Mitotic Homologous Recombination at Engineered Repeats in *S. cerevisiae* and in Novel Transgenic Mice

by

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Dedicated to my son,

Jacob,

his grandparents,

and my loving husband, Jason.

This work would not have been possible without

their unwavering love and support.

Preface

The work described in this thesis is the result of work done by the author in collaboration with several members of the Engelward laboratory. I am extremely grateful to all those who have contributed to this thesis project.

Chapter II was published in *DNA Damage* in August of 2002. All repair deficient strains were created by the author except for the *rev3* mutant strains, which were made by Maja Razlog. Amy L. Brock assisted the author in piloting and optimizing the DNA damage-induced recombination assays. Maja Razlog, with help from Tetsuya Matsuguchi and Asti Goyal, was primarily responsible for studies detailing the role of polymerase zeta and also contributed to studies of recombination in strains that overexpress *MAG1* and/or *APN1*. All other results were provided by the author.

Chapter III is currently in press [*Proc. Natl. Acad. Sci. USA*]. Karen H. Almeida, Molly S. Stitt, Tetsuya Matsuguchi and the author all contributed to construction of the recombination substrate. PCR screening to identify FYDR mice was done in collaboration with Rebecca Rugo and Foster Kerrison. F. Kerrison was also responsible for showing DNA damage-induced recombination in ear fibroblasts. Vidya Jonnalagadda created the FYDR mouse genomic library, identified the site of integration and determined the arrangement of the DNA in FYDR cells. The author was primarily responsible for all other work.

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Abstract

Although homologous recombination provides an efficient means for repairing and tolerating DNA damage, mitotic recombination between misaligned sequences can lead to loss of genetic information (*e.g.* deletions, translocations and loss of heterozygosity). Given that such genetic changes may promote tumorigenesis, it is critical to identify those genetic and environmental factors that render cells susceptible to homologous recombination. Our goal is to elucidate the mechanisms of DNA damage-induced recombination and to determine the role of DNA repair enzymes in modulating homologous recombination in eukaryotic cells.

Alkylating agents are abundant in our environment and are generated endogenously as normal metabolites. In addition to their mutagenic and cytotoxic effects, alkylating agents stimulate homologous recombination in eukaryotic cells. Removal of alkylated bases by DNA glycosylases, such as the Mag1 3-methyladenine (3MeA) DNA glycosylase, initiates the base excision repair (BER) pathway. To investigate the molecular basis for methylation-induced homologous recombination in *S. cerevisiae*, intrachromosomal recombination was measured under conditions where *MAG1* expression levels were varied. Cells lacking Mag1 show increased susceptibility to methylation-induced recombination, suggesting that unrepaired 3MeA lesions induce recombination. Overexpression of *MAG1* also elevates recombination levels, presumably due to the accumulation of recombinogenic BER intermediates.

To study the relative importance of specific DNA repair enzymes in modulating recombination in mammals, we have engineered transgenic mice that make it possible to quantify homologous recombination events in primary somatic cells, both *in vitro* and *in vivo*. The FYDR (fluorescent yellow direct repeat) mice carry two different mutant copies of an expression cassette for enhanced yellow fluorescent protein (EYFP) arranged in a direct repeat. Homologous recombination between these truncated sequences restores expression of *EYFP*. Using flow cytometry, spontaneous and DNA damage-induced recombination events were quantified in primary fibroblasts cultured from embryonic and adult tissues. In addition, recombination events that occurred *in vivo* were detected directly in disaggregated skin cells. Currently, FYDR mice are being crossed with mice carrying engineered defects to determine how specific gene traits modulate susceptibility

to mitotic recombination. Ultimately, this tool will help us better understand how environmental agents and specific genes influence cellular susceptibility to cancer-promoting recombination events in mammals.

Thesis Supervisor: Bevin P. Engelward
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List of Abbreviations

AP	apurinic/apyrimidinic
APRT	adenine phosphoribosyl transferase
BER	base excision repair
BIR	break-induced replication
bp	base pair
EGFP	enhanced green fluorescent protein
ES	embryonic stem
EYFP	enhanced yellow fluorescent protein
5-FOA	5-fluoroorotic acid
FACS	fluorescence activated cell sorting
FYDR	fluorescent yellow direct repeat
kb	kilobase
LOH	loss of heterozygosity
Mb	megabase
3MeA	3-methyladenine
7MeG	7-methylguanine
MEF	mouse embryonic fibroblast
MMS	methyl methanesulfonate
NEF	nucleotide excision repair factor
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
O^6MeG	O^6-methylguanine

PCNA	proliferating cell nuclear antigen
Pol ζ	DNA polymerase zeta
SC	synthetic complete
SCE	sister chromatid exchange
SDSA	synthesis dependent strand annealing
SSA	single-strand annealing

Chapter I

Introduction

Chapter I

1.1 Homologous Recombination and Human Disease

Homologous recombination plays an important role in maintaining genetic integrity. Our cells are constantly exposed to endogenous and exogenous DNA damaging agents that can disrupt the covalent structure of DNA and lead to the formation of single and double strand breaks (*e.g.*, endonucleases, free radicals, ionizing radiation). Homology directed repair provides an important strategy for repairing these lesions. More importantly, repair and lesion avoidance pathways that involve homology searching are integral to DNA replication (1-4). It is estimated that ~ 10 double strand breaks are formed each time the mammalian genome is replicated (1) and proteins that are essential for homologous recombination (*e.g.*, RAD51) are also essential for life (5-7). In addition, the inability to efficiently repair double strand breaks by homologous recombination has been associated with an increased risk of tumorigenesis. For example, cells that are deficient in *BRCA1* and *BRCA2* are unable to effectively repair double strand breaks by homologous recombination (8-10) and 25-50% of people who carry mutations in these genes develop breast and/or ovarian cancer by age 50 (11). It has been shown that impairment of homology-directed repair leads to chromosomal instability (12), and ultimately, such chromosomal instability is likely to contribute to tumorigenesis (10).

Although impairment of homologous recombination is clearly problematic, too much homologous recombination can also cause an increased risk of cancer. Processes that involve homologous recombination generally do not result in sequence changes.

Indeed, mammalian cells are 100-1000 times more likely to use a sister chromatid as a repair template than they are to use a homologous chromosome (13-15), and since sister chromatids are "identical", no sequence alterations are incurred [reviewed in (16)]. However, homologous recombination events between misaligned sequences or homologous chromosomes can lead to deletions, translocations and loss of heterozygosity (LOH) as described below.

Sequence repeats comprise a large fraction of the mammalian genome (17,18) and recombination between repeat sequences that are misaligned can lead to genetic rearrangements. For example, recombination between repeats on the same chromosome can lead to deletions or duplications and recombination between repeats on non-homologous chromosomes can result in translocations. A number of diseases are attributed to such deleterious recombination events, including DiGeorge syndrome and some cases of acute myeloid leukemia (17). In DiGeorge syndrome, a chromosomal rearrangement during development results in absence of the aortic arch (19). Acute myeloid leukemia can be caused by a partial duplication of the *ALL-1* gene that results from a recombination event between repetitive *Alu* sequences (20). *Alu*-mediated mitotic recombination events have also been implicated in other cancers, including some cases of chronic myelogenous leukemia, breast cancer and Ewing's sarcoma (21,22).

