps to minimize both strand exchange and branch migration in the presence

of heterology. Other work (e.g. Nicholson et al., 2000) showed that the substrate specificities of Msh2-Msh3 and Msh2-Msh6 for suppressing recombination in heteroduplex intermediates were similar to their specificities during mismatch recognition in MMR. Mlh1-Pms1 and Exo1 play relatively minor roles in suppressing recombination, suggesting that heteroduplex rejection may not require a strong need for transduction of a signal to downstream nucleases. Consistent with distinct functions for MMR and heteroduplex rejection, a recent study in which Msh2-Msh6 function was restricted to a specific stage in the cell cycle indicated a coupling of MMR but not heteroduplex rejection to DNA replication (Hombauer et al., 2011).

It is now accepted that MMR factors, RecQ helicases (Sgs1), and type III topoisomerases (Top3-Rmi1) are primarily responsible for disrupting heteroduplex intermediates, but how their activities are coordinated on various types of recombination intermediates is still under active investigation (Nicholson et al., 2000; Sugawara et al., 2004; Goldfarb and Alani, 2005; Mankouri et al., 2011; Lo et al., 2006; Watt et al., 1996; Raynard et al., 2006; Ira et al., 2003). Furthermore, there are multiple steps where heteroduplex rejection might occur such as during strand invasion, extension of the invading strand, annealing of newly replicated strands (SDSA), second-end capture, and branch migration (DSBR). To study heteroduplex rejection in a simplified system that could ultimately be studied *in vitro*, the Haber and Alani labs used an SSA-based assay (Sugawara et al., 2004; Goldfarb and Alani, 2005) in which there is only a single heteroduplex intermediate. This assay revealed that Msh2-Msh6 and Sgs1-Top3-Rmi1 were required to disrupt annealed heteroduplex intermediates between 3% divergent sequences by a conservative unwinding mechanism (Sugawara et al., 2004; Goldfarb and Alani, 2005; C. George and E. Alani, unpublished observations). Mlh1-Pms1, Exo1, and other helicases (e.g.

Srs2) did not have a role in rejecting the heteroduplex intermediate, and Msh2-Msh3 could not be tested due to its requirement for 3' non-homologous tail removal during SSA.

The current model for heteroduplex rejection during SSA is that base mismatches and insertion/deletion loops within heteroduplex intermediates are recognized by the Msh proteins which directly interact with Sgs1 to stimulate unwinding of the heteroduplex DNA (Figure 1.4). Top3-Rmi1 may be required to relieve supercoils during unwinding, to stimulate Sgs1 helicase activity, or to direct it to the ssDNA tails on the SSA intermediate. Several observations support this model. First, both yeast Sgs1 and human RecQ helicases can physically interact with Msh6 (Doherty et al., 2005; Saydam et al., 2007; Yang et al., 2004; A. Lyndaker and E. Alani, unpublished results), and RecQ helicase activity is stimulated by human MSH6 (Yang et al., 2004). Furthermore, *in vitro* Sgs1 binds to and unwinds 3'-tailed substrates similar to the structure of SSA intermediates and is strongly stimulated in the presence of Top3-Rmi1 (Cejka and Kowalczykowski, 2010; Cejka et al., 2010; Bennett et al., 1998; Bennett et al., 1999).

The molecular switch model has become an important hypothesis to explain how mismatch recognition and downstream steps in MMR are coordinated (Acharya et al., 2003). This model is supported by experiments suggesting that mismatch binding by Msh protein triggers an ADP -> ATP exchange that enables it to enter a sliding clamp diffusion mode (Kunkel and Erie, 2005; Jiricny, 2006). In one version of the model, mismatch recognition stimulates Msh complexes to move away from a mismatch site until it encounters a signal (e.g. a DNA nick or PCNA loaded at a nick). Based on such studies one can imagine that mismatch recognition during heteroduplex formation would stimulate Msh2-Msh6 to search for factors that modulate recombination. For example, during SSA, upon mismatch recognition Msh2-Msh6 may recruit Sgs1 to the 3' tail or encounter Sgs1 upon reaching the tail, after which the Sgs1

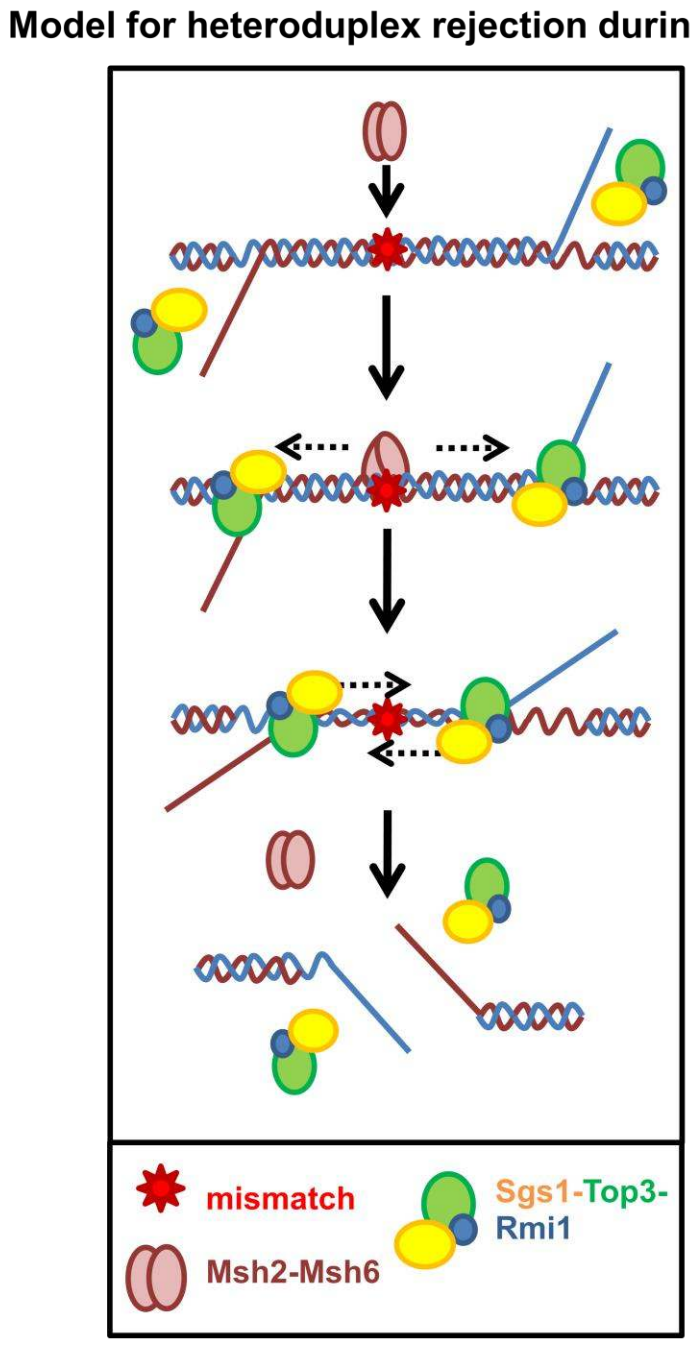

Figure 1.4. A model for how mismatch and double-strand break repair factors can collaborate to reject recombination between divergent DNA sequences during SSA. After annealing of divergent sequences, Msh proteins (i.e. Msh2-Msh6, pink ovals) can recognize base mismatches (red star) in the heteroduplex intermediate and changes conformation to begin a search for Sgs1-Top3-Rmi1 (yellow oval, light green oval, blue oval). Sgs1-Top3-Rmi1 can load onto the junction between the heteroduplex and the 3' non-homologous tail and is stimulated by Msh2-Msh6 to unwind the duplex.

helicase is activated. In the event of larger or more complex heteroduplex intermediates (i.e. strand invasions or Holliday junctions, Figure 1.5), Msh2-Msh6 may be more likely to encounter nicks, gaps, or an active replication fork and will instead stimulate Mlh1-Pms1, which could either participate in repair of the mismatch or modulate disruption of the heteroduplex through an alternative (possibly nucleolytic) mechanism. Such a model would be consistent with the modest role of Mlh1-Pms1 and Exo1 in heteroduplex rejection during the inverted repeat assay (Nicholson et al., 2000) but not the direct repeat assay (Sugawara et al., 2004). Current studies in our laboratory are aimed at confirming and distinguishing between these proposed models using a chromatin immunoprecipitation approach and to explore the role (if any) for PCNA in heteroduplex rejection during SSA.

In addition to rejection mechanisms that involve MMR factors, several helicases have been shown to prevent crossover formation during DSB repair and could thus prevent NAHR. Yeast Mph1 and human RTEL1, for example, dissociate D-loops to promote non-crossover rather than crossover repair of a DSB (Prakash et al., 2009; Barber et al., 2008). Srs2 discourages homologous recombination by dismantling Rad51 pre-strand invasion filaments (Krejci et al., 2003; Veaute et al., 2003). Also, in addition to its role in disrupting heteroduplex recombination intermediates (see above), Sgs1-Top3-Rmi1 and mammalian homologs suppress crossovers by dissolving dHJs (Ira et al., 2003; Ashton et al., 2011; Plank et al., 2006).

C. Regulation of recombination by cell cycle control: Limiting recombination to times when allelic templates are nearby. The initiating event of homologous recombination is end resection. Resected ends are unstable (Zierhut and Diffley, 2008) and need to be engaged with recombination factors as soon as they are formed to ensure that they can easily find an

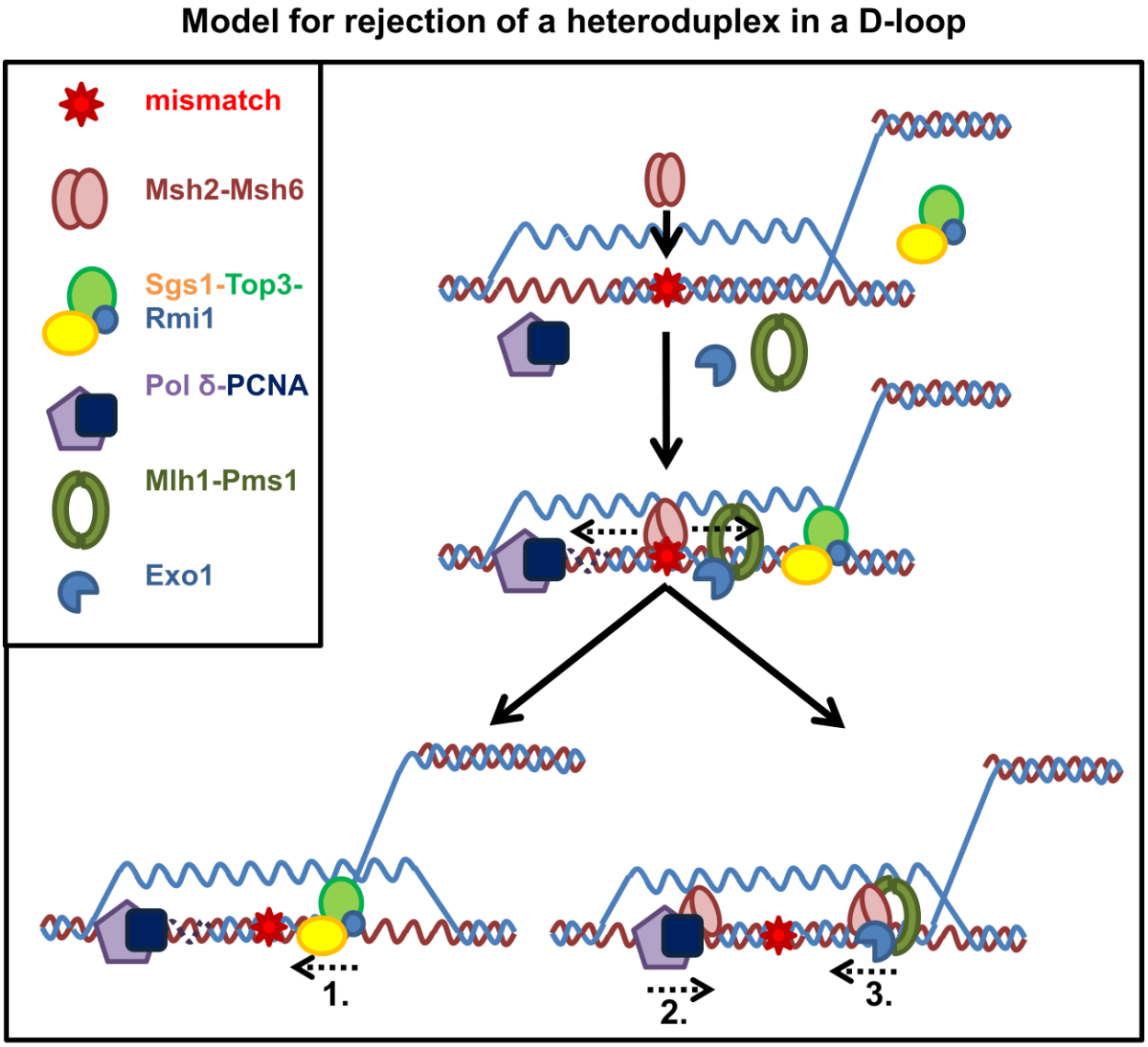

Figure 1.5. Heteroduplex rejection within a D-loop. Similarly to rejection during SSA (Figure 1.4), Msh2-Msh6 (pink ovals) will locate a mismatch (red star) and switch to searching mode. Msh2-Msh6 may either **(1.)** find Sgs1-Top3-Rmi1 (yellow oval, light green oval, blue oval, or another helicase such as Srs2, not shown) loaded at the duplex junction to stimulate unwinding, or instead may **(2.)** find an active replication fork (represented as Pol δ, purple hexagon) through a direct interaction with PCNA (dark blue square) to stimulate nucleolytic degradation. In a third alternative **(3.)**, Mlh1-Pms1(dark green ring) may accompany activated Msh2-Msh6 during the search and may recruit an exonuclease such as Exo1 (blue pac-man) to either stimulate repair of the mismatch, or to disrupt the D-loop by a nucleolytic mechanism.

appropriate complementary partner. Both yeast and mammals ensure that resection can only occur during the S and G2 stages of the cell cycle when allelic sequences in sister chromatids are in close proximity (Aylon et al., 2004; Ira et al., 2004; Jazayeri et al., 2006; Huertas and Jackson, 2009; Symington and Gautier, 2011; reviewed in Lee and Myung, 2009).

Cyclin-dependent kinases (CDKs) that control progression through cell cycle stages also limit resection to the S and G2 stages. In budding yeast, the primary catalytic subunit of CDKs Cdk1, controls resection in at least two ways. First, resection is inhibited by the association of the Rad9 DNA damage checkpoint protein with broken DNA ends, blocking assembly of the resection machinery. This association of Rad9 with DSBs requires methylation of histone H3-K79 by the Dot1 methylase. Histone methylation generally results in chromatin compaction that in this situation suppresses DSB resection, rather than repressing transcription (El-Osta and Wolffe, 2000). Resection is initiated during S and G2 in a Cdk1-dependent manner, and Cdk1 dependency can be bypassed in a *rad9*Δor *dot1*Δ mutant (Lazzaro et al., 2008). Presumably, a G2-phase expressed Cdk1-Clb modifies Rad9 to abolish the Rad9-histone interaction, releasing Rad9 from the DSB end and allowing resection. Alternatively, but perhaps not exclusively, a G1-expressed Cdk1-Cln may promote H3-K79 methylation and/or association of Rad9 with histone H3. The second level of control by Cdk1 is through direct modification of the resection machinery. Cdk1-Clb phosphorylates the Sae2 endonuclease which activates Sae2 activity in DNA end resection (Huertas et al., 2008). Regulation of resection through both Rad9 and Sae2 are functions conserved in the mammalian homologs 53BP1 and CtIP (Huertas and Jackson, 2009; Huyen et al., 2004).

During G0 or G1, resection is either absent or very limited. As a result, DSBs are typically repaired by non-homologous end joining (NHEJ; Zierhut and Diffley, 2008). Since

DSBs may not be in proximity to allelic sequences during G0 and G1, NHEJ is preferable so that use of a non-allelic complementary sequence for homologous recombination is limited. As long as only one DSB is present, NHEJ will repair the break without GCRs. Though NHEJ is non-conservative, mutations would be limited to the region of the DSB and would pose less risk than would a chromosomal translocation. Spontaneous DSBs occurring during the mitotic cycle are rare (Lettier et al., 2006), so the likelihood of two or more DSBs occurring simultaneously and thus the potential for NHEJ-mediated translocation is extremely rare, provided the cell is not exposed to DNA-damaging conditions or is pre-disposed to chromosomal instability.

In addition to activation of resection during S and G2, recombination factors including Rad51 and Rad52 are expressed at higher levels during S and G2 in yeast (Chen et al., 1997), and proteins targeted to the nucleus by Cdk1 include Dna2 and the dHJ resolvase Yen1 (Chen et al., 2011; Kosugi et al., 2009). Both Mus81-Mms4 and Yen1 are also regulated in coordination with the cell cycle to limit crossover formation during mitosis (Matos et al., 2011). Clearly, regulation of homologous recombination is very intimately involved with cell cycle dynamics. It remains to be determined whether Cdks can control expression, activation, or nuclear localization of any other recombination factors or proteins that disrupt heteroduplex intermediates.

D. Regulation of recombination by chromosome organization: Restricting availability of potential non-allelic templates in space (Figure 1.6). Chromosome pairing, also known as somatic pairing is a major form of regulation that restricts repetitive sequences in space (reviewed in Burgess et al., 1999). Work in budding yeast using fluorescence *in situ* hybridization analysis and recombination assays (Weiner and Kleckner, 1994; Burgess and

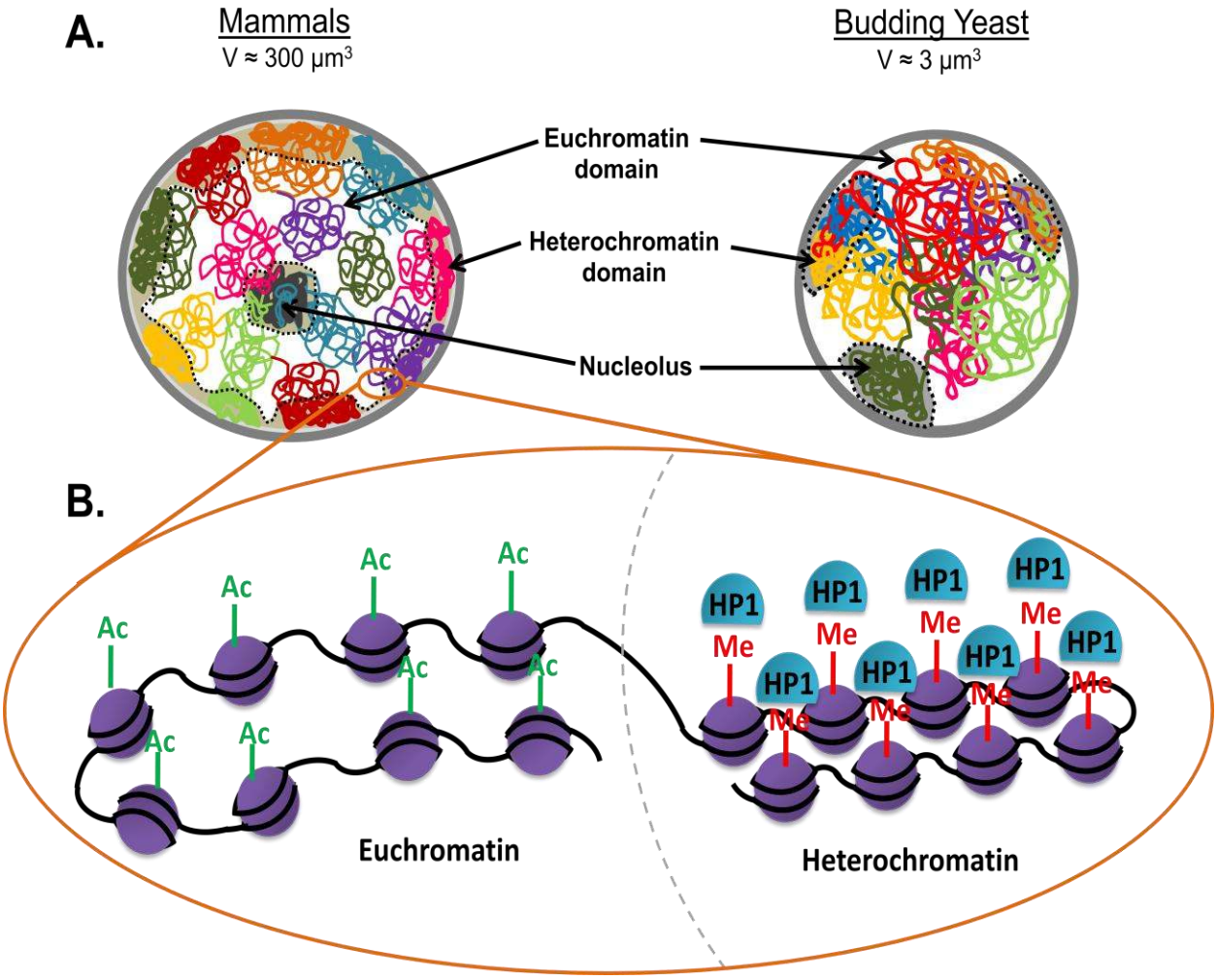

Figure 1.6. **(A)** Model for the nuclear organization of chromatin in mammalian and budding yeast nuclei. Fractal-globule models (Mirny, 2011) predict that individual chromosomes (distinguished by color) in mammalian nuclei (left) are folded into distinct, untangled territories with heterochromatin domains associated with the nuclear lamina and euchromatin in the center of the nucleus. The nucleolus is a distinct heterochromatin domain that houses ribosomal DNA which is distributed among multiple chromosomes in humans. The yeast nucleus (right), which is 100 times smaller than an average mammalian nucleus (3 versus 300 μm^3), is predicted to be less tolerable of a fractal globule model so that chromosomal territories are more closely entwined. 3C modeling by Duan et al., (2010) show protrusion of the rDNA locus on chromosome 12 into a distinct heterochromatin domain and also clustering of other heterochromatin regions such as centromeres and telomeres. **(B)** Heterochromatin is more compact than euchromatin and is associated with specific marks such as methylated (Me) histones and HP1 protein. Histones in euchromatin are usually acetylated (Ac). Nucleosomes (purple circles).

Kleckner, 1999; Burgess et al., 1999) has shown that chromosome homologs are paired in vegetative growth but this pairing is disrupted in S-phase and in G2-arrested conditions. This pairing ensures that allelic DNA sequences are closer to each other relative to similar sequences located on nonhomologous chromosomes. Burgess et al. (1999) suggest that "pairing may exist to promote juxtaposition of homologous regions within irregular genome complements." Such pairing could thus serve to restrict the availability of potential non-allelic substrates.