In addition to deletions, recombination between homologous chromosomes can result in loss of heterozygosity (LOH) events that can initiate or promote cancer (23). Since most people inherit two functional copies of each tumor suppressor gene, one allele from each parent, a mutation in one allele is generally inconsequential to a cell. However, in a cell that carries one wild type copy and one mutant allele of a tumor suppressor gene,

loss of the remaining wild type copy (LOH) can increase the likelihood of a cell becoming a preneoplastic colony. While LOH can occur through a number of mechanisms (Figure 1-1*A*), including point mutations, deletions, and aneuploidy, mitotic recombination has been estimated to be the underlying cause of LOH 25-50% of the time (24-26). Mitotic recombination can lead to LOH in several ways. Recombination between misaligned sequences on the same chromosome, such as *Alu* sequences, can result in deletions, while gene conversion can result in the complete replacement of the wild type allele by the mutant allele (Figure 1-1*A*). In addition, a reciprocal exchange followed by cell division can result in one cell that carries two wild type copies of the allele and one cell that has undergone LOH (Figure 1-1*B*).

Given that mitotic homologous recombination has been shown to give rise to deletions and cancer-initiating LOH events, it is not surprising that an increased susceptibility to homologous recombination is often associated with an increased risk of developing cancer. Indeed, a number of cancer syndromes are caused by defects in genes that modulate spontaneous homologous recombination (Table 1-1). For example, cells that are defective in the BLM helicase, which is responsible for Bloom's syndrome, have an elevated frequency of sister chromatid exchange (SCE) and contain large numbers of spontaneous chromosomal aberrations. Patients with Bloom's syndrome suffer from growth retardation, sensitivity to sunlight and a high susceptibility to infections. In addition, one in nine Bloom's patients develops malignancies by 20 years of age (27).

Recently, the cancer syndrome ataxia-telangectasia (AT) has provided a link between cancer predisposition, recombination and cell cycle arrest (Table 1-1). AT is an autosomal recessive syndrome caused by a mutation in *ATM*. Cells from AT patients

show an increased susceptibility to homologous recombination (28,29) and this increase in recombination is thought to underlie the chromosomal instability (30) and possibly, even the cancer predisposition observed in AT. Interestingly, the ATM protein has been shown to play a role in sensing DNA damage and activating damage response pathways by phosphorylating p53, NBS and BRCA1 as well as other proteins (30,31). While p53 is known to control cell cycle progression, it may also regulate homologous recombination through its interaction with RAD51. NBS and BRCA1 have been shown to be directly involved in the homology-directed repair of double strand breaks (9,32). Interestingly, defects in p53, BRCA1 or NBS can also increase one's risk of developing cancer (Table 1-1). Thus, it is becoming increasingly clear that maintaining normal levels of homology-directed repair is a critical aspect of tumor suppression (33).

1.2 Mechanisms of Homology-Directed Double Strand Break Repair in Eukaryotes

Much of what we know about the mechanisms of homologous double strand break repair has come from studies of *S. cerevisiae* [reviewed in (34)]. Indeed, studying these processes in yeast offers a number of advantages, including the ease with which genes can be targeted or disrupted (35,36). While *S. cerevisiae* cells have the capacity to repair breaks by non-homologous processes, homologous recombination is the predominant pathway of double strand break repair in yeast.

In mammalian cells, the repair of double strand breaks occurs by both non-homologous and homologous pathways. It has long been known that double strand breaks are repaired predominantly through non-homologous end-joining (NHEJ), a process that

involves recognition and ligation of the double strand ends (37). However, recent studies suggest that homologous recombination is also used to repair double strand breaks in mammalian cells, especially in late S and G2 phases of the cell cycle when an undamaged sister chromatid is available (16,31). Indeed, work by Liang *et al.* indicates that 30-50% of double strand breaks induced in mammalian cells may be repaired by homologous recombination (38).

The fundamental mechanisms of double strand break-induced homologous recombination are thought to be conserved from yeast to mammals (31,39). In general, double strand breaks may be repaired by three mechanisms of homologous recombination: single-strand annealing (SSA), gene conversion and break-induced replication (BIR) [reviewed in (34)], which are described in detail below.

Single Strand Annealing

A double strand break that occurs between or within directly repeated sequences is often repaired by single strand annealing (SSA). For SSA, the double strand ends on either side of the break are resected 5'-to-3'until a region of sequence homology is reached (Figure 1-2). Complementary single-stranded 3'overhangs reanneal and the non-homologous ends, frequenctly termed "flaps", are cleaved by structure specific endonucleases. Repair synthesis and ligation restores two continuous strands. Interestingly, SSA is efficient even if the repeated sequences are separated by as much as 15 kb and is almost 100% efficient when the homologous regions flanking the double strand break are greater than 400 bp in length (34). However, the process of SSA can

result in a significant loss of genetic information, since one copy of the repeated sequence and sequence information between the repeats are deleted.

Mechanisms of Gene Conversion

While SSA always results in deletions, gene conversion, which is defined as the nonreciprocal transfer of genetic information, can repair a double strand break without loss of sequence information [reviewed in (16)]. In order to repair a double strand break by gene conversion, duplex DNA that contains homology to the region surrounding the break (*e.g.* from a sister chromatid or homologous chromosome) must be available to serve as a repair template. In this process, information from the donor template is copied into the broken locus, making the repaired or recipient locus an exact copy of the donor.

The original model of double strand break repair was proposed by Resnick and Martin (40) and later elaborated by Szostak and colleagues (41). In this model, double strand break formation is followed by 5' to 3' recession of the double strand ends to form single-stranded 3' overhangs (Figure 1-3). The 3' ends invade an intact homologous duplex, most often a sister chromatid, and are then extended by the initiation of new DNA synthesis. This process leads to the formation of two Holliday junctions and these four-stranded branch structures are resolved in one of two ways. If both Holliday junctions are cleaved in the same direction, gene conversion will not be associated with an exchange of flanking markers. However, if the "crossed" strands of one Holliday junction are cleaved while the "continuous" strands are cut on the other Holliday junction, there will be an exchange of flanking markers, or a crossover event. Since in three dimensions the Holliday junction is perfectly symmetrical (*i.e.* "crossed" and

"continuous" strands can not be distinguished), this model would predict an equal number of crossover and noncrossover events. Indeed, the high frequency of crossover events that are often associated with gene conversion during yeast meiotic recombination strongly supports this mechanism of double strand break repair.

Although meiotic recombination intermediates are consistent with the Szostak model of double strand break repair (42), this model does not account for several observations in mitotic cells. First, only a small proportion of mitotic gene conversion events are associated with crossing-over. Studies in yeast have shown that only 10-20% of mitotic gene conversion events result in crossovers (43,44), and this proportion is even lower in mammalian cells (16). In addition, the Szostak model (Figure 1-3) predicts that both the recipient locus and the donor template should contain heteroduplex DNA following resolution of the Holliday junctions (see Figure 1-3, where a blue strand is annealed to a red strand). However, studies in *S. cerevisiae* have shown that while heteroduplex DNA is frequently found in the recipient, it is rarely found in the donor.

These inconsistencies led researchers to propose a model in which strand invasion and repair synthesis are coupled with strand annealing. This mechanism is frequently referred to as synthesis dependent strand annealing (SDSA) (34). Repair of a double strand break by SDSA is also initiated by the 5'-to-3' recession of the double strand ends to form single-stranded 3' overhangs (Figure 1-4). However, in this mechanism, only one end of the double strand break invades the intact DNA, resulting in the formation of a D loop. The strands in the D loop serve as a template for DNA synthesis by the invading strand. Once the missing sequence is restored, the newly synthesized strand can be extracted from the D loop and subsequently anneal to the 3' overhang of the non-

invading double strand end. Repair synthesis and ligation complete this process, similar to SSA. Since no Holliday junctions are cleaved, SDSA precludes crossing over. In addition, the duplex that serves as a donor of information is utterly unchanged, making this model consistent with there only being heteroduplex DNA in the recipient.