In addition to pairing of allelic sequences, non-allelic sequences are sequestered from each other via organization of the nucleus. The nucleus of lower and higher eukaryotic cells is composed of distinct but dynamic sub-compartments that confine intranuclear processes to a limited space. (Léger-Silvestre et al., 1999). Recent chromosome conformation capture studies in yeast and human interphase cells have modeled these compartments in three-dimensional space. This work shows that highly repetitive, primarily non-coding, DNA is organized into heterochromatin domains located near the nuclear periphery. In mammalian cells, chromosomes are organized into their own stable globular territories, away from other chromosomes that may share similar sequences (Tanizawa and Noma, 2011; Duan et al., 2010; Figure 1.6A). Fractal-globule models (reviewed in Mirny, 2011) predict that globular territories are the natural folded state of individual chromosomes within the environment of the mammalian nucleus; however it is possible that a mechanism, perhaps analogous to chromosome motion mechanisms seen in meiosis in a variety of organisms, prevents individual chromosomes or domains from being tangled or interlocked during movement of chromosomes (Wanat et al., 2008).

The nucleolus, which has historically been a sub-compartment of mysterious function, is now being recognized as an extensively heterochromatic domain that houses and protects the repetitive ribosomal DNA arrays from genome instability (Chiolo et al., 2011). These

observations are re-defining the term "heterochromatin;" rather than being defined *by* regions of gene silencing and characteristic marks such as methylated histones and the HP1 protein, they are being defined as regions of condensed repetitive DNA that are usually associated with these features (Peng and Karpen, 2008; Figure 1.6B). Heterochromatin is highly enriched for essential genomic features such as centromeres, telomeres, and ribosomal DNA, and is essential for their function and protection (Peng and Karpen, 2008). Heterochromatin is also enriched for satellites and transposable elements that are sequestered to prevent hyper-recombination (Slotkin and Martienssen, 2007).

At first the idea of repetitive DNA being confined to a tightly packed region is counterintuitive to global chromosome pairing mechanisms that prevent the close alignment of non-allelic sequences that could participate in NAHR. However, such an organization provides the advantage of sequestering DNA packaged into heterochromatin from non-allelic sequences located in distant areas of the genome, leaving it at risk for only small-scale intrachromosomal instability. The take-home message from studies done so far is that repetitive DNA resides inside a heterochromatic environment where recombination using a homologous chromosome is suppressed and recombination that is intrachromosomal or with a sister chromatid is promoted.

Heterochromatin displays very limited γH2AX foci (a DSB marker) compared to euchromatin following treatment with ionizing radiation (Costes et al., 2010); this observation suggests that heterochromatin is less accessible and possibly, less sensitive to DNA-damaging agents that initiate recombination. Such observations have led to the idea that genome stability of heterochromatin is maintained in a manner similar to heterochromatin-mediated gene silencing (Peng and Karpen, 2008; Osley and Shen, 2006).

Though chromatin dynamics involved in gene silencing have been extensively studied, chromatin dynamics in DSB repair is a very recent focus and is less clear. Like gene silencing, chromatin structure appears to regulate homologous recombination through two major mechanisms; histone modification and nucleosome remodeling (Peng and Karpen, 2008; Figure 1.7A). In yeast, modified histone components such as phospho-H2A and γH2AX appear at sites of DSBs coincidently with the chromatin remodelers SWI/SNF, RSC, and Ino80 (Shroff et al., 2004; Rogakou et al., 1999; Celeste et al., 2003; Shen et al., 2000; Chai et al., 2005). All three of these remodelers appear to be required for DSB repair in yeast (Chai et al., 2005; Shim et al., 2007; Morrison et al., 2004; van Attikum and Gasser, 2005; van Attikum et al., 2007; van Attikum et al., 2004), and similar roles for equivalent chromatin remodelers appear to be conserved in humans and *Drosophila* (Ogiwara et al., 2011; Park et al., 2010; Kusch et al., 2004). Histone modifications are thought to contribute to DSB-dependent chromatin remodeling by recruiting remodelers to the DSB (Ogiwara et al., 2011), and by modulating the localization of HR proteins to the break (Lazzaro et al., 2008; Osley and Shen, 2006; Oum et al., 2011; Tsukuda et al., 2009). In addition, RSC promotes loading of cohesins to the DSB site to hold sister chromatids close together, thereby restricting NAHR by promoting sister chromatid recombination (Figure 1.7A; Liang et al., 2007).

Chromatin remodeling surrounding DSBs within heterochromatin domains is critical for their repair by homologous recombination. Furthermore, if a DSB within a heterochromatic region has to be repaired using a homolog, it must be moved to a more euchromatic environment to do so. Recent work by Chiolo et al., (2011) in *Drosophila* showed that DSBs in heterochromatin could be resected quickly but had to be moved, by a yet to be understood mechanism, to the heterochromatin periphery and create a local environment more typical of

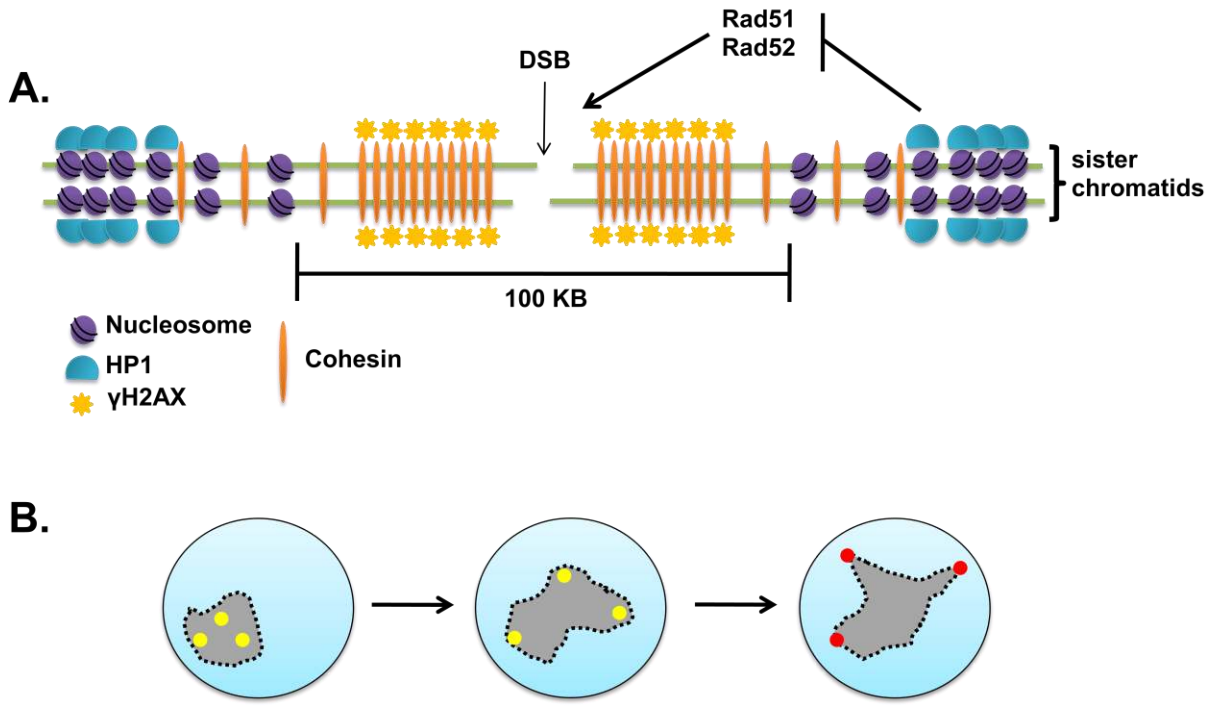

Figure 1.7. **(A)** DSB repair by homologous recombination requires chromatin modifications and nucleosome remodeling within approximately 50 KB on each side of the DSB to facilitate loading of HR proteins that are excluded from heterochromatin (i.e. Rad51 and Rad52). During S and G2 phase, break-induced loading of cohesin (orange lines) occurs within the region of the DSB to facilitate sister chromatid recombination and this is dependent on γH2AX (yellow stars) and the resection initiator MRX (not shown). This panel is based on Figure 2 of Lee and Myung (2009). **(B)** Model for movement of DSBs within heterochromatin to the heterochromatin periphery, as described by Chiolo et al., (2010). DSBs (yellow) within heterochromatin domains (gray) move, by an unknown mechanism, toward the periphery of the heterochromatin (dotted line) accompanied by a global expansion of the heterochromatin domain. Finally, DSBs protrude into the euchromatin domain (light blue) where the Rad51 protein (red) is available for homologous recombination.

euchromatin in order to have access to Rad51 for strand invasion (Figure 1.7B). Similarly, Torres-Rosell et al. (2007) showed that Rad52 was excluded from the nucleolus in yeast and had to be moved to an extranucleolar site for repair by HR. The reason for condensation of repetitive DNA into heterochromatin, then, may be to promote the use of non-conservative repair at times when homolog pairing is absent and non-allelic sequences are more available.

Closing thoughts

In this review, we outlined the numerous cellular and molecular mechanisms by which genome stability is maintained, particularly in the suppression of NAHR events that can create GCRs. GCRs are more destructive than other types of genomic alterations because they can disrupt multiple genes at once and alter chromosome organization, making cells more susceptible to DNA damage (Stankiewicz and Lupski, 2002). Furthermore, a chromosome that receives a large genomic alteration is often less stable, either due to altered chromatin status or as in the case of a dicentric chromosome, prone to further rearrangement (Schmidt et al., 2010; Hastings et al., 2009; Fournier et al., 2010). Multiple breakages and fusions of unstable chromosomes are thought to contribute to chromothripsis, the rapid bursts of large-scale genomic rearrangements that can be found in many cancerous tumors (Stephens et al., 2011).

Unicellular organisms such as budding yeast can better tolerate recombination during vegetative growth because, unlike multi-cellular organisms, a mutation that enhances the survival of a single cell can be passed to future generations and enhance survival of the species. Also a deleterious mutation in a uni-cellular organism will only eliminate a single cell (or small population of cells) without affecting survival of the species as a whole. In contrast, if a single

somatic cell in a multicellular organism receives a GCR, the organism could develop cancer. Such differences between multi- and uni-cellular organisms could explain why somatic mammalian cells favor non-homologous end joining as the primary mode of DSB repair, whereas vegetative yeast primarily use homologous recombination mechanisms such as SDSA (e.g. Guirouilh-Barbat et al., 2004; Mao et al., 2008).

The mammalian genome appears to have accommodated widespread repetitive elements by providing each chromosome a specific domain. However, in the more tightly packed yeast nucleus, individual chromosomes are in intimate contact, which could explain why yeast have a limited number of repetititve elements in its genome (Tanizawa and Noma, 2011; Duan et al., 2010; Figure 1.6A). For mammalian genomes, perhaps the positive functions provided by repetitive sequences (e.g. their presence in centromeric, telomeric and ribosomal DNA) are worth the risk of a rare NAHR event. Such a fitness cost could also be offset by a greater chance for genetic variability because the opportunity for recombination is restricted to prevent GCRs.

Here, we illustrate that multiple redundant forms of regulation - early and late recombination mechanisms, cell cycle control, and chromosome organization - collaborate to ensure the stability of repetitive DNA. Because of the essential nature of genome maintenance in adaptive evolution and prevention of disease, these mechanisms are likely to act redundantly. Support of this idea comes from analysis of chromosome stability in MMR-defective human and yeast cell lines. While specific assays have been used to show critical roles for MMR in preventing NAHR, MMR defective lines are in fact karyotypically stable (Heck et al., 2006; Snijders et al., 2003). However, it is likely that such redundant mechanisms are disrupted in some cancers where hundreds to thousands of GCRs can exist within a single tumor (Chen J-M et al., 2010; Stratton et al., 2009; Velculescu 2008).

Of the mechanisms discussed, genome organization may have the most important role in maintaining stability of repetitive DNA and genome stability in general. Large perturbations in genome organization could conceivably disrupt sequestered repetitive elements and compromise other genome protection mechanisms as well. In support of this, mutations within genes that establish and maintain global genome structure – cohesins, condensins, histone acetylases, histone deacetylases, and histone methylases – can cause massive genome instability that is suggested to drive some cancers (Strunnikov, 2010; Fraga and Esteller, 2005). In some cases, such as in the presence of condensin dysfunction, distinct regions of repetitive DNA can become more sensitive to breakage, increasing the opportunity for NAHR (Samoshkin et al., 2011). Beyond genome organization, the relative importance of the specific mechanisms that ensure stability of repetitive DNA will depend on the type of DNA damage, when it occurs during the cell cycle, and the relative importance of disrupted genes for the tissue and organism suffering the damage.

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CHAPTER II: RESEARCH STUDY I

The DNA damage checkpoint promotes recombination between divergent DNA sequences in budding yeast.[2]

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Abstract

In the early steps of homologous recombination, single-stranded DNA (ssDNA) from a broken chromosome invades homologous sequence located in a sister or homolog donor. In genomes that contain numerous repetitive DNA elements or gene paralogs, recombination can potentially occur between non-allelic/divergent (homeologous) sequences that share sequence identity. Such recombination events can lead to lethal chromosomal deletions or rearrangements. However, homeologous recombination events can be suppressed through rejection mechanisms that involve recognition of DNA mismatches in heteroduplex DNA by mismatch repair factors, followed by active unwinding of the heteroduplex DNA by helicases. Because factors required for heteroduplex rejection are hypothesized to be targets and/or effectors of the DNA damage response (DDR), a cell cycle control mechanism that ensures timely and efficient repair, we tested whether the DDR, and more specifically, the *RAD9* gene, had a role in regulating rejection. We performed these studies using a DNA repair assay that measures repair by single-

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[3] Amy Lyndaker contributed to this work in an intellectual capacity

strand annealing (SSA) of a double-strand break (DSB) using homeologous DNA templates. We found that repair of homeologous DNA sequences, but not identical sequences, induced a *RAD9*-dependent cell cycle delay in the G2 stage of the cell cycle. Repair through a divergent DNA template occurred more frequently in *RAD9* compared to *rad9Δ* strains. However, repair in *rad9Δ* mutants could be restored to wild-type levels if a G2 delay was induced by nocodazole. These results suggest that cell cycle arrest induced by the Rad9-dependent DDR promotes repair between divergent DNA sequences despite the potential for creating deleterious genome rearrangements, and illustrates the importance of additional cellular mechanisms that act to suppress recombination between divergent DNA sequences.

1. Introduction

The DNA damage response (DDR) plays a central role in ensuring that critical biological processes, such as immunoglobulin diversification, gamete development, and telomere homeostasis, occur with limited errors [1-3]. These processes rely on programmed genomic insults that are repaired in a highly regulated manner, and the DDR is essential for coordinating their repair with cell growth and division. Humans and mice with defects in the DDR exhibit increased genomic instability and can display increased incidence of cancer, neurodegeneration, immunodeficiency, or infertility (i.e. Ataxia telangiectasia, Nijmegen breakage syndrome, Down's syndrome, Alzheimer's disease [4]).

Genome stability is maintained by groups of proteins that recognize and repair DNA damage in the form of replication or recombination errors and chemically or radioactively-induced lesions [4-8]. DNA double-strand breaks (DSBs) are a cytotoxic type of DNA damage that can result from strand breakage associated with physical stress, ionizing radiation, endonuclease cleavage, stalled intermediates in DNA lesion processing, and replication fork collapse [9-16]. DSBs are often repaired by one of several forms of chromosomal recombination, and their timely and accurate repair is essential for avoiding the genomic rearrangements that can lead to disease.

In budding yeast the DDR is critical for promoting efficient repair of DSBs. Upon formation of a DSB, a single DNA strand is resected from each broken end in the 5' to 3' direction, exposing 3' single-stranded DNA (ssDNA). The ssDNA is immediately bound by RPA, followed by binding of complexes containing the Mec1/Tel1 PIKK protein kinases and Rad9 [17,18]. Rad9 is phosphorylated by Mec1/Tel1 and forms an oligomer which serves as a scaffold for Rad53, allowing for Rad53 autophosphorylation [19]. Rad53 is the central DDR

transducer which signals to many downstream effectors to promote localization of DNA repair factors to sites of damage and delays cell cycle progression to ensure that the damage is repaired before cell division [19-21]. If a DSB fails to be repaired, the cell will either remain terminally arrested at the G2/M stage of the cell cycle or will undergo break adaptation and die after several divisions [22-24].

Though the DDR has been widely studied, our understanding of all of its downstream steps is far from complete. One area that is not well understood is the role of the DDR in the choice of DSB repair pathway and the recognition of the correct repair template for homologous recombination. DSB repair may occur by the non-conservative non-homologous end joining pathway (NHEJ), or by one form of conservative homologous recombination (HR) including classical double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA), single-strand annealing (SSA), or break-induced replication (BIR), all of which initiate with strand invasion or annealing of homologous DNA sequences which are then used as templates for DNA synthesis to fill in sequence gaps [25]. The current understanding is that the DSBR and SDSA pathways are preferred during the late S or G2 stage of the cell cycle or meiotic pachytene when chromosomes are in close proximity to a sister chromatid or homologous chromosome. In contrast, NHEJ is functional at all cell cycle stages, and therefore is primarily responsible for repairing breaks during the G0 or G1 stages in mammals (though it plays a smaller role in DSB repair in yeast) when homologous chromosomes are unavailable [26,27]. Finally, SSA and BIR are specialized for repair of DSBs within repetitive DNA elements and when only one DSB end has a homologous template, respectively [28].

Little is known about how the DDR affects the choice of homologous repair template during HR. Despite the fact that approximately 50% of the human genome is composed of

repetitive DNA elements [29], homologous template choice for DSB repair is still chosen with remarkable fidelity [30]. Still, a number of genome rearrangements between repetitive elements have been described that have been associated with cancers and neurological diseases, including familial breast and ovarian cancer as well as Charcot-Marie-Tooth disease [31-34], highlighting the need to further understand the mechanisms that control HR. Some of the factors required for homologous template choice have been identified [35-42]; however, the role of the DDR in this choice has not been explored. Here, we tested a role of the DDR in homologous partner choice using SSA between direct DNA repeats as a model. Previous work using this model [35] has shown that SSA between 205 bp repetitive elements spaced 2.6 kb apart is repaired efficiently by annealing of complementary DNA on resected ssDNA ends, cleavage of the 3' tails derived from the intervening non-homologous sequences, and filling of gaps by DNA synthesis and ligation to create a double-stranded DNA (dsDNA) deletion product (Figure 2.1). However, SSA repair at the same locus is inefficient when the repetitive elements share less-than-perfect sequence identity, except when factors critical for disrupting the heteroduplex intermediate (Msh6 or Sgs1) are absent [35]. The process for disruption of divergent SSA intermediates, termed heteroduplex rejection, occurs by a conservative unwinding mechanism such that rejected intermediates still have the potential to repair correctly if the appropriate homologous template is available [36].

To determine whether the *RAD9*-dependent DDR is involved in the formation or rejection of heteroduplex SSA intermediates, we compared the effectiveness of heteroduplex rejection in a wild-type versus *rad9Δ* strain background. Unexpectedly, we found that heteroduplex rejection was less efficient in the presence of the DDR than in its absence; DDR promoted recombination between divergent sequences. Further analysis showed that a G2 delay occurred in wild-type strains that promoted divergent recombination, and inducing a synthetic G2 delay in *rad9Δ*

Figure 2.1. Heteroduplex rejection is enhanced in a *rad9Δ* strain. (A) The SSA assay. Two strains, A-A and F-A, possess a partial duplication of *URA3,* an HO cut site, and 2.5 kb of *λ* DNA upstream of the endogenous *URA3* locus. Induction of HO endonuclease expression produces a unique DSB between the repeats that is repaired efficiently by SSA in A-A, but inefficiently in F-A due to the rejection of the heteroduplex intermediate created by annealing of the divergent *URA3* repeats (3% divergence). SSA repair can be quantified by Southern blot using a probe downstream of the duplicated region of *URA3* to detect uncut (8.3 kb), cut (4.8 kb), and product (5.5 kb) species following HO expression. Successful SSA will promote cell survival, but cells that try to repair by SSA (perhaps multiple times) but ultimately fail will suffer the lethality caused by a persistent DSB. (B) Southern blot analysis of HO-induced DSB formation and repair at the indicated times following induction of HO expression by addition of galactose. Representative blots of 3-6 independent experiments are shown. (C) Quantification of the data presented in B, normalized to the loading control and plotted as the fraction of starting material that is repaired as SSA product (Uncut at T=0/Product at T=5). Product at T=0 was set to 0 and subtracted from product at each time point.

Figure 2.1 (continued)

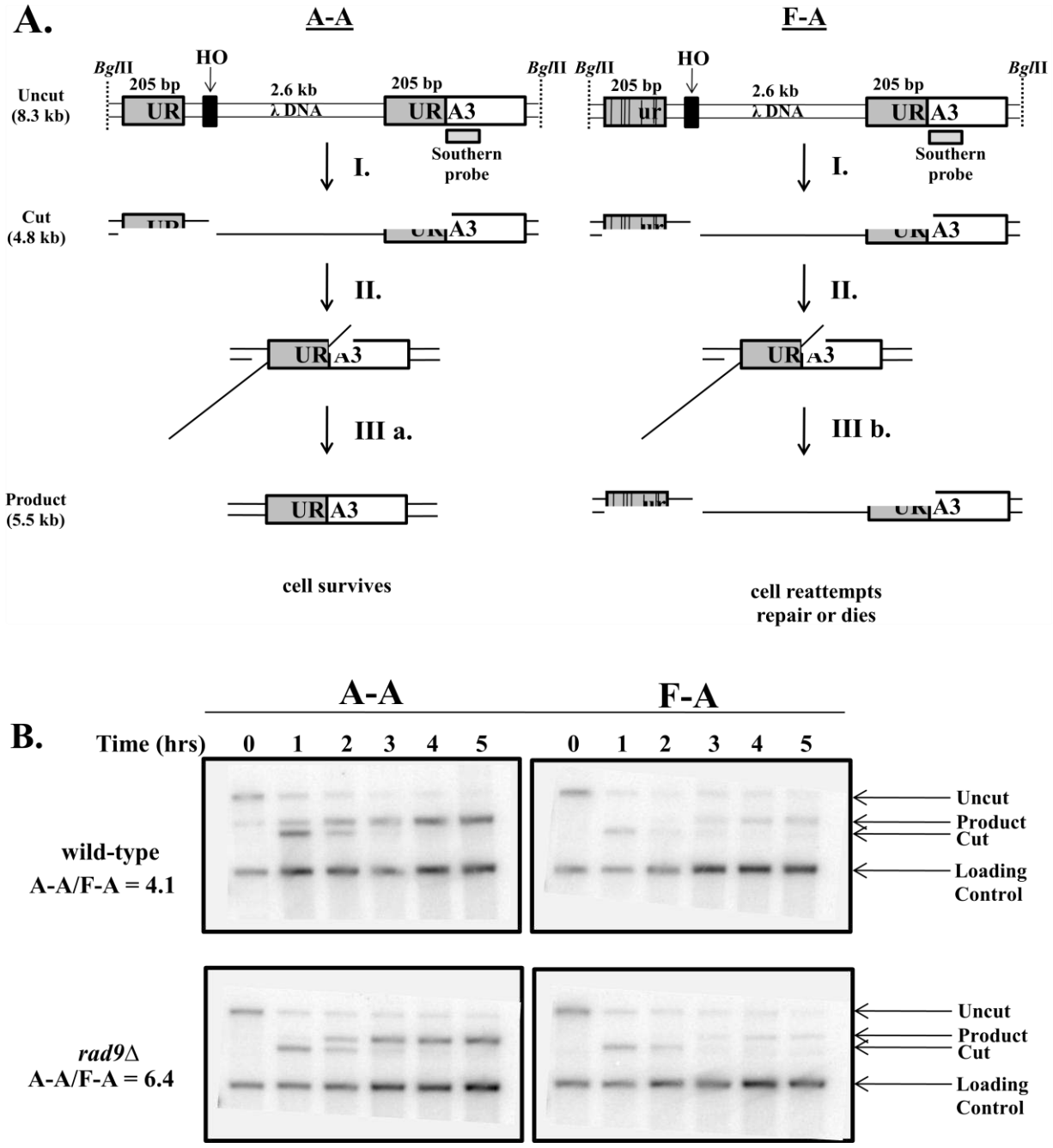
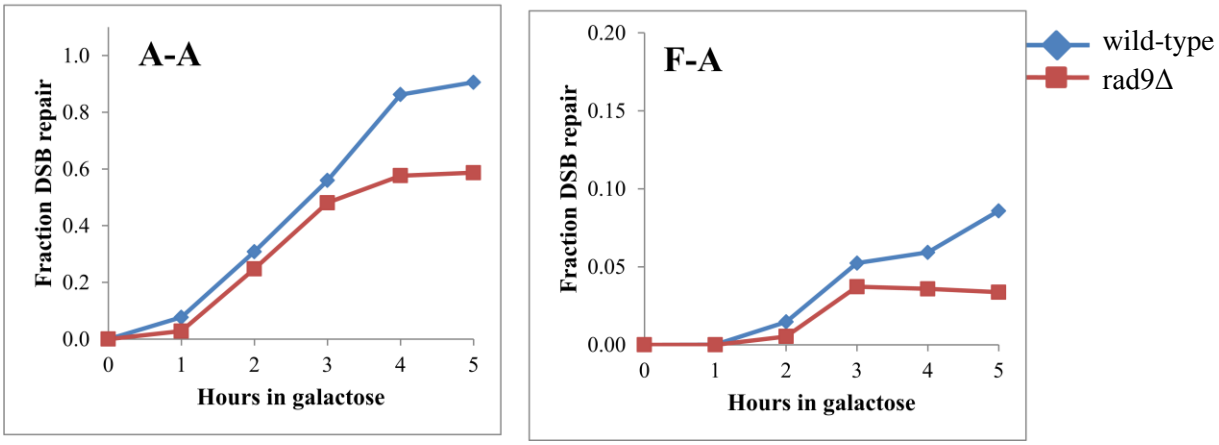


Figure 2.1 (continued)

C.



mutants by adding nocodazole was able to restore the wild-type level of rejection. These results are the first example of the DDR playing a potentially deleterious, rather than protective, role in genome stability via HR. This work also provides insights into how repetitive DNA can threaten the integrity of the genome and suggests a new explanation for why some disease-causing rearrangements could escape mechanisms that normally suppress them.

2. Materials and methods

2.1. Strains

Strains used in this study were identical to or derived from those used in Sugawara *et al.* [35] and Goldfarb and Alani [36]. These strains carry a duplication or triplication of the 5'-205 bp of the *URA3* gene (A) that is identical to or 3% divergent (F) from the wild-type sequence. The wild-type and parent strains were EAY1141 (A-A), EAY1143 (F-A), EAY1137 (A-A-A), and EAY1139 (A-F-A). Mutant derivatives were created by standard gene replacement using auxotrophic or drug resistance markers to create the following strains: EAY1392 (A-A *sgs1Δ::KANMX*), EAY1354 (F-A *sgs1Δ::KANMX*), EAY1387 (A-A *msh6Δ::KANMX*), EAY1388 (F-A *msh6Δ::KANMX*), EAY2375 (A-A *rad9Δ::KANMX*), EAY2376 (F-A *rad9Δ::KANMX*), EAY2566 (A-A *rad9Δ::TRP1 msh6Δ::KANMX*), EAY2567 (F-A *rad9Δ::TRP1 msh6Δ::KANMX*), EAY2404 and EAY2405 (A-A-A *rad9Δ::KANMX*), and EAY2551 and EAY2552 (A-F-A *rad9Δ::KANMX*).

2.2. Survival Assay

Strains were struck from -80˚C freezer stocks and, after 2-3 days, single colonies were inoculated into 5 ml Yeast Peptone Dextrose (YPD) [43]. After growth at 30˚C to saturation,

cells were collected, washed in distilled water, and inoculated to a final dilution of 1:25 to 1:100 in 5 ml YP-lactate (2%). Lactate cultures were incubated at 30˚C for 16-18 hours until mid-log phase growth ($O.D._{600}$ = 0.4-0.6), diluted to 1:2500 in distilled water, and 100 µl was plated on YPD and YP-galactose (2%). Plates were incubated at 30˚C for 2-3 days. SSA efficiency is presented as the ratio of colonies present on YP-galactose to YPD plates for each strain ± SD.

2.3. Southern Blot Analysis

Strains were struck from -80˚C freezer stocks and, after 2-3 days, single colonies were inoculated into 5 ml YPD. After growth at 30˚C to saturation, cells were collected, washed in distilled water, and inoculated to a final dilution of 1:100 or 1:200 in 250 ml YP-lactate (2%). Lactate cultures were incubated at 30˚C for 16-18 hours until mid-log phase growth ($O.D._{600}$ = 0.4-0.6). 40 ml of the culture was collected for the uninduced (T=0) time point and galactose (2%) was added to the remaining culture. Cultures were incubated for 5 hours, and 40 ml samples were collected at various points throughout the time course. Collected samples were pelleted and stored at -80˚C for at least 16 hours, after which chromosomal DNA was isolated and subjected to Southern blot analysis as described in Goldfarb and Alani, 2005 [36]. The amount of SSA product at 5 hours after galactose induction was calculated as a fraction of starting material (uncut band intensity at T=0). In the indicated time courses, nocodazole (Sigma M1404) was added to a final concentration of 15 µg/ml simultaneously with galactose. In these experiments, the overnight lactate cultures were incubated in the presence of 1% DMSO to facilitate the solubility of nocodazole in the cytosol.

2.4. Microscopy

Samples for assessing cell cycle stage by microscopy were collected simultaneously with Southern blot samples. 1 ml of culture was collected at each time point and fixed in 4% formaldehyde. Within 2 weeks of storage at 4˚C, cells were suspended in VectaShield (Vector Laboratories, Burlingame CA), mounted to glass slides, and in some cases stained with DAPI to visualize the nucleus [44]. The slides were viewed by both light and fluorescence microscopy (Zeiss 38HE filter) at 100x using an oil immersion lens and were scored for bud size and location of the nucleus: No bud, randomly-placed nucleus (G1); small bud, randomly-placed nucleus (S); large bud, nucleus at the bud neck (G2); large bud, nucleus spanning the bud neck (M); large bud or pair of enlarged divided cells with asymmetric nuclear distribution (failed nuclear division). A small bud was defined as being less than half of the size of the mother, and a large bud was more than half the size of the mother. In cases when DAPI was not used, G2, M, and failed nuclear division were difficult to distinguish and were scored as: bud less than size of mother (G2/M) and bud same size as mother (failed nuclear division). In the latter case, both mother and bud were most often enlarged.

2.5. Fluorescence Activated Cell Sorting (FACS)

Samples for FACS were collected simultaneously with Southern blot and microscopy samples. 1 ml of culture was collected at each time point, washed in distilled water, and suspended in 70% ethanol to fix cells. After 1-3 days at 4˚C, the ethanol was removed and prepared for flow cytometry as described in Lyndaker *et al.* 2008 [45]. Cells were processed immediately at the Cornell Flow Cytometry Core Laboratory.

3. Results

3.1. Divergent SSA recombination is decreased in the absence of the RAD9-dependent DNA damage response

We examined whether the DDR acts to suppress recombination between divergent sequences using a SSA assay [35,36]. In this assay, a reporter consisting of a duplication of the 5'- 205 bp of *URA3* followed by the recognition site for the HO endonuclease and 2.5 kb of λ DNA is positioned upstream of the *URA3* coding region (Figure 2.1A). The HO endonuclease is expressed under the control of a galactose-inducible promoter and upon expression, will cleave the dsDNA between the *URA3* repeats and at no other location in the genome. The resulting break has the potential to be repaired by SSA, and has little probability of being repaired by NHEJ or any other HR pathway [35]. In one of the two isogenic strains, the 5'-*URA3* duplication is identical to the endogenous *URA3* sequence (A-A), and in the second strain (F-A), the duplication contains seven polymorphisms with respect to the endogenous sequence such that the level of identity between the two repeats is 97%. By creating a deletion or mutation of a gene of interest in these two strains and comparing the success of SSA, we can determine whether that gene has a role in rejecting heteroduplex recombination intermediates when the repeat sequences are divergent.

We assessed the efficiency of SSA in the A-A and F-A strains by both physical and genetic analyses (Materials and methods). We induced DSB formation by adding galactose to mid-log phase cultures and collected and plated cells at time intervals for up to 5 hours following induction. By 5 hours, break repair is complete and cells with repaired breaks begin to grow. We isolated genomic DNA at each time point, digested the SSA locus with *Bgl*II, and detected the presence of recombination intermediates and products by Southern blot using a dsDNA probe

specific to a region downstream of the 5'-*URA3* repeats (Figure 2.1A). Consistent with previous analyses [35,36], we saw that in a wild-type strain, SSA was about 90% efficient in repairing a DSB between identical repeats, but could only repair about 20% of DSBs between the divergent repeats (cells that fail to repair the HO induced DSB do not survive [22]), such that the ratio of identical repair to divergent repair (A-A/F-A) was 3.3 in cell viability assays (Table 2.1) and 4.8 in physical assays (Table 2.2). In contrast, strains lacking genes required for rejection (*msh6Δ* or *sgs1Δ*) displayed equivalent SSA efficiencies in both the A-A and F-A strains (and therefore an A-A/F-A ratio closer to 1.0), indicating that recombination between divergent repeats was no longer being suppressed (Tables 2.1, 2.2) [36].

To determine whether the DSB-induced DDR is important for homologous template choice, we compared the efficiency of heteroduplex rejection, as measured by the A-A/F-A ratio, in a *rad9Δ* strain during SSA between the identical versus divergent repeats. We focused on *RAD9* because of its requirement for recognizing and responding specifically to DSBs and not to damage associated with replication stress [19]. Surprisingly, we found that loss of the DDR resulted in greater efficiency of heteroduplex rejection such that SSA repair between divergent repeats was promoted by *RAD9* (Figure 2.1, Tables 2.1, 2.2). Though the absence of *RAD9* had no effect on the initial rate of SSA, it did have a subtle, but significant, effect on the absolute number of repaired breaks (Figure 2.1C). Furthermore, as shown in Table 2.1, survival in the presence of galactose was unchanged by *RAD9* when heteroduplex rejection was disabled by deletion of *MSH6*, indicating that the decreased survival of F-A *rad9Δ* cells is due to the rejection of SSA between the divergent repeats.

Overall SSA product in *rad9Δ* was decreased by 38% when the break occurred between identical repeats (A-A), but was reduced to greater extent (56%) when the break was between the

Table 2.1. SSA repair efficiency* as determined by survival assays

Relevant genotype	A-A	F-A	A-A/F-A
wild-type	0.78 ± 0.14	0.23 ± 0.07	3.3
msh6Δ	0.76 ± 0.09	0.51 ± 0.01	1.5
rad9Δ	0.81 ± 0.11	0.13 ± 0.02**	6.4
rad9Δ msh6Δ	0.70 ± 0.12	0.53 ± 0.13	1.3

*Survival of indicated strains expressed as colony forming units on galactose/glucose 3-12 experiments.

**significantly different from wild-type base on Wilcoxon-Mann Whitney test ($p < 0.001$).

Table 2.2. SSA repair efficiency* as determined by Southern blot analysis

Relevant genotype	A-A	F-A	A-A/F-A
wild-type	0.86 ± 0.14	0.18 ± 0.04	4.8
*msh6Δ***	1.12 ± 0.26	0.86 ± 0.07	1.3
rad9Δ	0.53 ± 0.09***	0.08 ± 0.04***	6.6
wild-type + nocodazole	0.82 ± 0.13	0.25 ± 0.04	3.3
rad9Δ + nocodazole	0.72 ± 0.11	0.15 ± 0.01	4.8

*SSA product at T=5 hr following galactose induction relative to uncut at T=0 hr expressed as the mean ± SD for 3-11 experiments. Nocodazole was included in time courses as described in the Materials and methods.

** The *msh6Δ* data were previously reported in Goldfarb (2005).

***significantly different from wild-type base on Wilcoxon-Mann Whitney test (p = 0.009 (A-A), p = 0.03 (F-A)).

divergent F-A repeats (Table 2.2), indicating that although the DDR promotes overall DSB repair, it promotes divergent DSB repair to a greater extent than identical repair. This discrepancy may reflect saturation to maximum repair in the A-A strain, rather than an actual homology-directed decision. Interestingly, SSA repair between A-A repeats as determined by Southern blot did not perfectly correlate with cell survival, suggesting that the absence of *RAD9* may promote an alternative repair pathway for breaks between identical repeats that produces a product not detectable by our methods.

3.2. RAD9 does not affect homologous partner choice during SSA

To test whether *RAD9* has a direct role in the choice of an identical versus a divergent template for SSA repair, we utilized a variant of the SSA reporter in which an additional identical repeat is provided upstream of either the A-A or F-A locus [36]. In this situation, SSA can either occur between the proximal repeats creating a small deletion product or the distal repeats creating a large deletion product (Figure 2.2A). Thus, cells that reject heteroduplex DNA between the F and A repeats can still form A-A duplexes, complete SSA, and survive galactose exposure. We determined the preference for formation of duplexes with proximal or distal repeats by evaluating the amount of small and large deletion products by Southern blot. Both wild-type and *rad9Δ* strains showed the same 16:1 preference for forming homoduplexes with the distal repeat rather than forming heteroduplexes with the proximal repeat, and little preference for either template when both repeats were identical [35,36]. This result indicates that *RAD9* does not play a role in choosing a homologous versus divergent template.

Figure 2.2. *RAD9* does not play a role in homologous template choice. (A) A variation on the SSA assay in Figure 2.1A with an additional partial duplication of *URA3* 2.9 kb upstream of the first duplication. After strand resection (I.), the duplex intermediate can form between the endogenous *URA3* sequence and either the distal (IIa.) or proximal (IIb.) repeat. In the former case, a large deletion will result (IIIa.) and small deletion will result from the latter case (IIIb.) Both products can be detected and quantified by Southern blot and distinguished by their mobility on the gel (2.9 vs. 5.5 kb). (B) Southern blot detection of HO-induced DSB formation and repair by SSA as large deletion and small deletion products for wild-type and *rad9Δ* when all repeats are identical (A-A-A), or when the proximal repeat is 3% divergent (A-F-A). Representative blots are shown. (C) Southern blot quantification of small and large deletion products at 5 hours after galactose addition expressed as a fraction of total product (small + large). Averages of 3-4 independent experiments are shown along with the average small deletion/large deletion ratio for each strain ± SD.

Figure 2.2 (continued)

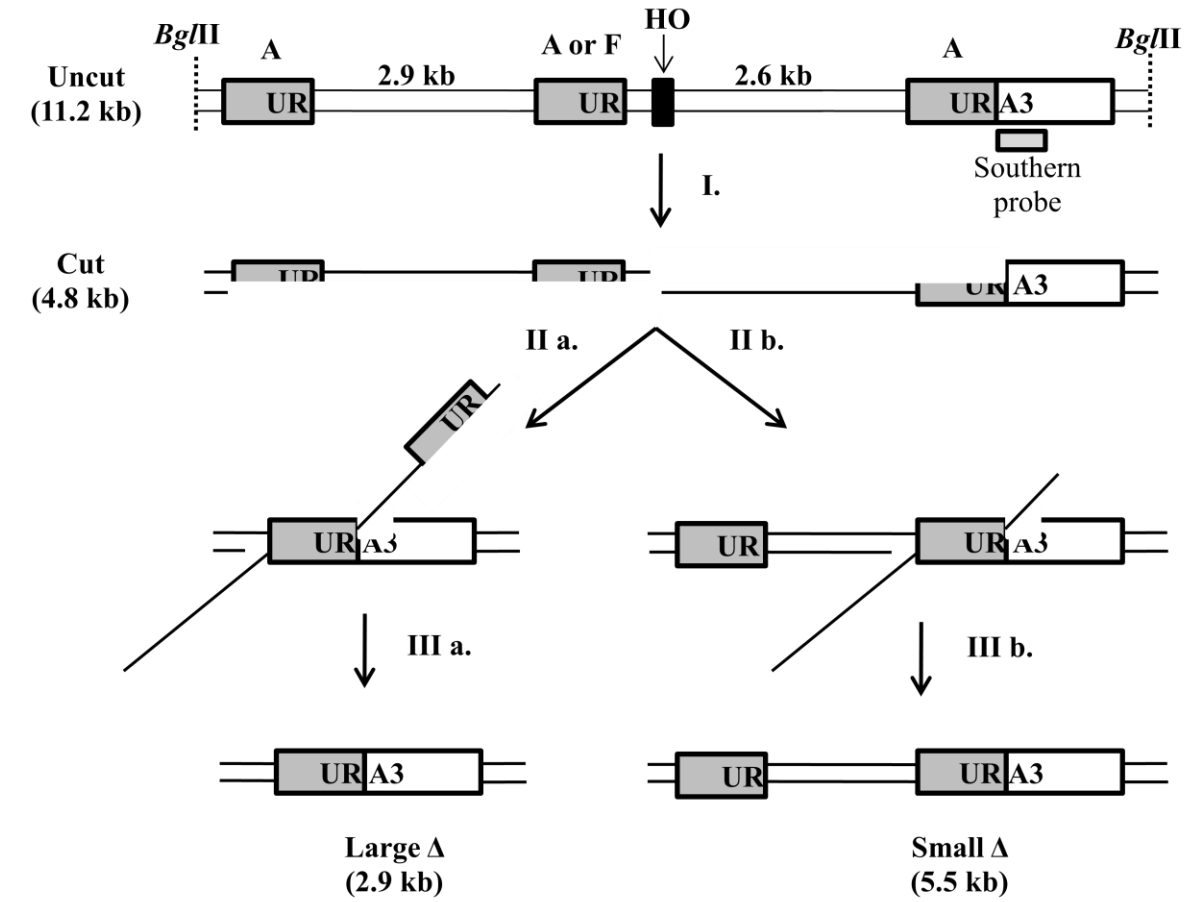
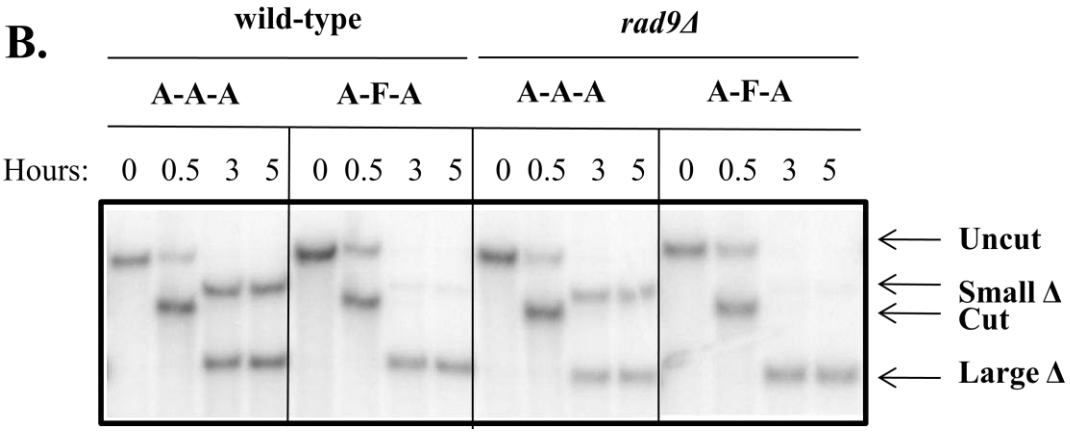


Figure 2.2 (continued)

B.



C.

Genotype	A-A-A Small Δ	A-A-A Large Δ	A-A-A Small/ Large	A-F-A Small Δ	A-F-A Large Δ	A-F-A Small/ Large
wild-type	0.45	0.55	0.83 ± 0.03	0.06	0.94	0.06 ± 0.02
rad9Δ	0.47	0.53	0.88 ± 0.08	0.06	0.94	0.06 ± 0.01

3.3. Heteroduplex rejection elicits a RAD9-dependent cell cycle delay

rad9Δ strains exposed to radiation-induced DSBs are inviable and do not show a cell cycle delay at the large-budded G2 stage of the cell cycle [46]. Weinert and Hartwell showed that the cell cycle delay induced by the DDR is important to promote repair of radiation-induced DSBs. Consistent with this and the observation that a single DSB within the genome can elicit a DDR [22,23], we found that induction of the HO-catalyzed DSB between the 5'-*URA3* repeats caused a G2 delay that was *RAD9*-dependent (Figure 2.3A *left*). The G2 delay was maintained indefinitely when the repeats were divergent, similarly to the arrest observed in the presence of a persistent DSB [22]; however, due to the delayed accumulation of anucleate cells ("failed nuclear division" phenotype observed by microscopy, data not shown) it appeared that at least some cells completed the first round of cell division and encountered chromosome segregation problems in subsequent divisions. G2-stage haploid cells are those defined as having 2n DNA content, after DNA replication but before mitosis. Fluorescence-activated cell sorting (FACS) confirmed that the delay at the large-budded stage was due to a delay of mitosis following replication, since a similar *RAD9*-dependent delay occurred at the 2n stage (Figure 2.3B, *left*).