Break-Induced Replication

Homologous recombination plays an important role in normal DNA replication in somatic cells (1,45). It has been estimated that ~10 double strand breaks are formed each time the mammalian genome is replicated and these double strand breaks are often repaired by homologous recombination (1). Replication induced double strand breaks can arise from replication across pre-existing nicks or gaps in the DNA backbone (Figure 1-5), or possibly from the collapse of a replication fork that is stalled at a DNA lesion (45). DNA lesions that block progression of the replication fork (*e.g.* interstrand crosslinks, 3-methyladenine) are potentially lethal if replication cannot be restarted.

Repair of broken replication forks can occur through a recombination-dependent mechanism known as break-induced replication (Figure 1-5) (BIR) (34). BIR is initiated by a 5'-to-3' exonuclease that resects the broken end to produce a 3' single-stranded overhang that can then invade the intact DNA to form a Holliday junction. The invading strand primes leading strand synthesis and resolution of the Holliday junction completes this repair process. Given that replication forks restarted by BIR can proceed all the way to the end of the chromosome, BIR may be the mechanism underlying long tract gene conversions in mitotic cells (which would be associated with LOH). In addition, if a double strand break occurs at a region that shares homology with sequences located on a

different chromosome, BIR may also give rise to nonreciprocal translocations. Furthermore, recent evidence suggests that this type of event may explain how mammalian tumor cells that lack telomerase are able to maintain telomeres (1).

1.3 Proteins Involved in Mitotic Homologous Recombination in Eukaryotes

Roles of Recombination Proteins in *S. cerevisiae*

In *S. cerevisiae*, homologous recombination is mediated by genes in the *RAD52* epistasis group. When mutated, each of these genes shows an increased sensitivity to X rays but not UV irradiation. Currently, 10 genes fall into this group: *RAD50-55*, RAD57, RAD59, *MRE11* and *XRS2* (31). A defect in any one of these gene products can suppress homologous recombination and sensitize cells to DNA damaging agents that induce double strand breaks (34).

Homologous repair of a double strand break is initiated by the Mre11/Rad50/Xrs2 complex, which functions in the 5'-to-3' recession of the double strand ends. The resulting 3' overhangs are then bound by Rad51, which like its *E. coli* homolog, RecA, forms a filament of oligomers along the single-stranded DNA and catalyzes homology searching, strand invasion and strand exchange (46). Interestingly, while RecA is indispensable for all mechanisms of double strand break-induced recombination in bacteria, Rad51 is not always required. Instead, interactions of Rad51 with other proteins, specifically Rad52, are essential to recombination and double strand break repair in *S. cerevisiae*. While Rad52 on its own cannot catalyze strand invasion, it appears to be critical for recognition of the double strand ends and for efficient annealing of the

invading 3' end to homologous sequences (1). Strand exchange is enhanced by Rad54, Rad55, Rad57 and the single stranded binding protein, RPA. A member of the Swi2/Snf2 family of chromatin-remodeling proteins, Rad54 is thought to "open up" the DNA for repair and recombination, while Rad55 and Rad57 may help "load" Rad51 onto the single stranded DNA. The proteins involved in migration and resolution of Holliday junctions (analogous to RuvABC in *E. coli*) have not yet been identified in eukaryotes. However, there is some evidence that the Mus81 endonuclease complex may cleave Holliday junctions formed from stalled replication forks (45).

It is noteworthy that since SSA does not involve strand invasion or exchange, Mre11/Rad50/Xrs2 and Rad52 are the only members of the *RAD52* epistasis group necessary for SSA, and dependence on Rad52 diminishes as the length of homologous regions flanking the double strand break increases from 1 kb (< 1% of cells recombine in the absence of Rad52) to > 10 kb (SSA is 100% Rad52-independent) (34). SSA also requires the nucleotide excision repair proteins, Rad1 and Rad10, which form an endonuclease that cleaves the 3' nonhomologous "flaps" that are created following reannealing of homologous regions.

Roles of Recombination Proteins in Mammals

The vast majority of recombination proteins are conserved from yeast to mammals (31,34). However, although they may share sequence homology and have similar biochemical properties, the phenotypes of the corresponding mutants are quite different (47). For example, while Rad52 is essential for the repair of double strand breaks in yeast, *Rad52*$^{-/-}$ mice are viable, fertile and show no hypersensitivity to agents

that induce double strand breaks. *In S. cerevisiae*, Rad51 is not essential for homology-directed repair (*e.g.*, Rad51 is not required for BIR) (34) and spontaneous recombination between heteroalleles is reduced only four to ten fold in *rad51* mutants (48). On the contrary, Rad51 is a critical protein in mammals. *Rad51*$^{-/-}$ mutant mice die early during embryonic development (5-7) and a disruption of *Rad51* in cultured cells is lethal (12). In addition, there are a large number of *Rad51* homologs in mammals, including *XRCC2* and *XRCC3*, both of which play a role in homology-directed double strand break repair (49,50). Another striking difference between yeast and mammalian cells is the requirement for Mre11 and Rad50. While yeast *mre11* and *rad50* null mutants grow normally, albeit slowly (34), disruption of *Rad50* in mice is lethal. Both *Rad50*$^{-/-}$ and *Mre11*$^{-/-}$ embryonic mouse cells are extremely sensitive to ionizing radiation and do not survive in culture (31).

1.4 Repair of Alkylation Damage in *S. cerevisiae*

Alkylating agents comprise the broadest class of DNA damaging agents. They are abundant in our environment (including food) (51) and are generated endogenously as a result of normal metabolic processes (27). Alkylating agents interact with nucleophilic sites in the DNA to create a broad spectrum of lesions, many of which are mutagenic and/or cytotoxic. Paradoxically, alkylating agents are both carcinogenic (because they induce mutations) and effective as cancer chemotherapeutics (because they are cytotoxic) (52).

As a model for alkylation damage, we have used the methylating agent methane methylsulfonate (MMS). MMS creates many different types of methyl lesions, including 7-methylguanine (7MeG) (80-85%), 3-methyladenine (3MeA) (9-12%), 3-methylguanine (3MeG) (0.3-0.7%), O^6-methylguanine (O^6MeG) (0.3%), 7-methyladenine (7MeA) (1.8%) and phosphotriesters (0.8%) (53). Among the different types of methylated bases, 3MeA and O^6MeG have the most serious biological consequences. 3MeA is toxic because it blocks DNA replication, whereas O^6MeG is point mutagenic because it tends to mispair during DNA replication (54,55). While several studies have addressed the effects O^6MeG on mitotic homologous recombination in eukaryotes [e.g. (56-58)], much less was known about the recombinogenic potential of 3MeA.

Base Excision Repair

Base excision repair (BER) is responsible for the repair of a wide variety of damaged bases, including alkylated, oxidized and deaminated bases, and it is the primary defense against lesions induced by MMS [reviewed in (59)]. BER can be initiated by a number of DNA glycosylases, each of which recognizes a specific damaged base or a subset of base lesions, and subsequently cleaves the glycosylic bond between the damaged base and the deoxyribose of the sugar phosphate backbone (Figure 1-6). In *S. cerevisiae*, the Mag1 3MeA DNA glycosylase is the major activity for removing alkylated bases (60). Cleavage of the glycosylic bond by Mag1 generates an apurinic/apyrimidinic (AP) site, which is then cleaved 5' to the abasic sugar by the major AP endonuclease, Apn1. Apn1 is responsible for >95% of the AP endonuclease activity in *S. cerevisiae* (61). The 3' hydroxyl group created by Apn1 is then extended one or

several nucleotides by DNA polymerase δ or ε (62,63). Finally the abasic deoxyribose is removed by the Rad27 flap-endonuclease, or possibly by a deoxyribose phophatase, to facilitate DNA ligation (59,64).

It is noteworthy that unrepaired BER intermediates, such as AP sites, single strand gaps and nicks, can themselves be mutagenic and recombinogenic. For example, yeast that are deficient in Apn1 AP endonuclease activity (*apn1*), and consequently are unable to process AP sites efficiently, have elevated rates of spontaneous mutation, presumably due to the accumulation of AP sites (65). Indeed, this mutator phenotype is suppressed in *apn1* cells that are also lacking Mag1 and induced in cells that overproduce Mag1, which suggests that AP sites accumulate when this DNA glycosylase is present, and that if these AP sites are not efficiently processed by Apn1, they can cause mutations (66). Genetic studies have revealed that AP site-induced mutations occur through a lesion bypass pathway in which polymerase ζ (Pol ζ) introduces mutant bases as it replicates past AP sites (67,68). Formed from a complex of Rev3 and Rev7 proteins, Pol ζ is a non-essential polymerase that helps cells tolerate DNA damage by facilitating DNA replication past DNA lesions that inhibit replication (69). Cells deficient in Pol ζ have an increased susceptibility to recombination caused by AP sites, presumably because inhibition of DNA replication induces homologous recombination (70).

Mag1 3-methyladenine DNA Glycosylase

3MeA is a toxic lesion that inhibits DNA replication *in vitro* (54). In *S. cerevisiae*, one of the main defenses against 3MeA is the Mag1 3MeA DNA glycosylase (60). Mag1 releases a variety of bases from DNA *in vitro* including 3MeA, 7MeA, 3MeG, 7MeG and

even normal bases (60,71-73). The AP sites generated by Mag1 are processed by downstream BER enzymes (Figure 1-6). Cells that are deficient in Mag1 are extremely sensitive to the lethal effects of a variety of alkylating agents including MMS, presumably because many of its substrates inhibit DNA replication (74). Additional disruption of genes required for homologous recombination makes such cells even more sensitive to methylation damage (75), suggesting that both BER and homologous recombination help prevent methylation-induced killing.

Although wild type levels of Mag1 protect cells against the cytotoxicity of methylating agents, an overproduction of Mag1 can elevate the spontaneous mutation rate and increase sensitivity to MMS (76). Contrary to what was observed for *MAG1*, overexpression of the *E. coli* 3MeA DNA glycosylase, *tag*, which almost exclusively repairs 3MeA and does not act on 7MeG or normal bases (73,77), does not induce spontaneous mutations or cause increased sensitivity to MMS. These results suggest that while removal of 3MeA is critical to cell survival in the presence of MMS, removal of 3MeA is not responsible for the mutator effect. Instead, high levels of Mag1 activity may overburden downstream BER enzymes by converting otherwise benign 7MeG lesions or even normal bases into mutagenic AP sites or other cytotoxic BER intermediates (78).

Nucleotide Excision Repair

In the absence of BER, nucleotide excision repair (NER) provides an alternative pathway for the repair of methylated bases. For example, *S. cerevisiae* deficient in both Mag1 and NER are far more sensitive to the toxic effects of MMS than are cells lacking

only Mag1, suggesting that NER can effectively remove potentially toxic lesions repaired by Mag1 (79,80).

NER is a multi-step process that involves damage recognition, DNA unwinding, and concomitant dual incision, resulting in the removal of a 25-30 base oligonucleotide containing the damage (Figure 1-7). In *S. cerevisiae*, cleavage of the DNA requires the concerted action of over a dozen proteins, which exist *in vivo* as multiprotein complexes termed NEFs (nucleotide excision repair factors) [reviewed in (81)]. Although the sequence of NEF assembly and the mechanisms underlying damage recognition are not yet fully understood, it is believed that both Rad14 (component of NEF1) and Rad4-Rad23 (NEF2) are necessary for recognition and binding of DNA damage. Recognition of DNA damage in transcriptionally inactive regions or in the nontranscribed DNA strand requires an additional component, NEF4 (Rad7-Rad16) (82,83). In the presence of ATP, NEF4 interacts with NEF2 to synergistically enhance damage binding (84). TFIIH-Rad2 (NEF3) and the single strand binding protein RPA are the last components to be recruited to the assembly. The Rad3 and Rad25 helicase activities associated with TFIIH result in local unwinding at the site of the lesion. Incision of the damaged DNA can then be accomplished by the endonucleases, Rad1-Rad10 and Rad2, that act in a structure specific manner to cleave the DNA 5' and 3' to the damage, respectively. These dual incisions result in the removal of an oligonucleotide 25-30 bases long that contains the damage (85). Intact duplex DNA is regenerated by the replication machinery [DNA polymerase δ or ε and proliferating cell nuclear antigen (PCNA)], which fills the gap and DNA ligase (Cdc9), which seals the nick (86).

Unlike BER, wherein components of the pathway are able to act independently of one another, NER requires that Rad14, RPA, Rad4-Rad23, TFIIH, Rad2 and Rad1-Rad10 act in concert to cleave the DNA and remove the damage. A loss of any one of these proteins results in a complete loss of incision at DNA damage (87). Thus, for example, *rad4* mutants are completely deficient in NER (88).

1.5 Alkylating Agents Induce Homologous Recombination

In addition to their mutagenic and toxic effects, alkylating agents also stimulate mitotic homologous recombination in both yeast and mammalian cells (89-91). Since alkyl lesions are not thought to be able to directly stimulate strand exchange, it is likely that they are processed into DNA intermediates that induce recombination, such as double strand ends or single strand gaps. In this work, we suggest two mechanisms by which methyl lesions may be converted into substrates for recombination.

Alkyl Lesions Induce Recombination by Blocking DNA Replication

One possibility is that replication blocking lesions, such as 3MeA, are converted into recombinogenic intermediates during S-phase. Indeed, Kadyk and Hartwell provide strong evidence that replication is required for unrepaired UV lesions to induce recombination (92), and Galli and Schiestl show the frequency of UV-induced recombination increases with time in S-phase (93).

Several models have been proposed for mechanisms by which an encounter between a replication fork and a replication-blocking lesion induces recombination. For

example, a lesion in the lagging strand could cause the replication machinery to stall, resulting in the formation of a single strand region, often referred to as daughter strand gap (Figure 1-8*A*). Both yeast and mammalian cells are known to use homologous recombination to repair daughter strand gaps (Figure 1-9) (94) (41). Likewise, if the lesion is in the leading strand, and lagging strand synthesis continues, single strand DNA in the leading strand may initiate recombination (3).

Alternatively, an encounter with a blocking lesion in the leading strand may cause the replication fork to collapse and result in the formation a recombinogenic double strand end (Figure 1-8*B*) (3,45,95-97). As described above (1.2 Mechanisms of Double Strand Break Repair in Eukaryotes), homology-directed insertion of a double strand end can repair the broken replication fork and restart replication (BIR; Figure 1-5) (1-3).