3.4. Nocodazole-induced cell cycle delay in rad9Δ mutants rescues divergent SSA recombination

To determine whether the absence of the G2 delay alone contributes to the enhancement of heteroduplex rejection, we attempted a synthetic rescue of the G2 delay in the *rad9Δ* strains. Microtubule de-stabilizing drugs have traditionally been used to reconstitute G2 delays in yeast strains lacking DNA damage responses [46]. Since the *RAD9*-dependent G2 delay appeared to initiate shortly after galactose induction and to reach a maximum by approximately 3 to 4 hours after induction, we reasoned that by adding an appropriate concentration of the microtubule de-

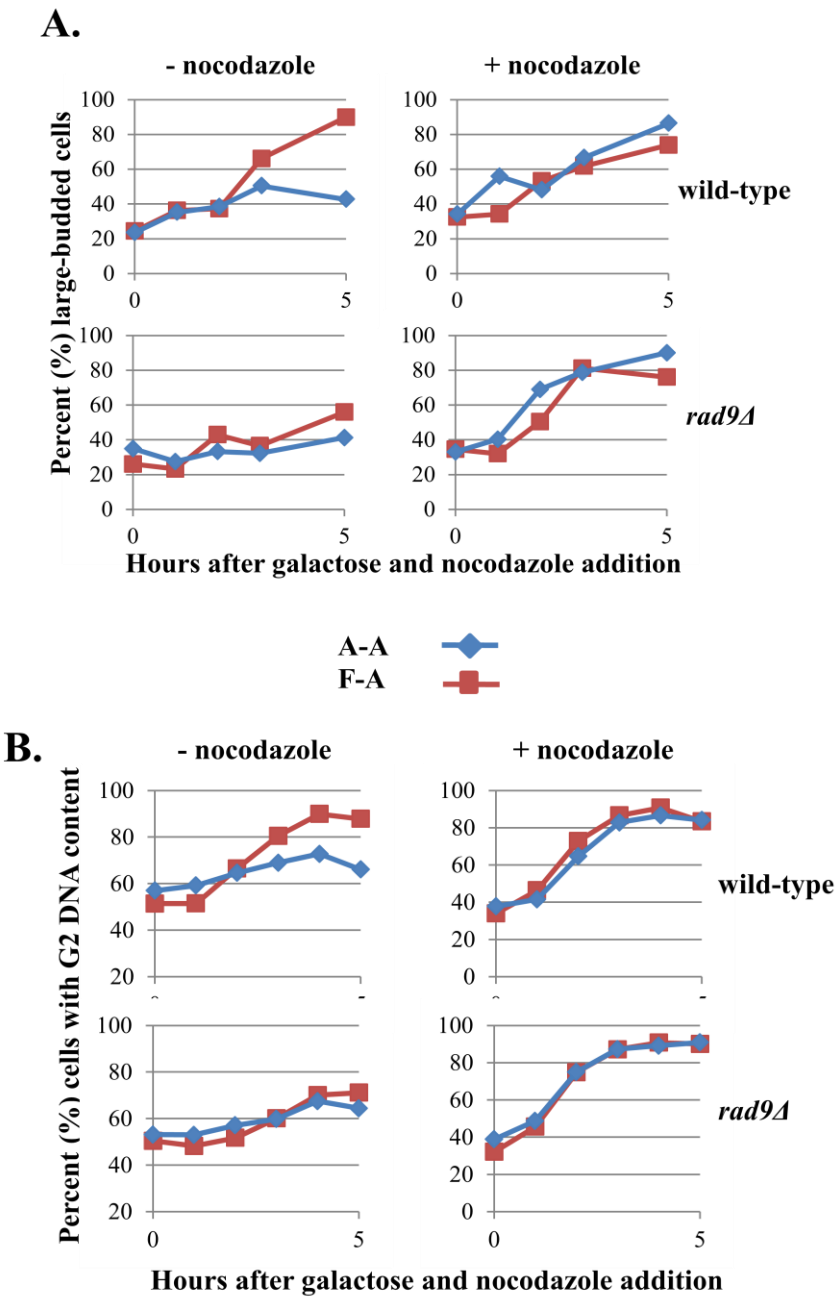


Figure 2.3. G2 delay alone is sufficient to promote homeologous recombination. (A) Cells were fixed in 4% formaldehyde at the indicated times following induction of HO expression by galactose addition and in the absence (*left*) or presence (*right*) of 15 ug/ml nocodazole (added simultaneously with galactose). The percentage of G2/M stage cells was determined by counting large-budded cells under light microscopy for wild-type and *rad9Δ* strains. Representative plots of 2-3 independent experiments are shown. (B) Cells were fixed in 70% ethanol at the indicated times following induction of HO expression by galactose addition in the absence (*left*) or presence (*right*) of 15 ug/ml nocodazole (added simultaneously with galactose). The percentage of G2/M stage cells was determined by flow cytometry. Representative plots of 2 independent experiments are shown.

stabilizer nocodazole simultaneously with galactose, we could induce a G2 delay that initiated and completed in a similar time frame to the delay normally imposed by *RAD9*. We repeated the SSA assays as usual, except that 15 μg/ml nocodazole was added at T=0. Analogously to the Weinert and Hartwell study [46] where the drug MBC protected *rad9Δ* mutants from ionizing radiation damage, nocodazole treatment enhanced the G2 delay in both wild-type and *rad9Δ* strains, regardless of the identity of the upstream 5'-*URA3* repeat (Figure 2.3A and 2.3B, *right*) and increased the fraction of DSBs repaired in all strains. The nocodazole-induced enhancement in repair in *rad9Δ* strains was larger for DSBs between divergent repeats (1.9-fold) than for DSBs between identical repeats (1.4-fold; Table 2.2). In the wild-type strains, nocodazole produced only a small enhancement in F-A repair (1.4-fold; Table 2.2), but had no effect on repair between A-A repeats. This difference adjusts the A-A/F-A ratio for *rad9Δ* to a ratio like that of wild-type in the absence of nocodazole (4.8; Table 2.2), while the rejection efficiency of wild-type in the presence of nocodazole was reduced even further (3.3; Table 2.2). These results indicate that the *RAD9*-dependent G2 delay alone is sufficient to promote SSA between the F-A repeats, and thus appears to allow greater opportunity for heteroduplex intermediates to escape rejection.

4. Discussion

DSBs are thought to be some of the most cytotoxic forms of DNA damage. The presence of even a single DSB within the genome induces a DNA damage response that can cause lethality if the DSB is difficult or impossible to repair [22]. However, improper repair can result in genome rearrangements and the potential for unequal nuclear division and chromosome loss [47]. How does the cell decide the ideal repair pathway for DSB repair? For the most part, this

choice depends on the cell cycle stage during which the DSB forms. For example, in mammals repair by HR occurs during late S and G2 because homologous templates are available and in close proximity to the break site, whereas during other stages of the cell cycle NHEJ takes on the primary role of DSB repair [27].

It is thought that all of the different modes of DSB repair work in both collaboration and competition with each other to ensure that DSBs are not left unrepaired, and preference decisions are coordinated with cell cycle control. For example, the decision between NHEJ and HR is modulated by control of DSB end resection by cyclin-dependent kinases (CDKs) [27,48]. Rad9 localization to DSB ends is thought to serve as a physical block to resection, requiring phosphorylation by S- and G2-stage expressed CDKs to be removed [49]. Rad9 has also been shown to be important for channeling mitotic HR to repair with the sister chromatid; *rad9* mutant yeast display increased chromosomal translocations [50].

Following the decision to repair a DSB by HR, the cell must then distinguish the correct homologous template for repair synthesis. This decision is made in part by control of Rad51 association to resected DSB ends. Loss of *RAD51* in yeast results in increased SSA-mediated translocations and it appears that Rad51 displacement from resected ends directs the choice to repair by SSA [51]. Consistent with this, Rad51 overexpression leads to enhanced gene conversion and genome instability in mice, illustrating the need for the proper balance between repair pathways [52]. The second line of defense against incorrect homolog choice is disruption of heteroduplex repair intermediates between non-identical sequences. Heteroduplex rejection requires the concerted action of mismatch recognition proteins, helicases, endonucleases, and topoisomerases [35-37]. Whether the DDR modulates localization or activity of Rad51 and heteroduplex rejection factors remains to be determined.

Here, we show that loss of the *RAD9*-dependent DDR enhances the ability of the cell to suppress SSA between divergent repeats (Figure 2.1, Tables 2.1, 2.2). The absence of *RAD9* did not affect the rate of SSA (ruling out the affect that *RAD9* loss may have on blocking end resection), nor did it play a direct role in a homology-directed decision (Figure 2.2B,C). Treating a *rad9Δ* strain with a nocodazole-induced cell cycle delay promoted SSA to a greater extent in F-A strains than A-A, shifting heteroduplex rejection (A-A/F-A) in *rad9Δ* mutants closer to that seen in wild-type strains (Table 2.2).

Our results show that the *RAD9*-dependent DDR can promote recombination between non-allelic sequences due to its ability to delay the cell cycle in G2 when HR repair is prominent. Observations in this paper and elsewhere [51] suggest that as long as normally-encountered levels or easy-to-repair types of DNA damage occur, the DDR can respond effectively and promote the most conservative form of repair possible in its present cell cycle stage. However, when higher-than-normal levels or difficult-to-repair damages are encountered, the conservative repair pathways become "overwhelmed," and instead of simply eliminating the damaged cells, the DDR chooses to promote cell survival via use of non-conservative repair mechanisms. For example, Argueso *et al.* [53] showed that acute levels of DNA damage in yeast result in an abundance of chromosomal translocations between unlinked repeats, and that SSA is primarily responsible for these translocations.

This strategy of promoting cell survival over the potential for mutagenesis is similar to the induction of translesion synthesis (TLS) observed when replication forks are damaged or stalled. Organisms ranging from bacteria to humans possess a collection of specialized error-prone DNA polymerases that step in when the high-fidelity polymerases encounter lesions that block their paths [54]. Although TLS promotes cell survival during replication stress it also

enhances the potential for base substitutions, microsatellite instability, and even gross chromosomal rearrangements [55-57]. We have shown in this study that the DDR surprisingly promotes error-prone recombination, and it is known that the DDR similarly promotes TLS. For example, DDR induction by stalled replication forks in *S. pombe* results in the up-regulation of the error-prone DinB polymerase and its recruitment to chromatin by the Rad9-Rad1-Hus1 (9-1-1) checkpoint complex [58,59]. In addition, mammalian polymerase η is dependent upon ATR for its role in recovery from UV irradiation, and the budding yeast S-phase checkpoint is responsible for suppressing TLS during exposure to methylmethane sulfonate [60,61]. However, the difference between these examples and the role of the DDR in rejection described here is that they occur through an active mechanism, i.e. a direct signaling event, rather than a passive effect of cell cycle delay. Although our study has not uncovered a more direct control of heteroduplex rejection by the DDR, we cannot rule out the possibility that rejection is controlled by checkpoint factors upstream of Rad9. In support of this, both Msh6 and Sgs1 are likely to be controlled by Mec1 rather than Rad9 since Msh6 was shown to be phosphorylated at two sites by Mec1 [62], and Sgs1 has several S/TQ motifs which are commonly targets of Mec1 [63]. Due to the requirement for Mec1 in SSA [64], we were unable to test rejection in a *mec1* strain using the SSA assay; however, studies with *msh6*-phosphorylation mutants are planned.

Approximately 50% of the human genome is made of repetitive DNA, much more than in the yeast genome [29], and it is estimated that the average human cell must repair about ten DSBs per day [65]. Disease-associated rearrangements between endogenous repeats have been described, but are not as common as these observations and work in yeast by Argueso *et al.* [53] would suggest. This lack of rearrangements between repeat sequences could be explained by the more frequent use of NHEJ in mammalian cells. Provided there is only limited damage, NHEJ

could promote repair with genomic modifications confined to the region of the DSB, instead of creating the potential for translocations that would result from non-allelic homologous recombination.

Perhaps the relatively low incidence of rearrangements between repetitive sequences in eukaryotes is better explained by their residence in tightly packed heterochromatin domains, which might limit their exposure to DNA-damaging agents as well as recombination factors [66-69]. Recent work by Chiolo *et al.* suggests that repeat recombination is prevented in heterochromatin because DSBs are recognized and processed for HR very rapidly, and are protected from Rad51 binding until the breaks are re-localized, in a checkpoint kinase-dependent manner, to a "safe," presumably repeat-free location at the heterochromatin periphery [70]. Earlier studies suggest that DSBs formed within heterochromatin are repaired more slowly than breaks within euchromatin, and heterochromatin may additionally be protected from DSB formation in other ways such as more effective scavenging of reactive oxygen species [71-73].

Even though the HO-induced DSB used in our SSA assay is not expected to reside within heterochromatin, unrepairable breaks in yeast (i.e. like those in the F-A construct) re-localize to the nuclear periphery in a checkpoint-dependent manner [74,75]. The presumed euchromatic nature of our system also may explain the lack of DDR regulation of heteroduplex rejection; checkpoint regulation of HR fidelity and partner choice may be more prominent in heterochromatic regions, as suggested by studies in human cells in which ATM signaling became increasingly important for DSB repair with increasing chromatin complexity [73], or may be specifically upregulated during late S-phase, when heterochromatic repeats become exposed during replication. In addition to the above strategies, certain cell types in higher organisms may have evolved other forms of checkpoint regulation to avoid non-allelic recombination, such as

minimizing time spent in G2-phase, down-regulating the DNA damage response, requiring a certain level of damage for full activation of the DNA damage response, or inducing apoptosis when persistent breaks or extensive damage is encountered [48,76-78].

Despite the many lines of defense against aberrant recombination in repetitive DNA, mammalian cells still may not be equipped to handle situations of acute DNA damage, such as overexposure to radiation or chemical mutagens, or when conservative repair pathways are compromised, as in pre-cancerous or tumor cells. This would allow some non-allelic recombination to slip through, and either cause disease or add more complexity to an already existing cancer.

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CHAPTER III: RESEARCH STUDY II

Protein-protein interactions that contribute to SSA and heteroduplex rejection in budding yeast.[4,5]

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Abstract

Instability of repetitive DNA puts cells at risk for frequent genome rearrangements, and heteroduplex rejection is an important process for maintaining stability of repetitive DNA. The Msh2-Msh6 heterodimer and Sgs1 helicase are well known for their role in suppressing homologous recombination between non-allelic DNA sequences, and both factors are required for unwinding of a heteroduplex intermediate during single-strand annealing (SSA) repair of a double-strand break between divergent repeats (Sugawara et al., 2004; Goldfarb and Alani, 2005). Despite genetic evidence for this requirement, little is known about how the activities of Msh2-Msh6 and Sgs1 are coordinated on a heteroduplex recombination intermediate and whether any other factors participate in the process. Since homologs of Msh6 and Sgs1 in mammals were shown to interact physically (Pedrazzi et al., 2003; Yang et al., 2004; Saydam et al., 2007), we asked whether the yeast proteins interact as well and here we show that they do. Additionally we surveyed non-essential proteins that are known to interact with either Msh6 or Sgs1, and revealed that interaction of Top3 and Rmi1 with Sgs1 contribute to heteroduplex rejection by stabilizing Sgs1 and may also serve a stimulatory role. We also show that the

[4] Amy Lyndaker performed the Msh2-Msh6 and Sgs1 CoIPs presented in this study

[5] Ujani Chakraborty performed the SSA assays with the *pol30* alleles presented in this study

PCNA replication clamp which interacts with Msh6 and is essential for mismatch repair is dispensible for heteroduplex rejection. Surprisingly, we discover an additional role for PCNA in SSA.

Introduction

Repetitive and non-allelic sequences of common ancestral origins pose a risk to eukaryotic cells, as they have the potential to recombine and form genome rearrangements that can lead to disease. Multiple cellular mechanisms contribute to suppressing homologous recombination (HR) events between non-allelic sequences in the genome. The organization of the interphase nucleus helps to limit physical interactions between distant regions of the genome, and cell cycle regulation of HR factors limits recombination events at times when distant genomic regions and non-allelic sequences tend to be unprotected or closer to each other in space (reviewed in George & Alani, 2012). Despite these lines of defense, physical interactions between non-allelic sequences can still frequently occur, and when damage or replication stalling occurs in the vicinity of these interactions, non-allelic homologous recombination (NAHR) can be initiated (reviewed in Liu et al., 2012). However, eukaryotes have evolved additional mechanisms to disrupt HR events that are initiated between non-allelic sequences, and these are termed "heteroduplex rejection," since these processes act by "rejecting" (by helicase-mediated unwinding, or perhaps, nuclease-mediated digestion) of HR intermediates that share imperfect homology ("heteroduplexes").

The single-strand annealing (SSA) pathway is a specialized type of HR that occurs between closely spaced repeat sequences (Lin and Sternberg, 1984; Fishman-Lobell et al., 1992). SSA is thought to be predominant form of DSB repair within highly repetitive regions of the genome, such as the ribosomal DNA (Kobayashi, 2006; Li et al., 2008), and limits unavoidable loss of genetic information to local deletions rather than large-scale rearrangements. SSA also serves as a useful tool for investigating heteroduplex rejection and other mechanisms that distinguish homology. Studies using an SSA cassette in which a 205-bp region of 97%

homology is assayed have identified a strong dependence on the Msh2-Msh6 complex that is more commonly known for recognition of nucleotide mispairs occurring during replication, and for the RecQ family helicase Sgs1 (Sugawara 2004, Goldfarb 2005) in disruption of heteroduplex intermediates during SSA.

Msh2-Msh6 and Sgs1 physically interact with a large array of proteins (Figure 3.1). Previously, Goldfarb and Alani (2005), determined that the Msh2-Msh6-interacting proteins Mlh1-Pms1 and Exo1, and Sgs1 interacting protein Srs2 did not contribute to rejection during SSA, although disruption of traditional HR did show a mild dependence on these factors in the presence of certain types of mismatched substrates (Nicholson et al., 2000). Here, we show that the yeast Msh6 and Sgs1 proteins interact *in vitro* and are likely to act cooperatively in heteroduplex rejection during SSA. We also test other proteins known to interact strongly with Msh2-Msh6 and Sgs1 to determine whether they influence SSA or heteroduplex rejection of an SSA intermediate. Using temperature-senstive alleles of the Top3- Rmi1 topoisomerase that is well known to form a complex with Sgs1 (Bennett et al., 2000; Fricke et al., 2001; Chang et al., 2005; Mullen and Brill, 2005; Chen and Brill, 2005), we found that it is required for heteroduplex rejection during SSA. While the rejection defect at the restrictive temperature can be attributed to destabilization of the Sgs1 protein, we also observe a minor rejection defect at the permissive temperature where Sgs1 protein appears to be stable, indicating that Top3-Rmi1 may stimulate heteroduplex rejection. Additionally, we show that the replicative clamp-loader PCNA which interacts with Msh6 via its Pol30 subunit does not influence rejection though it does play a role in SSA.

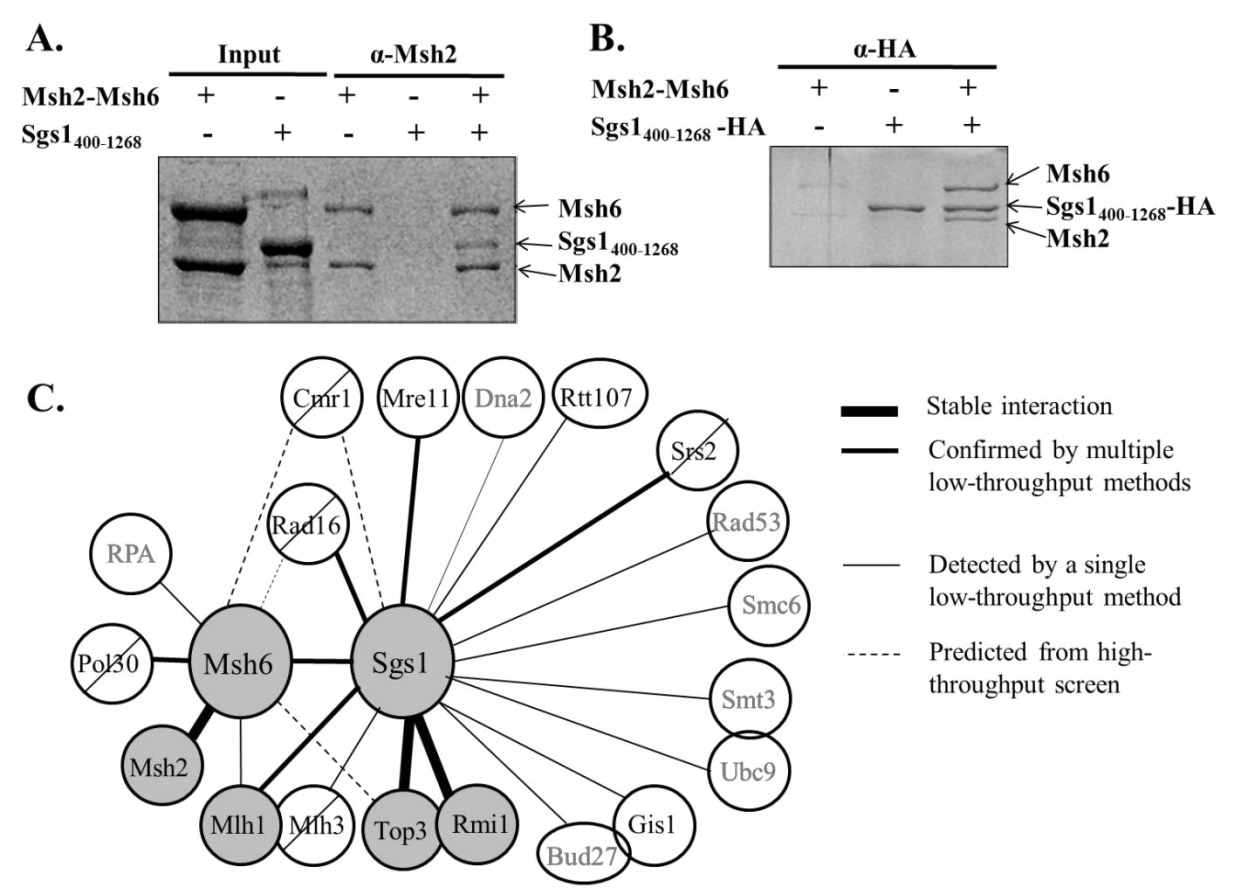


Figure 3.1. Msh6 and Sgs1 protein-protein interactions. Co-IP of $Sgs1_{400\text{-}1268}$ with Msh2-Msh6 **(A.)** and Msh2-Msh6 with $Sgs1_{400\text{-}1268}$-3HA **(B.)** *in vitro,* as appears in Lyndaker (2009) and described in Materials and Methods. **C.)** Protein-protein interaction profile of Msh6 and Sgs1. Line width indicates a rough confidence in detected interactions based on the number and reliability of experiments in which they are detected. Gray-filled proteins are confirmed to provide either an essential or stimulatory role in heteroduplex rejection during SSA as determined by this and previous studies (Sugawara et al., 2004; Goldfarb & Alani, 2005). Crossed-out proteins were tested for a role in heteroduplex rejection during SSA in this or the previous studies and were eliminated. Grayed-out proteins are essential for life and were not tested. Proteins that are touching each other are involved together in a common pathway. Interaction data was acquired using the BioGrid interaction database which includes both *in vivo* and *in vitro* methods of detection (Stark et al., 2006, 2011).

Materials and Methods

Strain construction

All strains used in this study bearing the SSA cassette are derived from EAY1141 and EAY1143 (Sugawara et al, 2004), and have the genotype *ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4::[THR4 ura3-**AorF**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT*, where the bold ***AorF*** represents the upstream repeat sequence which is derived from S288c in EAY1141 (A-A), or from strain FL100 in EAY1143 (F-A). Of the strains without the SSA cassette, EAY235 is a standard S288c strain of genotype *ura3-52 leu2Δ1 trp1Δ63*, and the *top3Δ* (EAY2402 or EAY2403) and *rmi1Δ* (EAY2623 or EAY2624) strains were made by integrating a *top3Δ::KANMX* or *rmi1Δ::KANMX* fragment obtained by PCR from the yeast deletion collection (Brachmann et al., 1998) into EAY235. Attempts to integrate these fragments into EAY1141 or EAY1143 were unsuccessful, indicating that they are likely to be toxic in the SSA assay strains.

The *top3^{ts}*(E447K S583L) allele was a gift from Rodney Rothstein (Wagner et al., 2006), and was amplified by PCR from strain J1022 (EAY2554, W303 background), fused to a *HPHMX* cassette, and integrated into the SSA strains. *rmi1* temperature sensitive alleles were isolated by transforming an *ARSCEN-LEU2-RMI1* plasmid library into *rmi1Δ* strains and selecting for failure to complement slow growth and sensitivity to methylmethane sulfonate (MMS) at 37°C, but not 23°C. The library was created by mutagenic PCR of pEAO243 (pJM7161 gifted by Steve Brill), and the mutagenized *rmi1* fragments were ligated back into unmutagenized pEAO243. Two candidates were chosen that had severe enough phenotypes to be easily observed, *rmi1^{ts}-2* (N103K W168R, L192S, F215Y) and *rmi1^{ts}-3* (L50I E60G N103K S137G, R211G, K236R). Both alleles were fused to the *KANMX* cassette and integrated into the

SSA strains to create A-A *rmi1^{ts}-2* (EAY3206, EAY3207, EAY3209), F-A *rmi1^{ts}-2* (EAY3210, EAY3211, EAY3212, EAY3213), A-A *rmi1^{ts}-3* (EAY3203, EAY3204, EAY3205), and F-A *rmi1^{ts}-3* (EAY3214).

The *pol30* alleles and *msh6-KQFF>AAAA* were provided to us by Richard Kolodner and Tom Kunkel, respectively (Clark et al., 2000; Lau et al., 2002). The *pol30* alleles were obtained by PCR from the following strains, linked to *KANMX*, and integrated into the SSA strains: RDKY3857 (*pol30-201*), RDKY3860 (*pol30-204*), and RDKY3872 (*pol30-216*), which are identified in our lab as EAY3304, EAY3305, and EAY3307, respectively. The *msh6-KQFF>AAAA* allele was obtained by PCR from pEAO216 (YIplac211 from Clark et al. 2000), fused to *KANMX* and integrated into the SSA strains. Sequencing confirmed that no other mutations were acquired during PCR.

SGS1-3HA was integrated into selected strains using the "pop-in" *LEU2* vector, pEAO252 (pJM1526 gifted by Steve Brill, Mullen et al., 2005) to create the following strains expressing the 3HA-tagged Sgs1 protein: A-A wild-type (EAY3249 and EAY3274), A-A *top3^{ts}* (EAY3269 and EAY3270), and A-A *rmi1^{ts}-2* (EAY3271 and EAY3272).

Purification and co-IP of Msh2-Msh6 and Sgs1$_{400\text{-}1268}$

The expression plasmid and strain for the 6-Histagged, HA-tagged soluble fragment of Sgs1 containing amino acids 400-1268 of 1447 was created in the Wang lab at Harvard University (Bennett *et al.* 1998) and obtained from the Lahue lab (University of Nebraska Medical Center). The expression plasmid (pRB222; pEAE265) was later put into the Alani lab expression strain, EAY33. The resulting EAY2339 Sgs1 expression strain was used for expression and purification of Sgs1$_{400\text{-}1268}$ as described by Lyndaker (2009). Briefly, six liters of

YP medium containing 2% lactate, 3% glycerol were grown in a 30°C shaker to $O.D._{600}$ of 0.3 - 0.4 and protein expression induced by addition of galactose to a 2% final concentration. Following a six-hour induction, cells lysates were prepared by the "liquid nitrogen popcorn" method and $Sgs1_{400\text{-}1268}$ purified using Ni-NTA column chromatography.

Msh2-Msh6 purification is also described by Lyndaker (2009). Briefly, *MSH2* and *MSH6* were co-overexpressed from *GAL10* promoters from plasmids pEAE9 and pEAE218 in the protease-deficient yeast strain EAY960 (EAY33 derivative), by addition of galactose (2% final concentration) to four liters of ura- trp- synthetic dropout medium + 2% lactate and 3% glycerol at $O.D._{600} = 0.7 – 0.8$ for 7 hours. Lysates were prepared by the "liquid nitrogen popcorn" method and Msh2 and Msh6 were purified by PBE94 anion exchange column chromatography similarly as decribed by Alani (1996).

CoIP with Msh2-Msh6 and Sgs1-3HA are as described in Lyndaker (2009). Equimolar amounts of purified Msh2-Msh6 and $Sgs1_{400\text{-}1268}$ proteins were incubated with 20 U of DNase I in 20 mM Tris pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$ for 30 minutes at room temperature. DNase I activity was confirmed by digestion of 1 µg of control DNA and agarose gel analysis. 1 µl of 12CA5 mouse monoclonal anti-HA antibody (Roche) or 0.5 µl of anti-Msh2 polyclonal antibody were added per reaction and incubated 1 hour at 4o C. Protein A agarose beads were suspended 1:1 (v/v) in 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA and 20 µl of the suspension were incubated with each sample for 1 hour. Beads were washed three times with 200 µl of 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% NP-40 and twice with 200 µl of 50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1% NP-40. Beads were boiled in SDS-PAGE loading dye for ten minutes, and samples were run on 8% SDS-PAGE gels followed by staining with Coomassie blue.

Single-strand annealing assays

SSA assays were performed similarly as described in Sugawara, et al. (2004), Goldfarb et al. (2005), and George et al. (2011). Briefly, cultures derived from single colonies were grown to mid-log phase in yeast peptone (YP), 2% lactate, diluted 1:2500 and plated (100 µl/plate) on both YP, 2% glucose and YP, 2% galactose. Plates were incubated at 37°C (restrictive temperature) or at room temperature (permissive, 22-23°C) SSA efficiency was determined by the number of colony forming units on galactose vs. glucose, and heteroduplex rejection efficiency was calculated as SSA efficiency of the A-A strain vs. F-A strain.

Southern blot analysis

SSA assays were performed similarly as in Sugawara, et al. (2004), Goldfarb et al. (2005), and George et al. (2011). Briefly, cultures derived from single colonies were grown to mid-log phase in yeast peptone (YP), 2% lactate, HO-DSBs induced by addition of 2% galactose, and 45 ml samples of culture were collected at indicated time points following induction. DNA was isolated from these samples, digested with BglII, run on 1% agarose, and transferred to a Hybond-XL (Amersham) membrane by the capillary transfer method. The membrane was probed with a α^{32}P-labeled dsDNA fragment of the *URA3* gene and exposed to a phosphoimager screen, to allow visualization of the uncut SSA locus, cut, and SSA products, which are distinguished by their mobility on the gel. Band intensity is quantified by the Imagequant software and normalized to the intensity of a probe that hybridizes to the *RAD10* gene. SSA efficiency is determined by the quantity of SSA product at 5 hr compared to the

uncut locus before break induction, and heteroduplex rejection efficiency was calculated as SSA efficiency of the A-A strain vs. F-A strain.

Drug resistance tests

Single colonies were inoculated into 5 ml YPD and incubated approximately 36 to 48 hours at room temperature (22°C) to bring the cultures to saturation. Saturated cultures were diluted in sterile water to O.D. $_{600}$ of 0.5 and 100 µl of each was transferred to a 96-well plate. They were subsequently serially diluted 5 times in 1:10 increments, and 5 µl of each were spotted onto YPD plates containing 7.5 mM erythrosine B and 0.02% methylmethane sulfonate, 20 mM hydroxyurea, or no drug. Plates were incubated at either room temperature for 4 days or 37°C for 3 days.

Western blot analysis

Single colonies of Sgs1-3HA expressing strains were inoculated into 5 ml YPD and incubated approximately 36 to 48 hours at room temperature to bring the cultures to saturation. Saturated cultures were diluted in 100 ml YPD to O.D. $_{600}$ of 0.2 and incubated at room temperature until mid-log phase growth (O.D. $_{600}$ of 0.5 to 0.6) and harvested by centrifugation for 10 min at 3000 rpm. Alternatively, cultures were transferred to 37°C upon reaching O.D. $_{600}$ of 0.4 to 0.5 and incubated for 1 hour prior to harvest. Cell pellets were suspended in 1 ml Lysis Buffer (50 mM HEPES pH 7.5, 1 mM EDTA pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholic acid, 1 mg/mL bacitracin, 1 mM benzamidine, 1 mM PMSF), lysed by glass

bead beating and, unless used directly, were flash frozen in a dry ice/ethanol bath and stored at -80°C. The lysates were suspended in 500 µl 3x Sample Buffer (30% glycerol, 3% β-mercaptoethanol, 6% SDS, 195 mM Tris pH 6.8, bromophenol blue) and 15 µl was loaded onto a 6% SDS-PAGE gel and run at 150 V for approximately 1.5 hours. Contents of the gel were transferred on a BioRad nitrocellulose membrane for 2 hours at 4°C and 100 V, or until transfer of the molecular weight marker was complete, blocked in 10% BioRad milk/TBST, and probed with HRP-conjugated α-HA antibody (Roche 12CA5) and secondary antibody α-mouse (Cell Signaling Technologies) both in 2% milk/TBST. HRP signal was detected using the Pierce ECL Western Blotting Substrate and exposed to Kodak BioMax Light film. Sgs1-3HA was determined to run at a mobility of ~200 kDa by its presence in strains integrated with the *SGS1-3HA-LEU2* vector but absence in parental strains.

Results

Msh2-Msh6 physically interacts with the core helicase domain of Sgs1

Evidence that human Msh6 physically interacts with RecQ helicases (Pedrazzi et al., 2003, Yang et al., 2004; Saydam et al., 2007), suggested that the yeast homologs could also interact and potentially function cooperatively in heteroduplex rejection. To determine if yeast Msh2-Msh6 and Sgs1 physically interact, we purified Msh2-Msh6 protein and a fragment of Sgs1, $Sgs1_{400\text{-}1268}$, which contains the helicase domain of Sgs1, but lacks other known or predicted domains (Mullen et al., 2000; Bennett et al., 1998, 1999). When Msh2-Msh6 was incubated with $Sgs1_{400\text{-}1268}$ and precipitated with α-Msh2 antibody, Sgs1 was found to co-precipitate (Figure 3.1A). Conversely, when $Sgs1_{400\text{-}1268}$ was tagged with HA and incubated with

Msh2-Msh6, the α-HA antibody also precipitated Msh2-Msh6 (Figure 3.1B). These results confirm that Msh2-Msh6 directly interacts with Sgs1, and that the interaction site on Sgs1 lies within amino acids 400 and 1268. Though this coIP does not distinguish whether Sgs1 interacts with Msh2 or Msh6, mammalian RecQ helicases are known to interact specifically with MSH6 and function with it in disruption of aberrant DNA structures during HR (Wang et al., 2000; Yang et al., 2004; Saydam et al., 2007). Thus, we infer that Sgs1 is most likely interacting with the Msh2-Msh6 complex via Msh6. Since Sgs1 is not known to participate in post-replication mismatch repair and Msh6 is not known to participate in any other pathways that require Sgs1, this interaction is likely to be important for heteroduplex rejection.

Survey of additional proteins that physically interact with Msh6 and/or Sgs1 for a role in heteroduplex rejection.

Following identification of an interaction between Msh2-Msh6 and Sgs1, we asked whether other proteins that interact with Msh6 or Sgs1 could play a role in heteroduplex rejection. We constructed a profile of known protein-protein interactions with Msh2-Msh6 and Sgs1 by searching the literature and utilizing the BioGrid protein-protein interaction database (Figure 3.1C, Stark et al., 2006, 2011). Sgs1 has a larger collection of interacting proteins than Msh6. Msh6 appears to interact nearly exclusively with replication and mismatch repair (MMR) components (Clark et al., 2000; Lau et al., 2002, 2003; Argueso et al. 2003; Shell et al., 2007; Chen et al., 2010), while Sgs1, in addition to its stable binding partners Top3 and Rmi1 (Gangloff et al., 1994; Bennett et al., 2000; Fricke et al., 2001; Chang et al., 2005; Mullen and Brill, 2005; Chen and Brill, 2007), shares interactions with proteins representing a wider array of

DNA repair processes, including DSB end resection (Mre11 and Dna2, Chiolo et al., 2005; Cejka et al., 2010; Niu et al., 2010), nucleotide excision repair (Rad16, Saffi et al., 2001), replication restart following damage (Rtt107, Chin et al., 2006), sister chromatid segregation (Smc6, Sollier et al., 2009), meiotic chromosome segregation (Mlh1-Mlh3, Argueso et al., 2002, 2003; Wang et al. 2002; Dherin et al., 2009), and the DNA damage response (Rad53, Bjergbaek, et al., 2005). Additionally, Sgs1 interacts with SUMO protein Smt3 and SUMO conjugating enzyme Ubc9, and sumoylation of Sgs1 is known to regulate telomere-telomere recombination (Sollier et al., 2009; Lu et al., 2010). Sgs1 and its human homolog WRN also interact strongly with Gis1, a histone demethylase, and its binding partner Bud27 (Tronnersjoe, et al. 2007).

Many of the proteins in the interaction profile for Msh6 and Sgs1 are encoded by essential genes and therefore could not be tested by deleting them (Figure 3.1C). We decided to focus only on interactions with non-essential proteins and those that were well-documented and likely to act in a rejection process. Previously, Mlh1 and Srs2 were tested for a role in heteroduplex rejection and were eliminated (Sugawara et al., 2004). The interaction between Pol30 and Msh6 had already been characterized and known to depend on the N-terminal linker domain of Msh6 and a conserved PIP (PCNA interaction peptide) motif within Pol30 (Clark et al., 2000; Shell et al., 2007). Though the catalytic member of the PCNA complex Pol30 is essential, viable Pol30-Msh6 interaction mutants as well as conditional mutants of *POL30* were readily available. Since Mre11 is required for end resection, we expected that SSA would be diminished in a *mre11* strain and decided not to pursue it. Even though *UBC9* and *SMT3* are essential genes, they are described to modify residues K621 and K831 on Sgs1 (Lu et al., 2010). However, attempts to mutate these residues as well as construct *rtt107Δ* and *gis1Δ* mutants in the SSA assay strains have so far been limited by technical issues.

Interestingly, Rad16 which interacts with Sgs1 is also predicted to interact with Msh6 (Gavin et al., 2002, 2006). We also noted that both Sgs1 and Msh6 were predicted to interact with a newly discovered protein Cmr1 which binds to damaged DNA (Gavin et al., 2002, 2006; Choi et al., 2012). We deleted *RAD16* and *CMR1* from strains bearing an SSA cassette with either identical (A-A) or 3% divergent (F-A) direct repeats. We utilized a SSA-based assay (described in Sugawara et al., 2004), to see if *rad16Δ* or *cmr1Δ* mutants were defective for disruption of a repair intermediate containing a region of heteroduplex DNA. Briefly, a DSB is induced between two 205-bp direct repeats that are either identical (A-A strain) or 3% divergent (F-A strain), and efficiency of SSA repair of the DSB in the two strains is compared. Following induction of a break between the direct repeats by galactose-inducible expression of the HO endonuclease, SSA will efficiently repair the break only when the repeats are identical (A-A) and confer cell survival (Table 3.1). The F-A heteroduplex is rejected by Msh2-Msh6 and Sgs1 so SSA is inefficient and the cells will die due to lethality cause by an unrepaired break. In *rad16Δ* and *cmr1Δ* strains, efficiency of SSA between A-A and F-A repeats was equivalent to the wild-type efficiency indicating that neither of these proteins has a role in SSA or heteroduplex rejection. Although *cmr1Δ* displayed a mildly better survival than wild-type when SSA occurred between the A-A repeats, the difference was not significant.

Top3-Rmi1 affect heteroduplex rejection by stabilizing Sgs1

Top3 is a type I topoisomerase that forms a stable complex with Sgs1 and depends on association with the small protein Rmi1 for its activity (Gangloff et al., 1994; Bennett et al., 2000; Fricke et al., 2001; Chang et al., 2005; Mullen and Brill, 2005; Chen and Brill, 2005). The

Sgs1-Top3-Rmi1 (STR) complex serves several roles in maintenance of genome stability such as DSB end resection, rescue of stalled replication forks, and dissolution of double Holliday junctions (Hickson and Mankouri, 2011; Mimitou and Symington, 2011). Strains lacking *TOP3* or *RMI1* are characterized by slow growth, hyper-recombination, and sensitivity to DNA damaging agents, and these phenotypes are often rescued by deletion of *SGS1*, indicating that Top3-Rmi1 is required for completion of Sgs1-mediated DNA transactions (Shor et al., 2002; Ui et al., 2005). However, it is clear that Sgs1 function does not absolutely depend on Top3-Rmi1 since Top3-Rmi1 is not required for DNA end resection, although it does serve a stimulatory role (Cejka et al., 2010). Since *top3Δ* mutants are known to suppress recombination between DNA sequences sharing limited homology (Wallis et al., 1989; Bailis et al., 1992), we thought that Top3-Rmi1 might participate with Msh6 and Sgs1 in heteroduplex rejection. To determine whether this was the case, SSA efficiency of *top3* and *rmi1* strains was determined using the A-A/F-A assay and was analyzed both by percentage of cells surviving the break or by quantification of SSA product in the cell population by Southern blot analysis.

Attempts to construct *top3Δ* and *rmi1Δ* in the A-A and F-A strains were unsuccessful, indicating that those modifications are likely to be lethal in the strain background used for SSA assays. Since constructing new SSA strains would be a time-consuming undertaking, we instead used temperature sensitive alleles of *TOP3* and *RMI1* that display *top3-null-* and *rmi1-null*-like phenotypes at 37°C, but not 22°C. The *top3^{ts}* allele shows slow growth and sensitivity to the DNA damaging agents methylmethane sulfonate (MMS) and hydroxyurea (HU) at the restrictive temperature, similar to *top3Δ* (Figure 3.2, Shor et al., 2002; Wagner et al., 2006). We obtained *rmi1^{ts}-2* and *rmi1^{ts}-3* by screening for *rmi1* mutants that failed to complement slow growth and MMS sensitivity of an *rmi1Δ* strain at 37°C but not 22°C. Both alleles are similar in phenotype

Table 3.1. SSA repair efficiency* as determined in survival assays

relevant genotype	temp	A-A	F-A	A-A/F-A
wild-type	22˚C	0.93 ± 0.09	0.25 ± 0.05	3.6
	25˚C	0.77 ± 0.13	0.28 ± 0.06	2.8
	30˚C	0.79 ± 0.11	0.24 ± 0.06	3.3
	32˚C	0.84 ± 0.07	0.22 ± 0.05	3.9
	37˚C	0.79 ± 0.09	0.25 ± 0.07	3.2
*msh6*Δ**	30˚C	0.87 ± 0.02	0.61 ± 0.06	1.4
*sgs1*Δ**	30˚C	0.79 ± 0.16	0.75 ± 0.18	1.1
rad16Δ	30°C	0.67 ± 0.10	0.21 ± 0.02	3.2
cmr1Δ	30°C	0.90 ± 0.10	0.19 ± 0.01	4.7
top3^{ts}	22˚C	0.70 ± 0.10	0.38 ± 0.01	1.9
	25˚C	0.74 ± 0.18	0.38 ± 0.08	2.0
	30˚C	0.75 ± 0.5	0.48 ± 0.05	1.6
	32˚C	0.78 ± 0.07	0.42 ± 0.04	1.9
	37˚C	0.67 ± 0.16	0.50 ± 0.14	1.3

Table 3.1 (continued)

rmi1^{ts}-2	22˚C	0.98 ± 0.16	0.58 ± 0.16	1.7
	25˚C	0.95 ± 0.13	0.60 ± 0.18	1.6
	37˚C	0.91 ± 0.15	0.64 ± 0.10	1.4
rmi1^{ts}-3	22˚C	0.77 ± 0.05	0.50 ± 0.21	1.5
	25˚C	0.83 ± 0.11	0.41 ± 0.10	2.0
	37˚C	0.96 ± 0.08	0.55 ± 0.08	1.8
msh6-KQFF >AAAA	30˚C	0.77 ± 0.08	0.21 ± 0.03	3.6
pol30-201	30˚C	0.56 ± 0.10	0.17 ± 0.03	3.2
pol30-204	30˚C	0.70 ± 0.08	0.21 ± 0.03	3.4
pol30-216	30˚C	0.48 ± 0.08	0.13 ± 0.02	3.7

*Survival of indicated strains expressed as colony forming units on galactose/glucose ± standard deviation for three or more experiments.

** *msh6Δ* and *sgs1Δ* data previously reported in Goldfarb and Alani (2005), shown for comparison.

to the *rmi1-1* mutant described by Ashton et al. (2011), and display similar phenotypes to the *top3*^{ts} allele, though with less severity (Figure 3.2).

The temperature sensitive alleles were integrated by replacement of the wild-type allele into the strains bearing the A-A and F-A cassettes and both SSA and heteroduplex rejection efficiencies were determined. In the wild-type strain, neither SSA nor rejection efficiency were affected at a variety of temperatures ranging from room temperature (RT, 22-23°C) to 37°C as determined by the cell survival assay (Table 3.1). However, in strains bearing the *top3*^{ts} allele or either one of the *rmi1*^{ts} alleles, heteroduplex rejection was reduced at all temperatures. These data reveal that, while these alleles are temperature-sensitive for slow-growth and sensitivity to DNA damage, they also have additional defects that are independent of temperature. Although heteroduplex rejection was reduced in all *top3* and *rmi1* mutants, it was never eliminated to the level that is typical for *sgs1Δ* (A-A/F-A = 1.1), indicating either a potential stimulatory but non-essential role for Top3-Rmi1 in heteroduplex rejection, or residual expression of Top3-ts or Rmi1-ts protein. Curiously, SSA appeared to be increased in the presence of the *rmi1*^{ts}*-2* allele at all temperatures and at higher temperature in the presence of the *rmi1*^{ts}*-3* allele, but the results were not significant based on a Wilcoxon-Mann Whitney test.