It is noteworthy that while daughter strand gap repair and BIR may be efficient means for tolerating lesions that block DNA replication, the damaged base itself is not repaired in the process and remains in the DNA. However, it is thought that many repair systems can not remove adducted bases in the context of a stalled replication fork (45). Therefore, bypass of the damaged base and restoration of duplex DNA are not only essential for preserving DNA replication, but are also essential for the eventual repair of the blocking lesion (3).

Alkyl Lesions are Converted into Recombinogenic Intermediates by BER Processes

Any alkyl lesion, including 3MeA and 7MeG, may be converted into a substrate for recombination through processing by BER enzymes. If the rate of adduct removal is greater than the rates of any intermediate step in the BER pathway, BER intermediates

may accumulate. Swanson and colleagues have provided substantial evidence that BER intermediates, such as AP sites, nicks and single strand gaps can stimulate strand exchange (70).

For example, incomplete BER of an alkyl lesion may result in a nick that when encountered by the replication machinery, results in the collapse of the replication fork to form a double strand end (Figure 1-8*C*). Again, homology-directed insertion of the double strand end reconstitutes the replication fork and allows replication to resume (Figure 1-5). Another possibility is that two lesions in close proximity, when partially processed by DNA repair enzymes, can form a double strand break (Figure 1-8*D*). A number of mechanisms by which homologous recombination can repair double strand breaks have been described (1.2 Mechanisms of Double Strand Break Repair in Eukaryotes). If the double strand break occurs during late S or G2 phases of the cell cycle, it is likely that a gene conversion between sister chromatids will repair the double strand break without any loss or exchange of sequence information.

If these mechanisms are biologically relevant, then alkylation-induced recombination may in fact be repair (or at least glycosylase) dependent and the recombination frequency may depend on the number of lesions and the activities of the different enzymes in the BER pathway. In addition, the ability of DNA glycosylases to modulate recombination will depend on the relative recombinogenicity of the lesions being removed versus the intermediate(s) that accumulate.

1.6 Use of Direct Repeat Sequences to Study Homologous Recombination in Eukaryotic Cells

Mechanisms of Recombination at a *his3* Direct Repeat in *S. cerevisiae*

A useful approach for studying recombination in eukaryotic cells is to engineer two mutant expression cassettes in a direct repeat and assay for restoration of wild type gene expression [reviewed in (34,98)]. Often, the direct repeat contains two inactive copies of a reporter gene, which when converted to a functional copy, allows the cell to grow in selective media.

In one such system in *S. cerevisiae* (99), two nonfunctional *his3* alleles, one truncated at the 3' end (*his3'Δ*) and the other truncated at the 5' end (*his5'Δ*), flank a *LEU2* cassette and plasmid sequence (Figure 1-10). A homologous recombination event between the two truncated alleles (which share 400 bp of homology) can restore a functional *HIS3* gene and allow cells to grow in the absence of histidine. Thus, the frequency of recombination events can be determined by scoring *HIS3* revertants per surviving cells. Spontaneous reversion of His3 function occurs at a frequency of 1 in 10^4 viable cells and exposure to certain DNA damaging agents can increase this frequency 100 fold (90).

Theoretically, full length *HIS3* sequence can be restored via several mechanisms, including intrachromatid exchange (loop out), SSA, unequal sister chromatid exchange (USCE) and gene conversion (Figure 1-10). However, loss of the *LEU2* cassette in ~99% of the recombinants suggests that gene conversion (which is a conservative process that is not associated with loss of sequences) is not a predominant mechanism underlying

spontaneous recombination at the *his3* repeat. Furthermore, more in depth analyses have shown that the majority of recombinants arise from SSA (99,100).

Direct Repeat Recombination Substrates in Mammalian Cells

Similar direct repeat recombination substrates have been used to monitor spontaneous and DNA damage-induced mitotic homologous recombination in mammalian cells (98,101). These systems are highly variable in terms of how recombinants are assayed, the types of recombination events that can be detected (*e.g.* gene conversion versus cross-over), the amount of homology shared by the two alleles and the locus of integration. Although the majority of systems rely on drug resistance (*e.g.* neomycin resistance) to select for recombinants, concomitant with the work described herein, Pierce *et. al.* developed an assay in which gene conversion can be detected by green fluorescence (49). Furthermore, some systems are designed to detect multiple types of recombination events [*e.g.* (102)], whereas others are designed to detect only gene conversion [*e.g.* (49)]. Given that the rate of gene conversion is directly proportional to the length of uninterrupted homology (103), variations in the length of homology shared by the repeated sequences can dramatically alter the rate of reversion. The recombination rate is also highly dependent on where in the genome the direct repeat has integrated. Recombination frequency varies among different loci by as much as 24 fold (104). Thus, it is not surprising that recombination rates reported for direct repeat substrates are highly variable, ranging from 5×10^{-8} to 5×10^{-3} per cell division (101).

Nevertheless, in mammalian cells, direct repeat recombination substrates have offered an effective approach for elucidating the effects of specific genes on

recombination susceptibility, for determining the predominant mechanisms of spontaneous recombination and for studying the effects of endonuclease-induced double strand breaks. In several studies, direct repeat substrates were stably transfected into mammalian cells that contained mutations in putative recombination genes in order to directly measure the effects of these genes on spontaneous homologous recombination. Indeed, such an approach helped to define the roles of Rad54 and the Rad51 homologs, XRCC2 and XRCC3, in modulating homologous recombination (49,50,105).

Bollag and Liskay have used direct repeats in altered configurations, where the order of the mutant alleles is alternated, to assess the relative contributions of various recombination mechanisms in mammalian cells (106). For direct repeat recombination substrates that are designed so that they do not allow recovery of SSA events, the majority of spontaneous recombinants were consistent with gene conversion (80%), while the remainder could be attributed to USCE. There was no evidence for recombination due to intrachromatid exchange (also known as "loop out") in these studies. Similar systems have been used to study the effects of double strand breaks induced *in vivo* by I-*Sce*I endonuclease treatment [reviewed in (16)]. Studies initiated in the laboratory of M. Jasin have shown that a double strand break, site-specifically introduced into a direct repeat recombination substrate by I-*Sce*I, can induce homologous recombination 2-3 orders of magnitude. Physical analyses of repair products indicate that 30-50% of I-*Sce*I-induced double strand breaks are repaired by homologous recombination (38).

Replication Slippage at Repeat Sequences

Previous studies have shown that direct repeats can be converted to single copies by a mechanism other than homologous recombination, such as slippage of the DNA polymerase during replication (107-109). Rearrangements that occur by replication slippage are highly dependent both on the size and proximity of the repeat elements. Studies in *E. coli* have show that while rearrangements between tandem duplications less than 300 bps in length may occur through a recombination independent mechanism, such as replication slippage, rearrangements between direct repeats greater than 300 bps are more dependent on homologous recombination (110). Similar findings have also been observed in eukaryotic cells, where rearrangements between long direct repeats are both Rad51 and Rad52 dependent (111). In addition to increased length, the presence of any intervening sequence between the repeat elements (even small insertions of a few hundred base pairs) significantly inhibits replication slippage (112).

In this work, we have used direct repeat substrates to measure homologous recombination in *S. cerevisiae* and mammalian cells (described below). Given that the direct repeats used in our studies are substantially longer than 300 bp and/or contain intervening sequences, restoration of transgene expression is most likely due to homologous recombination and not replication slippage.