While cell survival normally is an accurate reflection of SSA efficiency determined by Southern blot (Sugawara et al., 2004), analysis of SSA and heteroduplex rejection in wild-type and *top3*^{ts} strains at lower (23°C) and higher (37°C) temperatures using Southern blotting gave results conflicting with the cell survival data. First, SSA in general appeared to be increased in *top3*^{ts} at 37°C (product = 1.01 ± 0.07 for A-A and 0.66 ± 0.03 for F-A compared to survival = 0.67 ± 0.16 for A-A and 0.50 ± 0.14 for F-A, Figure 3.3D and Table 3.1). Secondly, heteroduplex rejection was clearly reduced in the *top3*^{ts} strains (A-A/F-A = 1.9, Figure 3.3D)

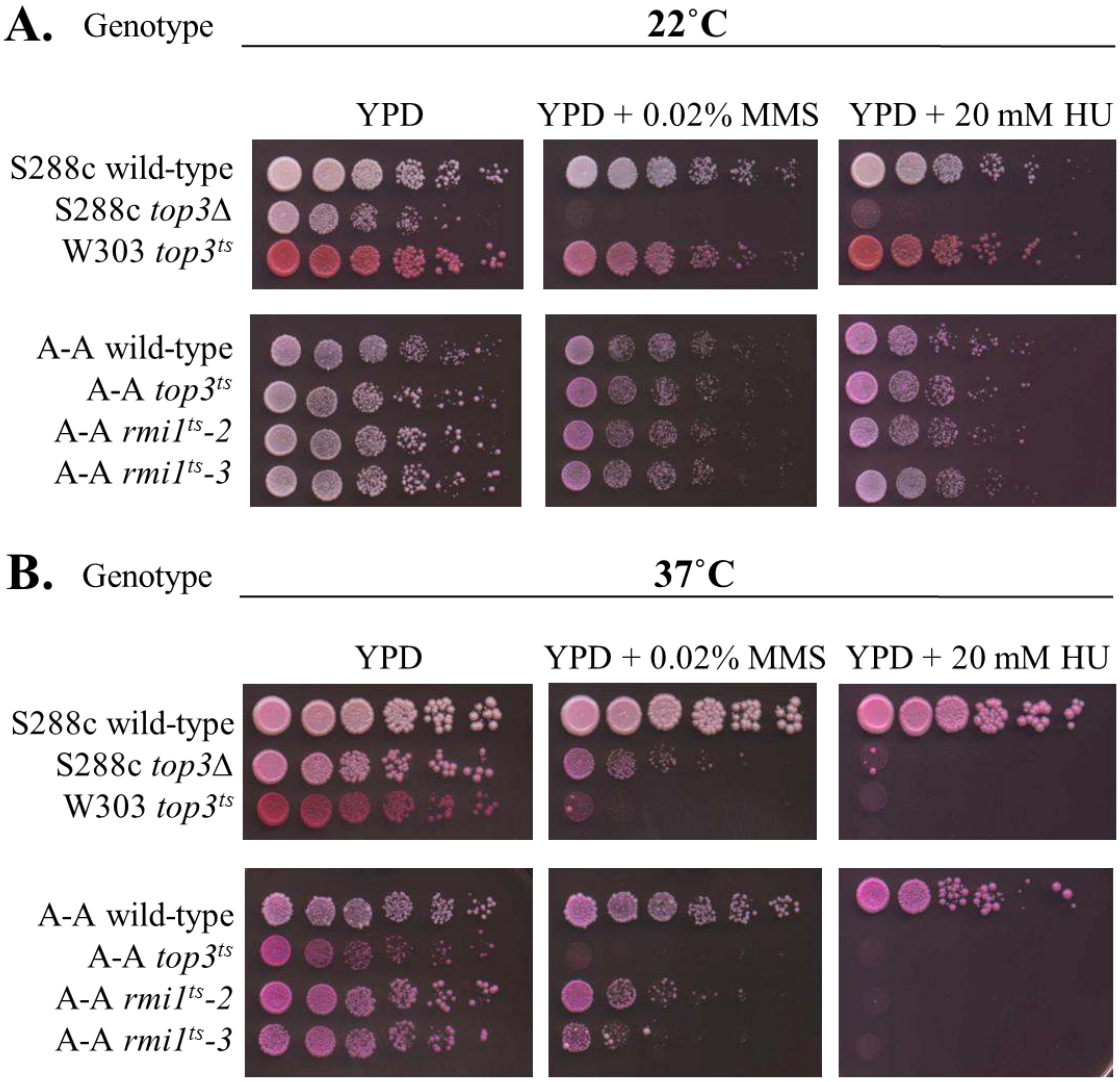


Figure 3.2. Temperature sensitive alleles of *RMI1* display *rmi1-null*-like phenotypes at 37°C but not 22°C, similarly to a previously characterized *top3*^{ts} mutant. 10-fold serial dilutions of the indicated strains spotted onto YPD plates containing 7.5 µm erythrosine B to stain dead cells pink and with or without the indicated DNA damaging agents. Both the *top3*^{ts} mutant (as described by Wagner et al., 2006) and two *rmi1*^{ts} alleles isolated in this study display slow growth, increased cell death, and sensitivity to DNA damaging agents at 37°C **(B.)** but not 22°C **(A.)**. Note that the W303 *top3*^{ts} strain appears pink due to an *ade2* mutation so that cell death by erythrosine B staining cannot be determined.

compared to wild-type (replicate blots and quantifications are in progress, A-A/F-A = 3.9, Figure 3.3C) at 37°C in agreement with the cell survival data. However, heteroduplex rejection appeared to be intact in the *top3*^{ts} strains (A-A/F-A = 3.9, Figure 3.3B) at 23°C, which is in opposition with the survival data that indicates a similar defect at both temperatures (Table 3.1).

Since Chang et al. (2005) showed that Sgs1 was de-stabilized in *top3Δ* and *rmi1Δ* strains, we wondered whether the *top3*^{ts} and *rmi1*^{ts} alleles may contribute to heteroduplex rejection by stabilizing Sgs1. To test this, we integrated the *top3*^{ts} and *rmi1*^{ts} strains with Sgs1-3HA and used immunoblotting with an α-HA antibody to evaluate the levels of Sgs1-3HA at 23°C and 37°C. Preliminary experiments show that Sgs1 stability is lost at 37°C but appeared to be at relatively normal levels at 23°C (Figure 3.4). Following optimization of the cell lysis and western blot protocols, we should be able to quantify Sgs1 levels at both temperatures, but optimization has proved to be difficult. At least in a qualitative sense, we can conclude that instability of Sgs1 explains the heteroduplex rejection defect at 37°C, and may also explain a partial defect at 23°C. The latter will need to be confirmed by quantitative analysis of Sgs1 levels if it can be achieved, and explanation for the discrepancy between cell survival and Southern blot analysis of heteroduplex rejection in the *top3*^{ts} strains. Analysis of rejection in the *rmi1*^{ts} strains by Southern blot may also reveal some clues to address these issues.

PCNA-Msh6 interaction is not required for rejection, but PCNA stimulates SSA

PCNA is a multi-subunit complex that is associated with replication forks and required for processivity of replication (reviewed by Kelman, 2007). The catalytic subunit Pol30 forms a stable interaction with the N-terminus of Msh6, and this interaction requires a characteristic

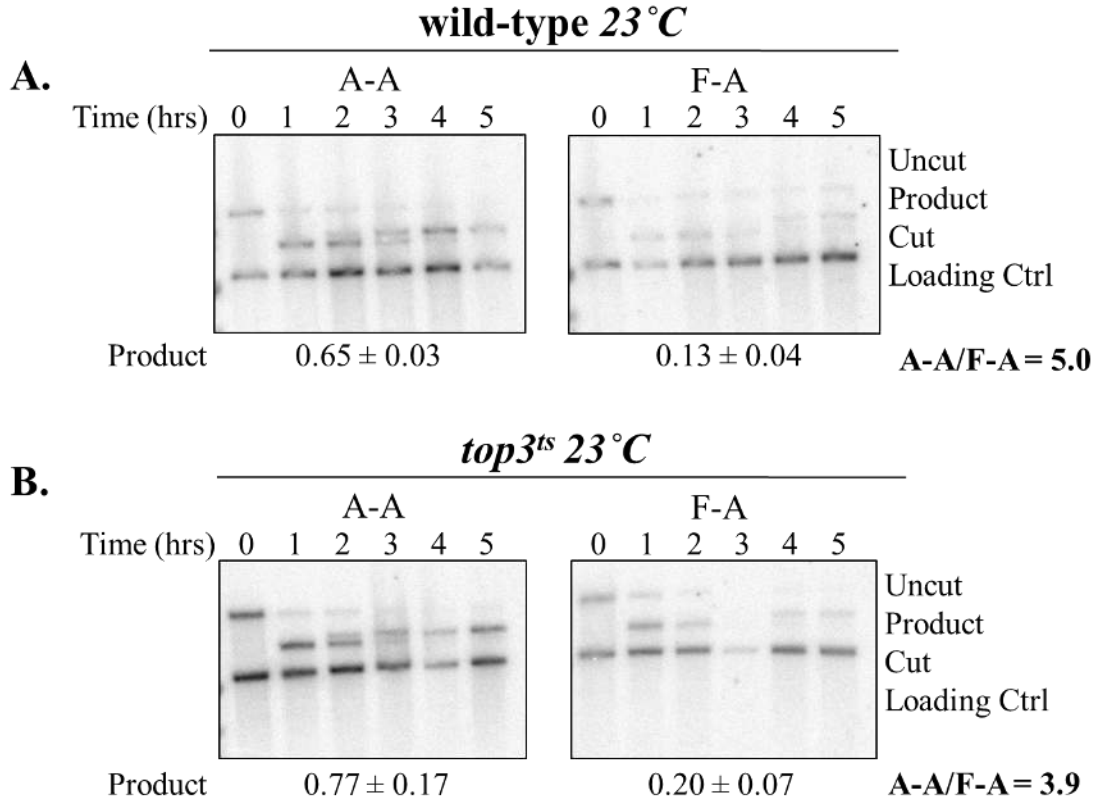
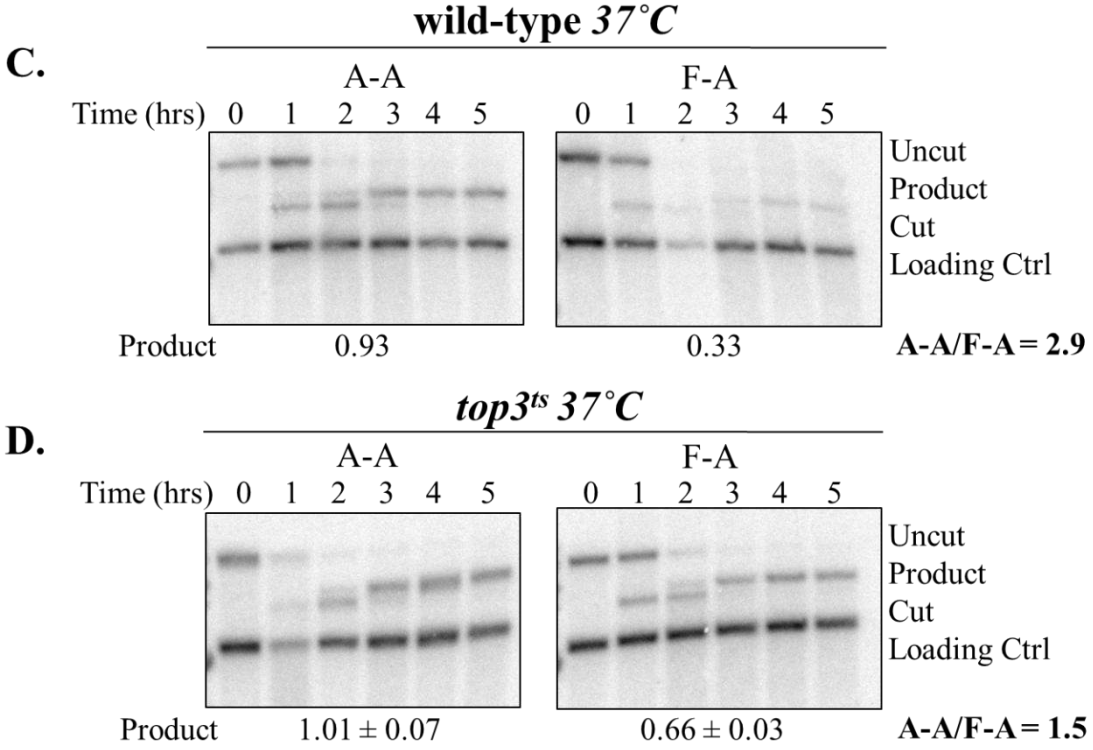


Figure 3.3. Heteroduplex rejection is defective in the *top3*^{ts} strain at 37°C, and partially defective at 22°C Southern blot analysis of heteroduplex rejection efficiency as described in Sugawara et al., 2004 of the indicated genetic backgrounds at the indicated temperatures; **A.)** wild-type at 22°C. **B.)** *top3*^{ts} at 22°C. **C.)** wild-type at 37°C. **D.)** *top3*^{ts} at 37°C. The times indicate hours following induction of the HO endonuclease expression and subsequent DSB formation by addition of galactose. The four bands from slowest to fastest mobility are the uncut SSA cassette, SSA final product, the cut SSA cassette, and the loading control. The amount of product at the 5 hr time point was quantified as a fraction of the uncut cassette at T=0, and the mean ± standard deviation is shown for 3 or more experiments. The A-A/F-A ratio is determined from the mean products and represents the heteroduplex rejection efficiency. Representative blots are shown.

Figure 3.3 (continued)



KQFF motif (Clark et al., 2000; Shell et al., 2007). Lau and colleagues demonstrated that interaction with PCNA enhances mismatch recognition by Msh2-Msh6 (Lau et al., 2002, 2003), prompting us to speculate that PCNA could stimulate heteroduplex rejection by maximizing Msh6 function. To determine if this was the case we used both a *MSH6* allele which cannot bind PCNA (*msh6-KQFF>AAAA*), as well as three *pol30* mutants; *pol30-216* which displays a range of moderate to severe phenotypes in the presence of DNA damaging agents, a milder allele *pol30-204* which fails to interact with Msh6, and *pol30-201* which maintains interaction with Msh6 but otherwise shares a similar phenotypic profile to *pol30-204* (Lau et al., 2002). In each of the three *pol30* mutants, the A-A/F-A ratio was in the wild-type range, indicating that heteroduplex rejection was intact and that Msh6 performs its role in rejection independent of PCNA (Table 3.1). Therefore, even though PCNA can stimulate mismatch recognition by Msh2-Msh6 in the context of MMR (Lau et al. 2003), it does not do so in the context of heteroduplex rejection. Interestingly and surprisingly, both *pol30-216* and *po30-201*, but not *pol30-204* had a significantly reduced SSA efficiency. One of two scenarios can explain these results; 1) PCNA function is important for stimulating the SSA pathway and this function is lost in *pol30-216* and *pol-201*, but maintained in *pol30-204*, or 2) Interaction of mutant forms of PCNA with Msh6 inhibits SSA. It will be interesting to further explore the role of PCNA in SSA.

Discussion

Homologous recombination can occur via a number of different pathways which varies based on many factors, such as the type of damage that initiates it, the local environment of the damage, the cell type in which it occurs, and the time it occurs with respect to the mitotic or

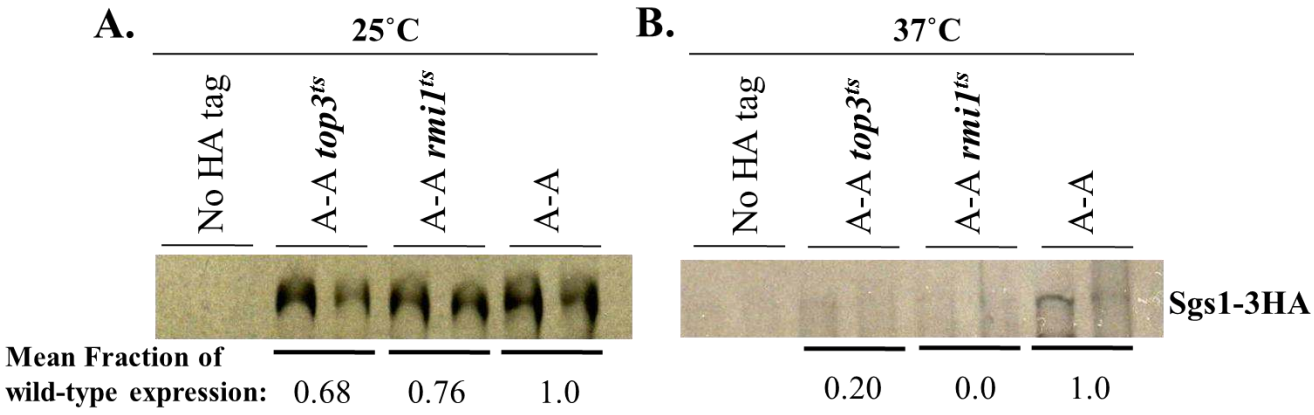


Figure 3.4. Sgs1 stability is lost in *top3*^{ts} at 37°C, but appears to be stable at 25°C. Preliminary western blot detection of Sgs1-3HA from cell extracts of strains of the indicated genotypes (*rmi1*^{ts} refers to the *rmi1*^{ts}*-2* allele) grown at **A.)** 25°C and **B.)** 37°C. Quantification of band intensities from the pictured blots are normalized to total protein concentration of the amount of lysate loaded onto the gel and are expressed as a mean fraction of the mean wild-type intensity at its respective temperature. Cultures were grown at in this experiment 25°C rather than 22°C due to error. Attempts to repeat these experiments had variable results with poor detection of Sgs1-3HA, though usually roughly equivalent levels at 22°C. Detection of Sgs1-3HA was always poor at 37°C even for wild-type, suggesting that Sgs1-3HA itself may be unstable at 37°C.

meiotic cycle (reviewed in George and Alani, 2012). Similarly, it is thought that the mode of heteroduplex rejection and/or factors involved may vary depending on some of the same factors and the type of HR pathway that is initiated. Because of its complicated nature, determining the mechanisms of rejection and factors involved has been difficult.

SSA between imperfectly homologous repeats consists of a single heteroduplex intermediate which arises by annealing of complementary single-stranded DNA (ssDNA) following 5' to 3' resection on opposite DSB ends. If the repeats are separated by a stretch of non-homologous sequence, the annealed region will be flanked by 3' ssDNA tails that would be, in the case of perfect homology, clipped off and deleted from the SSA repair product, but on a heteroduplex substrate may serve as a loading site for rejection factors. Previous studies in our lab have determined that the Msh2-Msh6 mismatch recognition complex and the Sgs1 helicase are required for unwinding of a heteroduplex SSA intermediate between 205 bp repeats of 3% sequence divergence (Sugawara et al., 2004; Goldfarb and Alani, 2005). The Mlh1-Pms1 and Exo1 proteins that interact with Msh2-Msh6 during MMR did not play a role in rejection of the SSA heteroduplex, nor did the Srs2 helicase which interacts with Sgs1 following DNA damage checkpoint activation and is also involved in suppressing HR (Chiolo et al., 2005). Here we show that three additional DNA repair factors that are known or predicted to interact with Msh6 and/or Sgs1 (Pol30/PCNA, Rad16, and Cmr1) are dispensible for rejection. Top3-Rmi1, though required for stability of Sgs1, may also lack a specific function in heteroduplex rejection, though this observation needs to be confirmed.

Absense of *TOP3* or *RMI1* results in loss of Sgs1 stability (Chang et al., 2005) and integration of a catalytic mutant of *TOP3* (*top3-Y356F*) was not attempted because it is known to be lethal to haploid cells (Wagner et al., 2006), so we expected the only way to investigate a role

for Top3-Rmi1 in the rejection mechanism *in vivo* was by using conditional mutants, in the hopes that such mutants would not affect Sgs1 stability. In our own hands and others (Wagner et al., 2006; Ashton et al. 2011), DNA damage sensitivity and growth phenotypes were only seen in the *top3*^{ts} and *rmi1*^{ts} strains at 37°C, so it was surprising to us to see equivalent reductions in heteroduplex rejection at 23°C and 37°C when using the cell survival assay. Since Sgs1 was clearly expressed (though we could not be certain whether to the wild-type level) at 23°C, we thought this could indicate a specific function for Top3-Rmi1 in rejection which we imagined could be stimulating binding to DNA or relieving supercoils that could arise from unwinding. However, Southern blot analysis of heteroduplex rejection clearly indicated a temperature-sensitive defect in rejection in the *top3*^{ts} mutant.

Interpretation of this discrepancy is puzzling, but by looking more closely we can see that the source of the discrepancy is in the data collected at 23°C. While Southern blots indicate that 80% of F-A cells do not repair the break, only 60% will die in response to the break. This says that a percentage of cells in which the F-A intermediate are rejected will still survive. I can imagine two possible explanations for this. First, the result may be an artifact of experimental protocol. More specifically, we may not be detecting all of the SSA product by choosing to quantify the 5 hour sample. Growth rate is slower at a lower temperature, and it may take longer for cells to express the HO endonuclease and thus DNA breakage and repair are delayed. If we collected samples at later time points, we may quantify a larger amount of SSA product and discover that rejection is indeed defective in the *top3* mutant as the survival data indicates. Though we could not prove significance with our small data set, SSA product in the F-A wild-type strain appears to be reduced by half at 23°C compared to 30°C (reported in Goldfarb and Alani, 2005 and George and Alani, 2011), which would support this idea. If this is true, then a

more careful quantification of SSA product formation at late times following break induction (i.e. by using qPCR rather that Southern Blot) and more reliable quantification of Sgs1 levels may be able to assign a specific requirement for Top3-Rmi1 to heteroduplex rejection separate from providing Sgs1 stability. So far, using immunoblotting to quantify Sgs1 levels has proven to be difficult, but should be attainable with some troubleshooting (see Appendix for suggestions).

The second possibility is that a fraction of the cells that have rejected F-A intermediates can undergo another form of break repair that is usually blocked by *TOP3*, ensuring their survival. One could perform PCR and sequencing on surviving colonies to determine whether this is happening; however this approach would be very difficult since we would not know what type of repair product to look for. Since rejected ends in our system lack homology to any other sequences in the genome, I would expect such a repair pathway to be one that does not require homology such as NHEJ. There are no reports that I can find, however, indicating *TOP3* as a regulator of NHEJ.

Since Lau et al., (2003) showed that PCNA promoted the ability of Msh2-Msh6 to locate mismatches during MMR, we thought it may also assist this activity during heteroduplex rejection. The study by Lau et al. showed that PCNA would hand-off Msh2-Msh6 to the mismatch by keeping it close to the replication fork, and wondered whether it may also serve to localize Msh2-Msh6 in a different context. We tested this by looking at both a Msh6 mutant that fails to interact with PCNA (*msh6-KQFF>AAAA*) and a PCNA mutants incapable of interacting with Msh6 (*pol30-204*). Both mutants completed heteroduplex rejection at wild-type levels, confirming that PCNA does not stimulate mismatch recognition during heteroduplex rejection. This provides evidence that the PCNA-Msh6 interaction serves only to tether Msh2-Msh6 to the

replication machinery where mismatches might occur, but PCNA does not in general help to recognize mismatches.

In addition to the *pol30* interaction mutant, we simultaneously tested two other *pol30* mutants, *pol30-201,* which was intended as a control for *pol30-204* since it appeared to act similarly in other assays even though the interaction with Msh6 was maintained, and *pol30-216*, which was a more severe mutant. It was surprising to see a decrease in SSA in these mutants, since PCNA has never been shown to participate in SSA. The fact that SSA is decreased but not eliminated in these mutants, indicates a stimulatory role, rather than a requirement. Human PCNA is known to stabilize binding of various polymerases to the 3'-OH of a DNA template, and to stimulate elongation (Maga and Huebscher, 1995; Einolf and Guengerich, 2000; Maga et al., 2002), so it is likely to be stimulating the DNA synthesis step of SSA. In fact, human PCNA has been shown to stimulate DNA synthesis during microhomology-mediated end joining, which is a process similar to SSA that is instead initiatied by terminal micro-satellite sequences on ssDNA ends and lacks the step requiring cleavage of non-homologous 3' ssDNA tails (Crespan et al., 2012). This would suggest that PCNA would act downstream of tail removal during SSA.

The difficulties we faced with modifying the A-A and F-A strains have precluded the ability to test a number of other factors we wanted to test. Currently, we have been unable to delete *MPH1*, another helicase which is known to suppress recombination (Banerjee et al., 2008; Prakash et al., 2009), and an initial attempt to delete *RTT107* marked with *KANMX* resulted in a number of G418-resistant colonies that retained the wild-type *RTT107* locus, similar to the results of trying to knock out *TOP3, RMI1,* or *MPH1*. It is still possible that the *gis1Δ* and sumoylation mutants *sgs1-K621R* and *sgs1-K831R* could be constructed. The latter we tried to create by Quickchange mutagenesis, but did not get the PCR to work after two tries. The

mutants should be attainable with troubleshooting. For the former, we tried to amplify a *gis1Δ::KANMX* fragment from the deletion collection, but could not see a PCR product even when primers as far as 400-bp outside the coding region were used. The primers were able to amplify wild-type *GIS1* from other strains, and we were able to PCR other genes from the genomic DNA isolated from the *gis1Δ* deletion collection strain. These indicate a discrepancy with the amount of *GIS1* sequence that is reported to be deleted in the deletion collection strain, and what is actually deleted. A *gis1Δ* fragment will have to be constructed by PCR of the selective marker using primers with ends homologous to *GIS1*.

Finally, we hoped to compliment this work with a chromatin immunoprecipitation (ChIP) approach to detect recruitment of Msh2-Msh6 and Sgs1-Top3-Rmi1 to the heteroduplex intermediate. This approach, though we believe an attainable goal, proved to be more complicated and time consuming than expected. Progress made with ChIP is reported in the Appendix.

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CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

Current understanding of heteroduplex rejection

Rejection of heteroduplex recombination intermediates is an area that largely has not been explored in mechanistic detail. While a number of studies have determined protein and minimal sequence requirements for suppression of HR, the individual heteroduplex intermediates that are acted upon in those situations are unknown due to the variety of HR pathways that may be employed in each case (Bailis and Rothstein, 1990; Sugawara, 2000; Nicholson, 2000; Schildkraut, 2005; Waldman, 2008; Jain et al., 2009). SSA has been a very useful model for determining more specifically the minimal protein machinery for rejection of a specific heteroduplex, and for determining some mechanistic details (Sugawara et al., 2004; Goldfarb and Alani, 2005; George and Alani, 2011). SSA is a very efficient and simplified form of HR that consists of a single duplex intermediate (Fishman-Lobell et al., 1992). Adaptation of a widely used SSA cassette (Sugawara et al., 1997) in yeast to compare repair efficiency when the duplex is either identical or 3% divergent has been used to confirm that the mismatch recognition complex Msh2-Msh6 and Sgs1 helicase work cooperatively to conservatively unwind duplex SSA intermediates that form between divergent sequences (Sugawara et al., 2004; Goldfarb & Alani, 2005). Those studies and the ones presented in this dissertation suggest that the components involved in heteroduplex rejection are few. In yeast, Msh2-Msh6 and Sgs1-Top3-Rmi1 appear to be the minimal machinery for these processes, though all potential factors have not been tested and it also cannot be ruled out that other components might be necessary for rejection of non-SSA heteroduplexes. Since these proteins are highly conserved across species, and mutation of their homologs in humans cause diseases characterized by frequent genome

rearrangements (reviewed by Cheok et al., 2005; Ouyang et al., 2008; Singh et al., 2009; Silva et al., 2009), it is likely that their function in rejection is conserved as well. These complexes are also central to many other genome maintenance processes (Reenan et al., 1992; Johnson et al., 1996; Zhu et al., 2008; Bernstein et al., 2009; Lydeard et al., 2010; Mankouri et al., 2011) and interact genetically and physically with numerous other proteins (Gangloff et al., 1994; Bennett et al., 2000; Clark et al., 2000; Fricke et al., 2001; Saffi et al., 2001; Argueso et al. 2002, 2003; Lau et al., 2002, 2003; Wang et al. 2002; Bjergbaek, et al., 2005; Chang et al., 2005; Chen and Brill, 2005; Chiolo et al., 2005; Mullen and Brill, 2005; Chin et al., 2006; Shell et al., 2007; Tronnersjoe, et al. 2007; Dherin et al., 2009; Sollier et al., 2009; Cejka et al., 2010; Chen et al., 2010; Lu et al., 2010; Niu et al., 2010), though all the interactions tested so far do not appear to have any direct role in recognition or disruption of a simple heteroduplex.

It seems that heteroduplex rejection, at least in the context of SSA, is likely to be a ubiquitous process that can occur during any stage of the cell cycle and is not regulated by checkpoint mechanisms (George et al., 2011; Hombauer et al., 2011). In other words, Msh2-Msh6 and Sgs1 are poised to unwind any heteroduplex if and when they occur, and the only limitations placed on rejection are contextual; the relative availability of ssDNA intermediates to Msh2-Msh6 and Sgs1 or a proper homologous substrate, and the amount of time available for finding them. If that is the case, then limiting availability to a homologous substrate would be expected to enhance the efficiency of heteroduplex rejection. Indeed, the SSA cassette used by ourselves and the Haber lab, was designed such that no better homologous substrate occurs in the genome other than within the SSA cassette, and when time for finding the sole homologous substrate in this context is lessened by inactivation of the DNA damage response, heteroduplex rejection is enhanced (George et al., 2011).

Unanswered questions about heteroduplex rejection during SSA and limitations of the SSA cassette and strains

The strains bearing the A-A and F-A cassettes are not typical wild-type strains and have been difficult to modify to our desires. They are derived from W303 background, and contain a number of genetic modifications that are required for the SSA assay (Sugawara et al, 1997, 2000, 2004). The A-A and F-A cassettes were integrated into the *URA3* locus and is marked by a *THR4* gene. The HO endonuclease site is deleted from the mating locus and exists only within the SSA cassette. The HO endonuclease is under the control of the *GAL4* promoter and this is marked with a *NATMX* cassette. The strains are sick compared to typical wild-type strains, with a growth rate of approximately 2-3 hours per doubling, though the reason for sickness is unknown. Modification at the mating locus causes them to be incapable of mating, and in any case they only exist as the α mating type. The result is that genomic modifications can only be done by HR-mediated integration; however, only two auxotrophic markers are available for strain modifications, *LEU2* and *TRP1*. The *leu2* and *trp1* mutations are not full deletions, but point mutations, and they can (and do) revert. Drug cassettes, *KANMX* and *HPHMX,* have also been used for marking integrations, but are not always successful, presumably recombining with the *NATMX* cassette rather than at the desired locus.

A few lines of evidence suggest that the A-A and F-A strains possess an unknown mutation that affects DNA repair. The first being a high rate of *leu2* and *trp1* revertants that arise during attempts to integrate modifications using either of these markers. The second was noted by the morphology of colonies on a plate. Though colonies are generally smaller than for a typical wild-type strain, they will become larger with time and will adopt a jagged rather than round shape that is typical of strains with recombination defects. Lastly, at least four genes

involved in DNA repair (*TOP3, RMI1, MPH1,* and *RTT107,* data not shown) were unable to be knocked out despite not usually being lethal modifications, suggesting a synthetic lethality with another unknown genetic modification. Furthermore, attempts to delete these genes with a *KANMX* marker often resulted in a large number of G418-resistant colonies, that when screened by PCR, revealed presence of *KANMX* in the genome but its absence from the desired locus, suggesting a propensity for the cassette to recombine elsewhere in the genome. It is likely that it only replaced the *NATMX* at on the *GAL4-HO* through shared homology between the two cassettes, though this was never confirmed.

The inability to create *top3* and *rmi1* deletions complicated attempts to determine whether there is a role for these proteins in heteroduplex rejection. It is quite clear that Top3-Rmi1 is required for stability of Sgs1 (Chapter III; Chang et al., 2005), and in that in this respect is necessary for heteroduplex rejection, but whether it has a specific functional role is unclear and has been difficult to determine using the current system. Attempts to delete *TOP3* or *RMI1* from the SSA strains were unsuccessful, and expression of the catalytic mutant *top3-Y356F* is known to be lethal (Wagner et al., 2006). An *sgs1* mutant lacking the Top3 interaction domain (*sgs1-ΔN644*) was defective for heteroduplex rejection, however the deleted region in this construct is quite large, and one cannot be certain that other functional domains are missing (Goldfarb and Alani, 2005). Furthermore, it is not known whether this allele is expressed at wild-type levels and I have been unable to establish a reliable method for determining the level of Sgs1 expression in my hands. In any case, I expect that Top3-Rmi1 is not essential for heteroduplex rejection, though it may play a minor stimulatory role, similar to its function during Sgs1-mediated end resection (Cejka et al., 2010, see Chapter III for further discussion).

Since the SSA strains and A-A and F-A cassettes are difficult to modify, use of these constructs for establishment of more specific sequence requirements would be a tedious task. Nevertheless, it would be useful to find the minimal and maximal repeat size, number, and sequence identity required for recognition and unwinding, and whether the necessary protein machinery differs with these variables. Doing so may give us an idea of the relative likelihood of any particular endogenous non-allelic sequences recombining with another, helping to narrow down the regions of the genome at most risk for chromosomal rearrangements. Based on the MEPS described by Jinks-Robertson (1993), reviewed in Waldman (2008), and introduced in Chapter I of this thesis, it appears that approximately 200 bp of uninterrupted perfectly homologous sequence is required for efficient recombination in yeast, and this number varies a bit depending on the species. However, MEPS is a function of all biochemical and cellular restrictions placed on HR, and is not a measure for heteroduplex rejection in particular. One would expect that increasing the homology or length of adjacent repeats would limit heteroduplex rejection, and the opposite would in enhance it. Furthermore, if different types of mismatches occur within heteroduplexes such as multiple adjacent mismatches and insertion/deletion loops, Msh2-Msh3 would be expected to recognize them rather than Msh2-Msh6 (Nicholson, 2000). Additionally, palindromic or hairpin-forming sequences would be more likely recognized by Srs2 (Dhar and Lahue, 2008). Msh2-Msh3 is not known to interact with Sgs1, but does interact with nucleases (Schmutte et al., 2001; Li et al., 2013), so that degradation of such heteroduplexes would be more likely. Furthermore, it makes sense that heteroduplexes which occur within more complex structures such as DSBR or SDSA intermediates, may be difficult to disrupt by unwinding alone and may require cleavage or digestion steps. In order to test some of these possibilities and determine the upper and lower

homology limits, a more tractable cassette would need to be created in which a range of sequence homologies and repeat lengths could be easily constructed.

The influence of distance between repeats on SSA is better understood, though not on heteroduplex rejection. SSA is fully efficient between repeats spaced up to 5 kb apart, and those at larger distances have a greater tendency to be recombine via traditional HR mechanisms (Jain et al., 2009). The time-limiting factor for SSA is the rate of resection and strand annealing cannot occur until both repeats are resected (Fishman-Lobell et al. 1992; Sugawara and Haber 1992), so it would make sense that heteroduplex rejection would be limited by the rate of resection as well. The SSA cassette contains 2.6 kb of non-homologous sequence between the A-A and F-A repeats that must be resected, and SSA is largely inefficient in the context of the F-A repeats. If one were able to increase the distance between the repeats to various lengths up to 5 kb, I would expect rejection to become increasingly less efficient since there would be less time for heteroduplexes to form and thus be recognized. Conversely, shortening the distance is expected to maximize rejection efficiency, although 2.6 kb appears to be approaching this threshold since 70% to 80% of heteroduplexes are successfully rejected in this context (Sugawara et al., 2004).

Though the SSA cassette is ideal for dissecting an individual SSA event, it is ectopically integrated into a non-repetitive region of the genome and is not reminiscent of natural repetitive sequences. Repetitive sequences of the genome tend to be sequestered within heterochromatic regions, and it is thought that their stability is largely influenced by chromatin condensation status (Torres-Rosell et al., 2007; Chiolo et al., 2010; George and Alani, 2012). In fact, Torres-Rosell et al. (2007) showed that HR factors in yeast are restricted from heterochromatic regions, suggesting that repair pathways, and thus rejection mechanisms may differ depending on the

amount of chromatin compaction in the region of the break. It would be interesting to test whether tendency for SSA and/or heteroduplex rejection are dependent on the location of the DSB in the chromatin landscape and the function of condensin proteins. Interestingly, Sgs1 was reported to physically interact with Smc5-Smc6, a condesin-like complex (Sollier et al., 2009). I would suggest obtaining mutants of Smc5-Smc6 and perhaps Smc2-Smc4 (condensin) and Smc1-Smc3 (cohesin) if available to determine if these factor influence SSA or rejection. Though deletion of *SMC5* or *SMC6* is lethal, well-characterized temperature-sensitive mutants are available (Cost and Cozzarelli, 2006;). Furthermore, if one could design a cassette that could be placed in different regions of the genome, one could determine whether dependence on the Smc proteins varies as a function of chromatin compaction status.

Finally, the heteroduplex intermediate that occurs during non-allelic SSA is quite different from other potential heteroduplexes that may form during traditional HR, although a similar intermediate is predicted to occur during the SDSA pathway. During the DSBR pathway, heteroduplexes may occur during strand invasion (D-loop formation), double Holliday junction formation, or branch migration (see Chapter I for description of DSBR). While, annealing of the SSA intermediate requires only Rad52, these more complicated structures require a number of additional factors and would be so simple to dismantle. Due to the vast complexity of HR pathways and our inability to accurately predict the pathway(s) that will be employed at any particular DSB, dissecting the mechanisms involved in heteroduplex rejection in these contexts would be extremely difficult to do *in vivo.* While recombination reporters can tell us which proteins suppress non-allelic recombination *in vivo,* they cannot tell us at which step and the mechanism by which they are acting. Development of an *in vitro* rejection system is absolutely necessary to determine the individual contributions of each of these factors (see below).

Suggestions for construction of new cassettes

Before further studies of heteroduplex rejection are continued in our lab, it is my suggestion to develop a careful plan for constructing new reporter cassettes. Since the goal is to ultimately understand the role that heteroduplex rejection plays in overall genome stability, it would be best to design a set of isogenic cassettes to compare suppression of non-allelic recombination among the major DSB repair pathways; DSBR vs. SDSA vs. SSA. Furthermore, while Southern blot analysis is probably the most accurate way of assessing efficiency of the individual pathways, it would be useful to develop cassettes using reporters that are more quickly and easily observed. I suggest the use of fluorescent markers such as GFP. For example, Mao et al. (2008) and Shao et al. (2012) are using cassettes like these in mammalian cells that produce a functional GFP only if the break is repaired by a particular pathway such as SSA. This allows rapid determination of repair type that may be observable in live cells by fluorescence microscopy or could be detected by FACS. One can also select for loss of a fluorescent marker that occurs between the repeats. My suggestion is to use such markers with isogenic cassettes in which the repeats are either in direct or inverted orientation to compare rejection during traditional HR vs. SSA.

For future strain construction, I would start with a healthier strain background with full deletions of marker genes rather than point mutations (S288c). The researcher should construct the cassettes on an integration vector in such a way as to anticipate future modifications. For example, one should make sure there are ample unique restriction sites to swap out repeats of differing sizes or sequence divergence, as well as to keep a plasmid bearing the repeat sequence alone for its mutagenesis and easy movement into the integration vector. Also, if one could do the same for the intervening non-homologous region if one wants to vary the distance between

repeats, for example. One should anticipate integration of the cassette into any site in the genome they choose and perhaps keep an uptag and downtag that could be fused to the desired sequences and used for PCR and integration of the cassette. If the cassette itself, repeats included, do not contain any sequence that is endogenous to the yeast genome, one would not have to worry about unwanted recombination events at the cassette during strain construction or the assay itself. It would be easy to do this if the selection marker were a drug resistance gene like KAN or NAT and the recombination reporter were something like GFP.

Finally, it would be helpful to maintain both a and α mating types of each strain to allow constructions by mating. Doing this would also open up the possibility of analyzing rejection in diploid cells or during meiosis. In order for such an option to be possible, the HO endonuclease cannot be used. Fortunately, we are at the advantage of having new and useful tools for DSB inductions. TALENs are an up and coming technology that have so far been successful in plants, zebrafish, xenopus, and several mammals (Carlson et al., 2012; Dahlem et al., 2012; Ding et al., 2012; Lei et al., 2012; Tong et al., 2012; Ansai et al., 2013; Zhang et al., 2013). These endonucleases can be engineered to recognize and cut any sequence of interest. By using TALENs, we would not even have to clone a cut site into the cassette, but could engineer an enzyme that will cut a sequence that is already present. Also, we could obtain multiple TALENs that will cut at different places within the cassette without having to alter the cassette itself. Additionally, we could co-cut with two or more TALENs simultaneously if we wanted. Finally, by putting the TALENs under the control of a strong drug-inducible promoter rather than the *GAL4* promoter, DSB induction could be more instantaneous and could be performed in rich media. This would eliminate the fermentative growth phase that is required prior to galactose induction which has been the most time-limiting factor in the SSA assay we have been using.

Developing an in vitro rejection system

The advantage of an *in vitro* system for heteroduplex rejection would be the ability to test individual DNA substrates one at a time and to add-in, leave-out, or do order of addition with protein factors. One could determine on and off rates for proteins on particular substrates and reaction kinetics and effects of nucleotides or other DNA binding proteins of interest on these parameters. Since different types of DNA substrates can be constructed that resemble D-loops, dHJs, or other HR intermediates, one could determine which intermediates heteroduplex rejection machinery are likely to be acting on *in vivo.*

Previous attempts by a former student in our lab Amy Lyndaker to reconstitute heteroduplex rejection *in vitro* were unsuccessful (Lyndaker, 2009). However, the major limitation at that time was that full-length Sgs1 protein had not yet been successfully purified, and the partial Sgs1 construct that Amy was using, $Sgs1_{400\text{-}1268}$, had only a weak helicase activity. Currently, two methods are reported for successful purification of full-length Sgs1, as well as the complete Sgs1-Top3-Rmi1 (STR) complex, and these proteins display robust helicase activity *in vitro* (Cejka, 2010; Niu et al., 2010). Sgs1 was purified by expression in insect cells, which is a system that has recently been established in our lab. Moreover, activity of Sgs1 on a wide array of DNA constructs that are reminiscent of various HR intermediates has already been characterized in these studies. If one wanted to set up an *in vitro* rejection system, one would have to first establish the purification protocol in the lab and to synthesize DNA constructs like those already tested in other labs, except with or without strategically placed mismatches. Protocols for purification of Msh2-Msh6 for these assays has already been established in the lab and is not difficult and reagents for making some of the DNA constructs are already present in the lab and were used by Amy (Lyndaker, 2009). Experiments would consist of DNA binding

assays by gel shift to determine whether Msh2-Msh6 stimulates binding of Sgs1 to mismatched DNA, and helicase assays to determine whether Msh2-Msh6 stimulates helicase activity of Sgs1 on mismatched DNA. Amy's work has begun to establish the conditions for these assays, but there is still significant optimization to do.

One potential challenge to consider is that synthesis of significantly large enough DNA constructs may be difficult. Amy was unable to see co-binding of Msh2-Msh6 and $Sgs1^{400\text{-}1268}$ to her DNA substrates. However, the footprints of both proteins together (Bennett et al., 1999; Kijas et al., 2003) are expected to take up a space that is larger than the substrate that she used. Her substrate consisted of 40 bp of dsDNA with a 25 bp 3' ssDNA tail and a mismatch was placed 20 bp away from the dsDNA/ssDNA junction. Since Msh2-Msh6 should take up about 12 bp on either side of mismatch and $Sgs1_{400\text{-}1268}$ would take up 12 bp of dsDNA, the footprints would overlap on the 40 bp substrate. Furthermore, full-length Sgs1 along with bound Top3-Rmi1 is significantly larger than $Sgs1_{400\text{-}1268}$ for which the footprint was determined. Depending on how Sgs1-Top3-Rmi1 sits at the dsDNA/ssDNA junction , I predict that a substrate with at least 40-50 bp between the mismatch and dsDNA/ssDNA junction would be necessary, and possibly a much larger substrate would be needed if a majority of the STR complex masks the dsDNA side of the junction. If linear substrates of this length cannot be constructed, one may have to use circular substrates. One also needs to consider the risks of trying to work with Sgs1. Sgs1 is a large and rather insoluble protein, though less insoluble in the presence of Top3-Rmi1 (Bennett et al., 1998; Cejka et al., 2010; Niu et al., 2010). It is difficult to purify and to maintain its stability *in vitro.* While others are successfully doing this, it is certainly not expected to be a simple or straightforward undertaking. One should expect taking on such a project to be challenging and a major time commitment.

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APPENDIX

METHOD DEVELOPMENT: A chromatin immunoprecipitation approach to detect recruitment of rejection factors to a heteroduplex SSA intermediate

Carolyn M. George and Eric Alani

In an attempt to further understand the mechanism by which Msh6 and Sgs1-Top3-Rmi1 act during heteroduplex rejection, I began developing a chromatin immunoprecipitation (ChIP) method to detect recruitment of these proteins to the SSA intermediate in the A-A and F-A strains following induction of the HO-DSB. Due to time constraints and difficulties faced during assay development, I was unable to complete this work. With more time and additional troubleshooting, I believe this method can be successful and provide an opportunity to answer many questions regarding timing and order of events during heteroduplex rejection. Here, I give an account of the progress I made so far, so that a future member of the lab can pick up where I left off.

Rationale

The studies presented in this dissertation and elsewhere (Sugawara, et al., 2004; Goldfarb & Alani, 2005) establish Msh2-Msh6 and Sgs-Top3-Rmi1 as the primary, and likely, the sole components required for rejection of a heteroduplex intermediate during SSA. The evidence for a physical interaction between these complexes (Lyndaker, 2009; Chapter III), suggest that they act together in the process, and their biochemical activities suggest a mechanism by which Msh2-Msh6 recognizes heteroduplexes and stimulates Sgs1-Top3-Rmi1 to unwind them.

However, no direct evidence exists to confirm this model. The ChIP approach discussed here, as well as previous attempts to reconstitute the heteroduplex rejection assay *in vitro* (Lyndaker, 2009), were aimed at providing this confirmation. In both cases, the strategy was to first determine whether Msh2-Msh6 was recruiting Sgs1 to sites of heteroduplexes, and then to determine whether it was stimulating its activity on these substrates. Once this relationship was established, the assay could be used to answer additional questions, such as: "What residues or domains on these proteins are important for recruitment or stimulation?," "How does the presence of other proteins affect recruitment or stimulation (i.e. Top3-Rmi1, Rad1-Rad10, Rad52, RPA)," and "What is the order of events and how quickly could the reaction occur following break induction?"

Materials and Methods

The assay is based on the ChIP assay described by Li et al. (2008) for detection of Rad1 to the SSA intermediate of the A-A strain. The assay protocol was based on the method described by Goldfarb and Alani (2004).

SSA time course and cross-linking: The SSA time course is performed similarly to described previously (George, et al., 2011; Chapter II), only using strains that are integrated with a 3HA-tagged Sgs1 or Rmi1 protein (Table A.1). Following induction of the HO-DSB with galactose, 45 ml samples are collected at various time points and incubated for 15 minutes, with gentle rocking, at room temperature with 4.5 ml of 37% formaldehyde to cross-link. 2.5 ml of 2.5 M glycine is added to quench cross-linking and incubated for 5 minutes with gentle rocking. The cells were pelleted by centrifugation at 3000 rpm for 5 minutes and placed on ice. Cell pellets were washed twice in 20 ml ice cold TBS buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl) and

Table A.1: Strains to use for ChIP at SSA locus

Strain numbers	Genotype
EAY3249, EAY3274	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**A**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT SGS1::**SGS1-3HA-LEU2***
EAY3222-24	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**F**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT SGS1::**SGS1-3HA-LEU2***
EAY3269-70	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**A**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT **top3^{ts}::HYG** SGS1::**SGS1-3HA-LEU2***
EAY3271-72	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**A**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT **rmi1^{ts}-2::KAN** SGS1::**SGS1-3HA-LEU2***
EAY3275, EAY3440-42	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**A**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT **msh6Δ::KAN** SGS1::**SGS1-3HA-LEU2***
EAY3266, EAY3443-47	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**F**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT **msh6Δ::KAN** SGS1::**SGS1-3HA-LEU2***
EAY3319-20, EAY3454-56	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**A**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT **RMI1::RMI1-3HA-TRP1***
EAY3321-22, EAY3457-58	*ho HMLα matΔ::leu2::hisG hmr-3Δ. mal2 leu2 trp1 thr4 [THR4 ura3-**F**(205bp) HOcs URA3-**A**] ade3::GAL-HO::NAT **RMI1::RMI1-3HA-TRP1***

suspended in 0.5 ml Lysis Buffer (50 mM HEPES pH 7.5, 1 mM EDTA pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholic acid, 1 mg/mL bacitracin, 1 mM benzamidine, 1 mM PMSF). At this point, the cells were either flash frozen in a dry ice/ethanol bath and stored at -80˚C, or were used immediately for cell lysis.