1.7 Existing Mouse Models for Detection of Homologous Recombination

Prior to this work, there were only a few systems available to detect mitotic homologous recombination in mammals, and these systems are described below.

p^{un}/p^{un} Mice

The p^{un}/p^{un} mouse carries a naturally occurring 70 kb tandem repeat in the *p* gene, which controls pigmentation in skin, hair and retinal epithelium (113-114). Normally, these mice have pink eyes and light gray fur, due to the reduced expression of melanin in these tissues. A homologous recombination event can restore wild type melanin expression by deleting one copy of the 70 kb repeat. If this event occurs early in development, clonal expansion of recombinant cells can result in the appearance of dark patches in the skin, hair or retina. Studies utilizing p^{un}/p^{un} mice have been pivotal in demonstrating that many types of DNA damaging agents induce recombination *in vivo* (115-117).

However, there are serious limitations in using the p^{un}/p^{un} mouse system to study recombination. Since clonal expansion of recombinant cells is necessary for the detection of pigmented spots, only recombination events that occur prenatally can be observed. Consequently, in order to measure the effects of DNA damaging agents on recombination, mice must be exposed to the agent *in utero* and the timing of exposure must be very carefully controlled as recombination rates fluctuate during development (118). Since the *p* gene controls pigmentation, only those cell types that express melanin can be assayed. Additionally, large numbers of mice are required in order to achieve

statistically significant data. For example, in order to show that an Atm deficiency increases spontaneous homologous recombination in mice, over 700 mice were analyzed for fur spots (119). To determine the effects of a specific DNA damaging agent, over 100 animals must be treated for each dose studied and fur spots must be analyzed in the resulting offspring (116,120). With so many animals to be housed, dosed and analyzed, studies employing p^{un}/p^{un} mice tend to be both expensive and time consuming.

APRT+/- Mice

Another system for detecting spontaneous and DNA damage-induced recombination events in mammals is the *APRT*+/- mouse in which loss of heterozygosity (LOH) at the *APRT* gene is scored by selection with 8-azaadenine (121-123). Since LOH can be caused by many mechanisms, including point mutations, deletions, aneuploidy and mitotic homologous recombination, identifying clones that have undergone a recombination event requires a combination of PCR, G-banding and chromosome painting (124). In addition, only cells that can be extracted from mice and grown in culture, such as ear fibroblasts and T-cells, are assayable using this system.

Mice that Carry Engineered Direct Repeat Recombination Substrates

Despite the proven efficacy of direct repeat substrates for studying mitotic homologous recombination in mammalian cells (98,101), only a few animal models have been created that exploit direct repeats and none are suitable for the detection of mitotic recombination events in somatic cells of mature animals. Bonnerot and Nicolas engineered a transgenic mouse in which a recombination event between duplicated

sequences in the coding region of the *lacZ* gene could result in the expression of β-galactosidase, and this mouse has been used to identify cell lineages specifically in the embryonic myotome (125). In contrast, transgenic mice engineered by Murti *et al.* allow for the detection of spontaneous and DNA-damage induced recombination events that occur in the germline of male mice (126,127). In this system, spermatids that have undergone a gene conversion event between two mutually defective *lacZ* alleles (the recipient allele contains a 2 bp insertion and the donor allele is truncated at both the amino and carboxy termini) can produce functional β-galactosidase activity and be visualized by histochemical staining or flow cytometry.

Similar lacZ-based mouse models, which also detect recombination events that occur in the male germline, were created in the laboratory of M. Jasin (128,129). One of these mice carries two copies of the *lacZ* recombination substrate arranged as a palindrome and has been integral in monitoring the frequency and type of rearrangements that occur at palindromes *in vivo* (130,131). However, the instability of this palindromic substrate has prevented the establishment of a stable mouse line and consequently, has limited its application as a tool to investigate the genetic and environmental factors that modulate recombination *in vivo*.

1.8 Specific Aims of This Thesis

Mitotic homologous recombination can provide an efficient means for repairing and tolerating DNA damage. In eukaryotic cells, pathways that involve homology searching are integral to DNA replication (1-3). However, recombination between misaligned sister chromatids or between homologous chromosomes can lead to deletions,

translocations and loss of heterozygosity, events that are known to promote cancer. Consequently, increased susceptibility to recombination is a known risk factor for cancer [*e.g.* (26,29,132,133)]. Furthermore, defects in genes essential for homology-directed repair result in a decreased susceptibility to homologous recombination and an increased susceptibility to cancer. Although mitotic recombination may be critical to the process of tumorigenesis, we do not yet fully understand the molecular basis for how DNA lesions induce mitotic recombination in eukaryotic cells. The ultimate goal of our research is to elucidate the mechanisms of DNA damage-induced recombination and to determine the role of DNA repair enzymes in modulating homologous recombination in yeast, mammalian cells and mice.

Alkylating agents are ubiquitous DNA damaging agents that are mutagenic, cytotoxic, and recombinogenic. Consequently, cells have evolved highly effective means for repairing alkylation damage, such as the base excision repair (BER) pathway. BER is initiated by a DNA glycosylase, such as the *Saccharomyces cerevisiae* Mag1 3-methyladenine (3MeA) DNA glycosylase, which recognizes the alkylated base and removes it from the DNA backbone. Although wild type levels of Mag1 can protect cells against the lethal effects of a variety of alkylating agents (74), an overproduction of Mag1 actually sensitizes cells to the cytotoxic effects of methylating agents (76), presumably by creating an overabundance of unrepaired BER intermediates. There is now substantial evidence that BER intermediates, such as abasic (AP) sites, nicks and single-strand gaps can stimulate mitotic homologous recombination (70,80). In this work, we study the role of Mag1 in modulating methylation-induced recombination in *S. cerevisiae*. In doing so, we test the hypothesis that alkylated bases can be converted into

substrates for recombination through processing by BER enzymes. We also investigate the possibility that methyl lesions themselves may induce recombination, possibly by blocking DNA replication.

Analogous studies to explore the genetic factors that modulate susceptibility to homologous recombination in mammals have been hindered by the lack of effective tools for measuring recombination events *in vivo*. A common approach to studying homologous recombination in eukaryotic cells is to engineer two mutant expression cassettes and assay for restoration of transgene expression (98,99,101). Despite their proven efficacy, few animal models have been created that exploit direct repeat substrates to study homologous recombination *in vivo* (126,128,134) and none are suitable for the detection of mitotic recombination events in somatic tissues of mature animals. To this end, we have created transgenic mice that allow for the detection of spontaneous and DNA-damage induced homologous recombination at an enhanced yellow fluorescent protein (*EYFP*) direct repeat. We show that the rate of spontaneous recombination can be quantified in embryonic and adult primary fibroblasts and that recombination events can be detected in skin *in vivo*. These mice can be crossed with mice carrying a variety of engineered genetic defects to reveal how specific DNA repair processes affect susceptibility to mitotic recombination in mammals, and as such offer a powerful new tool for studies of mitotic homologous recombination. A mechanistic understanding of DNA damage-induced recombination, facilitated by studies using mice that we have developed, may help us better understand the complex relationship between DNA damage, recombination and carcinogenesis.

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Table 1-1. Cancer syndromes associated with defects in homologous recombination.

Disease	Gene	Protein Function	Cellular Phenotype	Clinical Phenotype	Ref.
Bloom's syndrome	*BLM*	Homolog of *E. coli* RecQ DNA helicase; 3'-to-5' helicase	Slow growth, ↑ frequency of SCEs, photosensitivity, chromosomal instability	Stunted growth, immunodeficiency, skin lesions, predisposition to cancer	(27, 30, 132, 135)
ataxia telangiectasia	*ATM*	Kinase; substrates include p53, NBS and BRCA1 in response to DNA damage	Chromosomal instability, radiosensitivity, ↑ rates of homologous recombination	Neurologic degeneration, immunodeficiency, predisposition to lymphoid cancer in childhood	(17, 28-30, 119)
Werner's syndrome	*WRN*	Homolog of *E. coli* RecQ DNA helicase; 3'-to-5' helicase and exonuclease; may resolve recombination intermediates	Limited cell divisions, hypersensitivity to DNA damage, chromosomal instability, ↑ spontaneous mutation rate, aberrant mitotic recombination	Premature aging, predisposition to non-epithelial solid tumors and, to a lesser extent, leukemia and carcinomas	(30, 136, 137)
Nijemengen break syndrome	*NBS*	Functions in a complex with MRE11 and RAD50; 5'-to-3' resection of double strand ends in homology-directed repair	Chromosomal instability, hypersensitivity to ionizing radiation, decreased homology-directed repair	Immunodeficiency, predisposition to lymphoreticular tumors	(31, 32, 34)

Table 1-1 (cont). Cancer syndromes associated with defects in homologous recombination.

Disease	Gene	Protein Function	Cellular Phenotype	Clinical Phenotype	Ref.
Rothmund-Thomson	*RECQL4*	Homolog to *E. coli* RecQ DNA helicase	Chromosomal instability, aneuploidy	Growth deficiency, photosensitivity, premature aging, immunodeficiency, predisposition to malignancy, especially osteogenic sarcomas	(33, 138-140)
Fanconi's anemia	8 complementation groups, *FA-A – FA-G*	Interaction with BRCA1 suggests role in homology-directed DNA repair	Chromosomal instability, hypersensitivity to cross-linking agents; ↑ susceptibility to recombination	Progressive bone marrow failure, congenital abnormalities, predisposition to acute myelogenous leukemia and other malignancies	(30, 141-143)
Li-Fraumeni syndrome	*TP53*	Regulates cell cycle progression, apoptosis, and homologous recombination via its interaction with RAD51	Chromosomal instability in dermal fibroblasts, ↑ frequency of gene amplification, conflicting reports of p53's effect on homologous recombination	Early onset cancer, most prominent are carcinoma of the breast, sarcomas, brain tumors, leukemia and lymphoma	(30, 144-147)

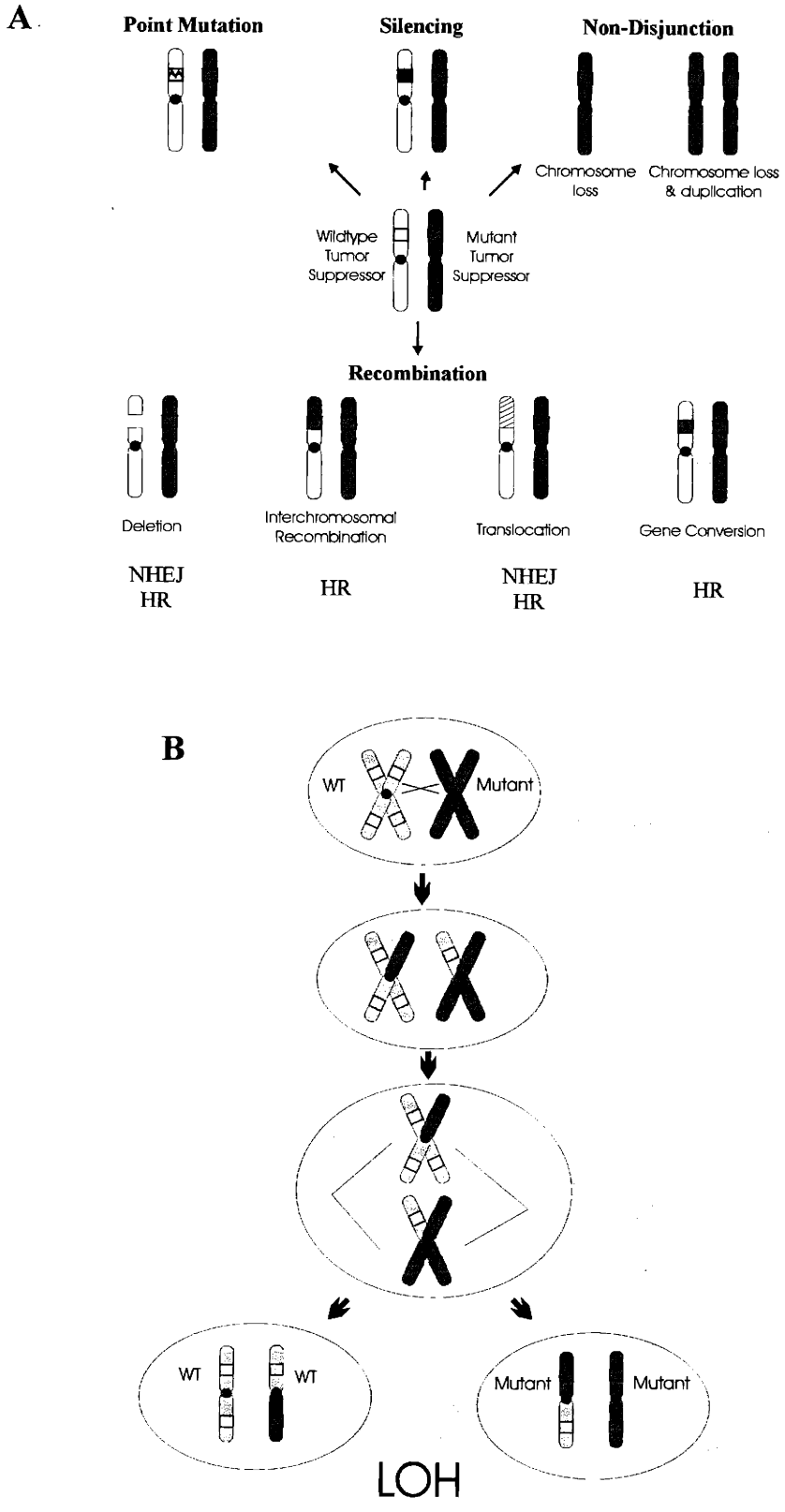

Figure 1-1. Mechanisms of loss of heterozygosity (LOH). (A) Loss of a wild-type tumor suppressor gene (LOH) can be caused by point mutations, aberrant silencing, non-disjunction or genetic changes that arise from nonhomologous end-joining (NHEJ) or homologous recombination (HR). (B) Schematic of one mechanism by which mitotic homologous recombination can lead to LOH.