Cell lysis and sonication: If stored at -80˚C prior to lysis, cell suspensions were thawed on ice. Approximately 0.5 ml of acid-washed glass beads were added and cells were mixed by vortexing at 4˚C until a majority of the cells were lysed. Cell lysis was monitored by observing a 10 ul sample of extract under 100X light microscopy to confirm the presence of "ghosts," those cells appearing as a faint outline with no discernable organelles. The duration of vortexing was generally about 40 minutes. Glass beads were removed by poking a hole in the bottom of the tubes with a red hot 22_G1 needle and collecting lysates by 1 minute centrifugation. Cell lysates were sonicated in 2 or 3 ~10 second pulses at setting 1.5 on the Branson Sonifier 250 to shear the DNA. Pulses were peformed at 4˚C and samples were placed on ice for 2 to 3 minutes between each pulse. Shearing was monitored by running 10 ul of sonicated lysate on a 1% agarose gel, and was considered successful if the DNA appeared as smear ranging from ~ 100-1000 bp with maximum intensity focused at 500 bp. Sonicated lysates were either flash frozen in a dry ice/ethanol bath and stored at -80˚C, or were used immediately for immunoprecipitation (IP).

Immunoprecipitation: Each sample was thawed on ice if stored at -80˚C prior to IP and 50 ul was added to 250 ul 1xTE/1%SDS. These were the "Input" samples and were stored at -20˚C until DNA extraction. Also, $1/10^{th}$ of the original lysate was removed and stored at -20˚C for western blot analysis. 1 to 5 ug of the α-HA antibody (Roche, 12AC5) was added to the remainder of each sample to IP either Sgs1-3HA or Rmi1-3HA and were incubated ~ 1hr at 4˚C

on a rotisserie mixer. Meanwhile, Protein G- agarose beads (Roche) were prepared according to manufacturer instructions to create a 50% slurry with Lysis Buffer. 35 ul of 50% slurry was added to each sample and they were incubated for another hour at 4˚C on the rotisserie. Beads were pelleted by 1 minute centrifugation at 3000 rpm and the cleared lysate was removed with a 26_G3/8 needle and stored at -20˚C. The beads were washed in a series of 5 minute, 4˚C incubations in 1 ml Lysis Buffer on the rotisserie. Just before the final wash the beads were suspended in a small amount of Lysis Buffer (300 ul or less) and $1/10^{th}$ of them were removed and stored at -20˚C for western blot analysis. Success of the IP was determined by western blot analysis (usually on the following day) with the aforementioned -20˚C samples.

Elution and extraction of DNA: Beads remaining from the IP were washed as before, once with High Salt Lysis Buffer (same as Lysis Buffer but with 500 mM NaCl), once with Wash Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 250 mM LiCl, 0.5% Nonidet-P 40, 0.5% sodium deoxycholic acid), and twice with 1xTE. To elute the DNA from the beads, the beads were suspended in 100 ul Elution Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) and incubated 15 minutes at 65˚C. The beads were pelleted by 1 minute centrifugation at 13,000 rpm and the supernatant was transferred to a new tube. 150 ul of 1xTE/0.67% SDS was added to the beads and they were incubated another 15 minutes at 65˚C and pelleted again. This supernatant was combined with the previous supernatant ("eluate"), and the beads were discarded. For cross-link reversal, the eluate was incubated overnight at 65˚C. The next day, 250 ul TE containing 0.08 ug/ul glycogen and 0.4 ug/ul proteinase K was added to the eluates and they were incubated at 37˚C for 2 hours. DNA was extracted from these and the "Input" samples from above by standard phenol:chloroform extraction.

Semi-quantitative PCR: The primers for PCR are designed to amplify the region of the SSA locus that will become the 3'ssDNA tail/dsDNA junction in the annealed intermediate. Positive control primers are designed to anneal to a nearby region just upstream of the SSA cassette which resides at the *URA3* locus, and negative control primers anneal to the *PRE1* gene that is further away on the chromosome. We ordered primer pairs identical to those described by Li et al. (2008), where JC1/2 are AO2379 and AO2380, JC3/4 are AO2381 and AO2382, JC5/6 are AO2383 and AO2384, and the PRE1 primers are AO2851 and AO2852 (Table A.2 and Figure A.1). All JC primer pairs should give an approximately 100 bp product on both Input and ChIP DNA. PRE1 primers should give a 250 bp product only with Input DNA. The IP signal is determined by the ratio of PCR signal from ChIP DNA to Input DNA and is normalized to the western blot signal. The IP signal for the JC5/6 pair is expected to be constant across all SSA time points, but the IP signal for JC1/2 and JC3/4 may increase with increasing time points, indicating recruitment of the HA-tagged protein to that site.

Progress and Future Directions

Western blot detection of Sgs1-3HA and Rmi1-3HA: Detection of Sgs1-3HA is challenging due to its low expression level and the natural instability of the protein (Bennett et al., 1998). I have been able to detect Sgs1-3HA with varying success (Figure A.2A). The Sgs1-3HA band is expected to appear at approximately 165 kDa but actually runs at a mobility closer to 200 kDa. Using the HiMark Protein Standard (Invitrogen), it is easiest to identify it as a band that runs approximately halfway between the 171 and 238 kDa markers. Identity of this band as Sgs1-3HA is based on its presence in strains confirmed to have an Sgs1-3HA integration by PCR and sequencing, but absence in strains that have not been integrated with Sgs1-3HA (compare to EAY1141 in Figure A.2).

Table A.2: Primers for ChIP at SSA locus

Alani number	**Name** (from Li, et al)	**Target**	**Orientation**	**Sequence**
AO2379	JC1	*URA3*	Sense	5' GCCCAGTATTCTTAACCCAACTGCAC 3'
AO2380	JC2	pUC19	Antisense	5' CAGCTGGCGTAATAGCGAAGAGGCCC 3'
AO2381	JC3	λ phage	Sense	5' CCTTAGTAGTTGGTAACCTGACAAAGG 3'
AO2382	JC4	*URA3*	Antisense	5' CCTTCTGTTCGGAGATTACCGAATC 3'
AO2383	JC5	pUC19	Sense	5' GAGACGGTCACAGCTTGTCT 3'
AO2384	JC6	pUC19	Antisense	5' GCATCTGTGCGGTATTTCACA 3'
AO2851	5'PRE1	*PRE1*	Sense	5' CCCACAAGTCCTCTGATTTACATTCG 3'
AO2852	3'PRE1	*PRE1*	Antisense	5' ATTCGATTGACAGGTGCTCCCTTTTC 3'

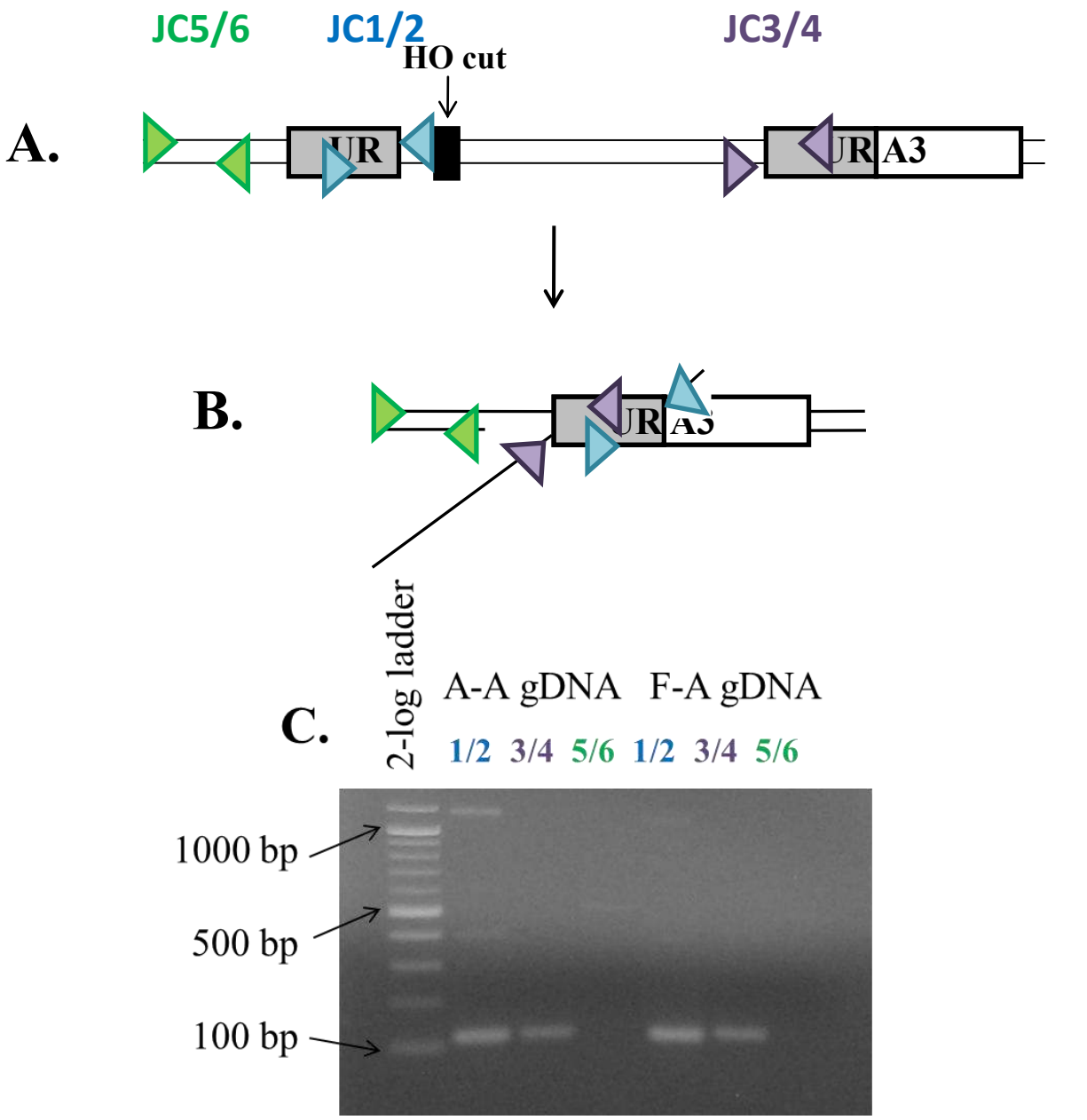

Figure A.1. Orientation and approximate location of ChIP primers on the full-length SSA locus **(A)** and duplex intermediate **(B)**. Repeat sequence (gray boxes), HO cut site (black box), non-homologous yeast sequence (white boxes), foreign DNA sequences (pUC or λ, black lines), control primer set (green arrowheads), upstream (long) ssDNA tail/duplex junction primer set (purple arrowheads), downstream (short) ssDNA tail/duplex junction primer set (blue arrowheads) **C.)** Example of PCR using the 3 primer sets on genomic DNA isolated from the A-A and F-A strains. JC1/2 and JC3/4 give the expected products of 110 bp, but no product could be obtained using JC5/6

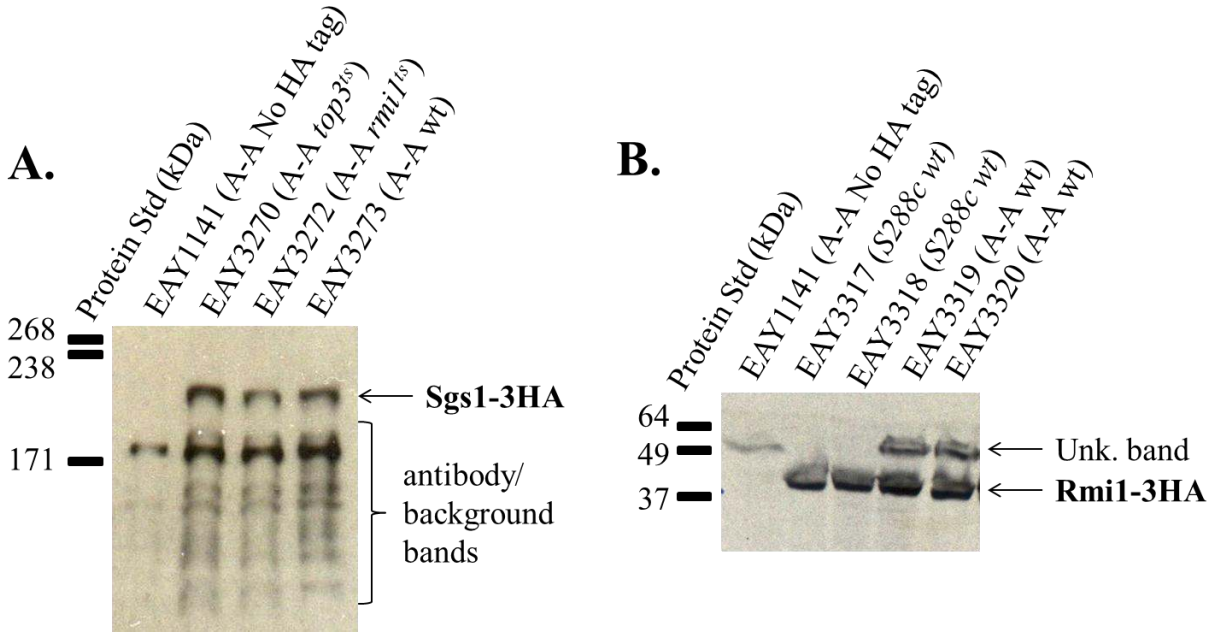

Figure A.2. Western blot detection of Sgs1-3HA **(A)** and Rmi1-3HA **(B)**. With the exception of strain EAY1141, all strains have an integration of either a Sgs1-3HA:LEU2 or Rmi1-3HA:TRP1 "pop in" vector confirmed by PCR and sequencing.

Detecting Sgs1 by western blotting generally requires between 0.1 to 1 ng/ul α-HA antibody (1:1000 to 1:10,000 of 5 mg/ml 12CA5, Roche) with 1:4000 to 1:5000 HRP-linked α-mouse IgG secondary antibody from Cell Signaling Technologies, although I have not been able to identify an ideal condition because success of western blot detection seems to rely more strongly on yield and cleanliness of protein extracts and amount of loaded protein. All blots thus far have not been free of unidentified and non-specific bands and particularly a band at ~170 kDa (notably, where Sgs1 is expected to run based on size). This band is the approximate size of undenatured IgG, indicating that smaller amounts of antibody during blotting should reduce this signal, and that denaturation of proteins is not fully efficient. During a less successful blot, this band may appear very distinctly and wash out the 200 kDa Sgs1-3HA signal. It cannot be ruled out however, that there is Sgs1 protein running at 170 kDa that is masked by the "antibody" band, and that the band at 200 kDa is a modified Sgs1 that runs at a higher mobility. If this is the case, it may be useful to try using Sgs1 with a larger epitope tag.

Attempts I have made to construct A-A and F-A strains bearing Sgs1 with different tags have so far been unsuccessful, but should only require optimization of the integration protocol (perhaps different selective markers) to be acquired. Along these lines, I had trouble integrating Sgs1-3HA using a *KANMX* marked integration fragment (XhoI-BamHI of pEAI206), but was easily able to intregate the same construct using a "pop-in" vector with a *LEU2* marker (pEAO252). Currently, I have an Sgs1-FLAG construct on a "pop-in" *TRP1* vector (pEAO253), and one should be able to acquire an Sgs1-13myc from one of a few labs (i.e. Sang Eun Lee). Both of these tags are not significantly larger than 3HA, though, so one may consider trying to construct something with a larger modification. The risk to this approach though, is that ChIP

with epitope-tagged protiens is most often performed using HA, and the utility of other tags in ChIP is not known.

As mentioned, optimization of protein extraction is another necessary area to focus on for improving western detection. Since I have been having variable success with bead-beating, I attempted to prepare extracts using the "liquid nitrogen popcorn" method that has been used successfully for detecting other large and unstable proteins in our lab. However, this method did not appear to improve stability following a single attempt. Mizraei et al. (2011) had considerable success detecting Sgs1 in TCA precipitated extracts, so this is a method to consider. Once an ideal protein extraction method is established and a stronger more stable signal is achieved, I expect the issues with non-specific bands will be diminished and ideal blotting conditions will be more easily determined.

Rmi1 is a much smaller and more stable protein than Sgs1 and its detection by western blotting has not been difficult. In A-A and F-A strains integrated with Rmi1-3HA by a "pop-in" vector, a sharp band near 37 kDa was detected with α-HA antibody that was not present in non-integrated strains (Figure A.2B). Rmi1 itself is expected to be 26 kDa and 30 kDa with the 3xHA, so Rmi1, like Sgs1, runs slower than expected based on size.

Cell lysis and DNA shearing: One factor contributing to poor extraction of Sgs1-3HA, is likely due to the long vortex time needed for cell lysis. Ideally, cell pellets and lysates should be kept frozen as often as possible to avoid degradation of the Sgs1 protein. Though protease inhibitor is present in the lysate, Sgs1 still seems to be sensitive to degradation. I have attempted lysing the cells for only 10 minutes, but only a small percentage (~ 10-20% estimated by visual inspection) of the cells were lysed. Due to the low abundance of Sgs1, I expected that I would need at least

a majority of the cells to be lysed in order to detect a signal on the western, and so I decided to use longer vortex incubations. However, I now believe a better strategy is to attempt to maximize lysis in as short a time as possible by adjusting cell/bead ratio. I suspect that cell pellets in previous preps (derived from 45 ml of O.D.600 = ~0.5 cultures) are too large to be efficiently lysed by 0.5 ml beads in 0.5 ml Lysis Buffer. I suggest using smaller pellets or a larger volume of beads and Lysis Buffer. One may also experiment with other protease inhibitors in the buffer. I had been using PMSF, but Roche protease inhibitor cocktail tablets may be more effective.

Thus far, I have not discovered any difficulty with shearing of the DNA since mobility on 1% agarose appears as a smear from 100 bp to 1000 bp (data not shown), though running whole cell lysates as I did to monitor shearing is not ideal since. I suggest, rather running a small amount of the Input DNA extracts that has been treated with RNase A. It does not appear that undershearing should become an issue, but one should take care to avoid overshearing, as PCR reactions may not work on smaller fragments.

Immunoprecipitation: So far, IPs have been hit or miss and may depend on success of cell lysis and protein extraction. Sgs1-3HA IP has been successful on at least two occasions (Figure A.3), and so far has worked best using 1 ug of α-HA antibody. In both cases, successful IP was apparent by depletion from the lysate, however Sgs1 was only detectable in the IP fraction when the whole IP sample was loaded on the gel. During the ChIP procedure, only $1/10^{th}$ of the sample was loaded on the gel and in this case, Sgs1 was not detectable by western. One should determine whether it is possible to generate enough IP sample for both western detection and ChIP, since the western signal will be used to normalize the ChIP signal.

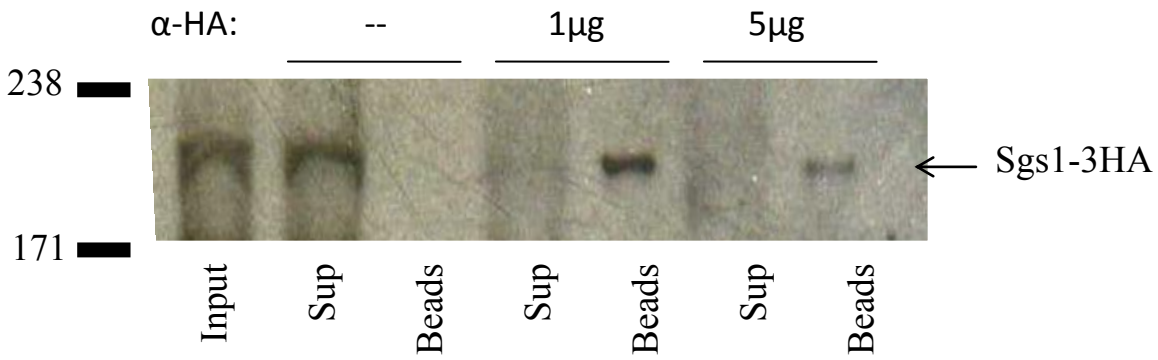

Figure A.3. Immunoprecipitation of Sgs1-3HA. Input is the cell lysate prior to IP. Sup is the lysate cleared after IP, and beads contain the Protein G beads with antibody and immunoprecipitated proteins.

IP of Rmi1-3HA was only attempted one time. During this attempt, it appeared that IP was partially successful, as it was detected in both cleared lysate and IP samples (data not shown). Antibody bands are also detectable in the IPs as well as a small band just above the major IgG band could be antibody-bound Rmi1. The suspected presence of antibody-bound proteins on westerns may indicate inefficient denaturation of samples.

DNA elution and semi-quantitative PCR: So far, DNA extraction and PCR have been successful using the Input samples, however, I have been unable to determine whether ChIP DNA elution was successful due to failure of PCR reactions. During DNA extractions from both Sgs1-3HA and Rmi1-3HA ChIP eluates, pellets were either extremely tiny or not visible at all and DNA concentration is too low to be detected by gel electrophoresis or spectrometry. This does not cause alarm though, since ChIP DNA is often at a very low concentration.

PCR analysis at this point is only preliminary. Using a 1:10 dilution of Input DNA, I could detect PCR products using the PRE1 primer set and JC1/2 and JC3/4 primer sets, however the JC5/6 primer set never gave a product in any PCR reaction including control genomic DNA derived from strains EAY1141 and EAY1143, indicating the primer stocks may be defective. Attempts to PCR the same region with other primers taken from our lab collection were also unsuccessful on a single attempt, but this may only be due to routine PCR issues. Due to the small size expected for PCR product, I noted that it may be difficult to determine a true PCR product from a primer dimer and suggest that a no template control always be included during analysis.

PCR from both Sgs1-3HA and Rmi1-3HA ChIP samples did not yield signals with any primer sets, nor at a number or dilutions tested. I concluded that there is either none or too little

DNA in these samples, which could be due to failure of the ChIP procedure. Overshearing of DNA, IP failure, failure to cross-link, or to reverse cross-links are all situations that could result in lack of PCR product with ChIP samples. These steps, however, will be difficult to optimize until lysis, IP, and blotting procedures are first optimized.

Closing thoughts

Though I expected developing this ChIP assay would take time and be labor intensive, I believe I largely underestimated the time it would take. Until IP and western blot detection of Sgs1 is running smoothly, it is not worth attempting to incorporate ChIP. Once this is achieved though, I would encourage one to continue to develop this assay, though I would caution them to expect it will take many months and to keep it as a side project rather than a primary focus.

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