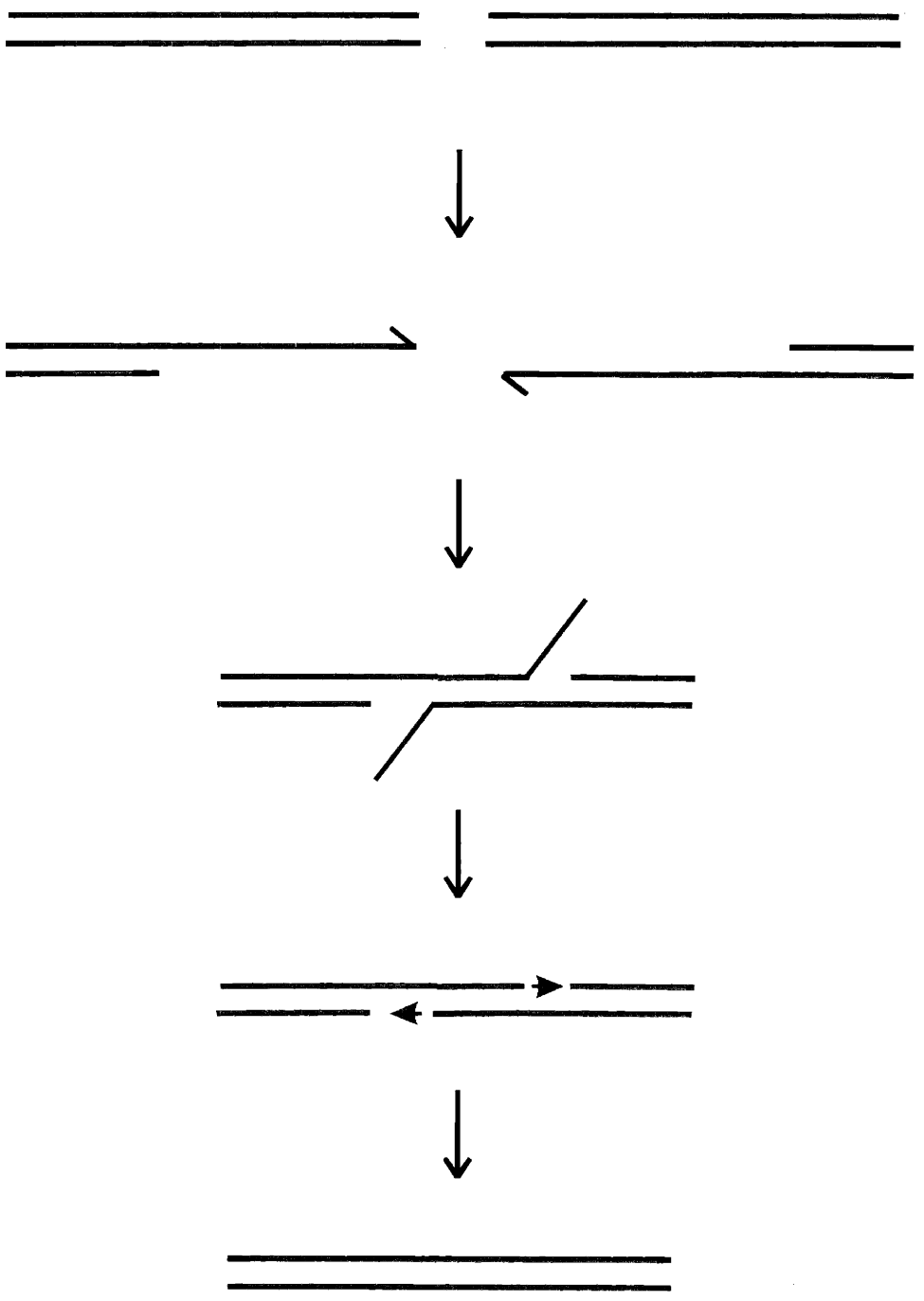

Figure 1-2. Model of single strand annealing. A double strand break occurs between two repeated homologous sequences (red). 5' ends of the DNA are recessed until a region of homology is reached. Homologous regions are reannealed and overhanging flaps are cleaved. DNA synthesis and ligation restores intact duplex DNA. Adapted from (34).

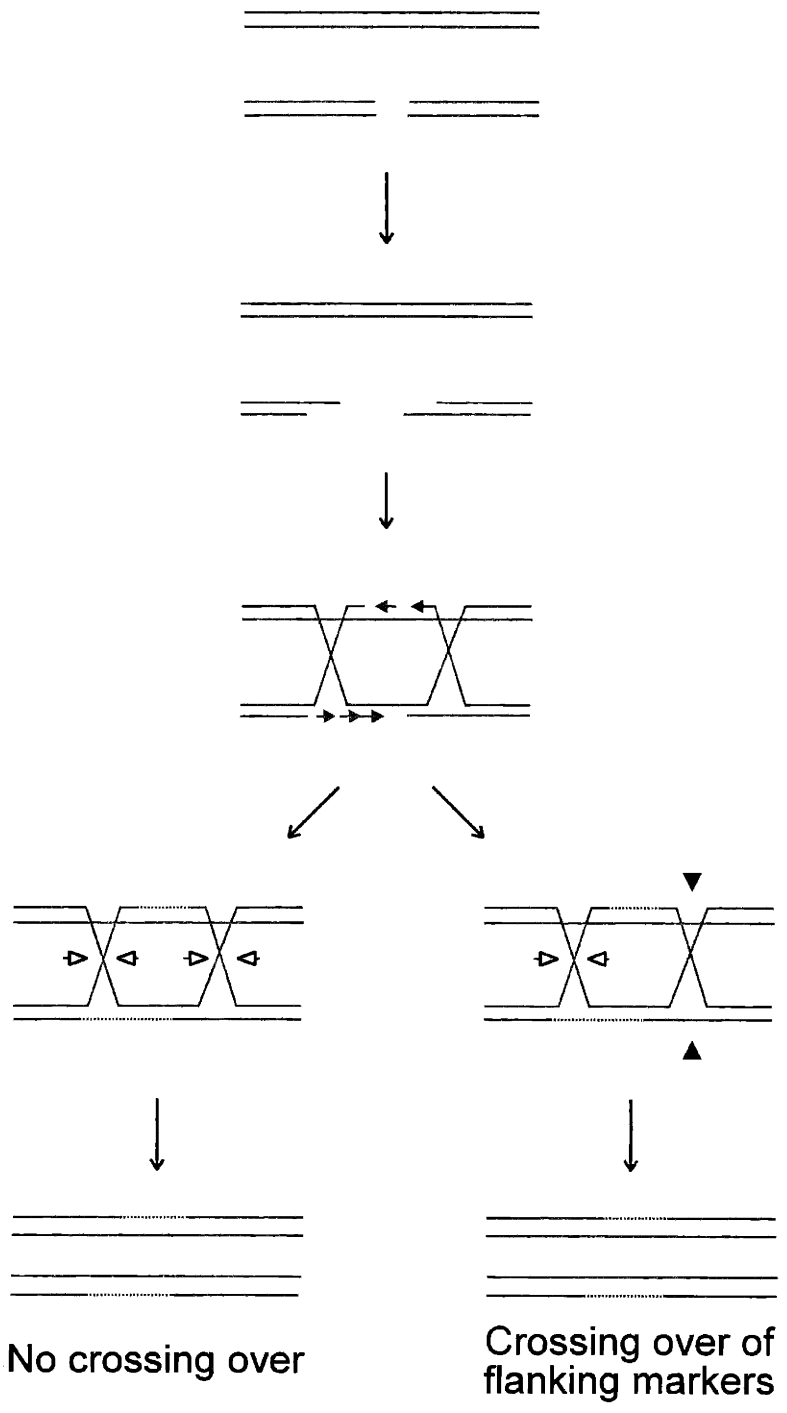

Figure 1-3. Double strand break repair model proposed by Szostak *et al.*(41). 5'-to-3' resection of the double strand ends creates 3' overhangs that can invade a homologous duplex and initiate repair synthesis. Holliday junctions are resolved by cutting the "crossed" (open arrowhead) or "continuous" (closed arrowhead) strands. Depending on the way each Holliday junction is cleaved, crossover or noncrossover products can result. Adapted from (1).

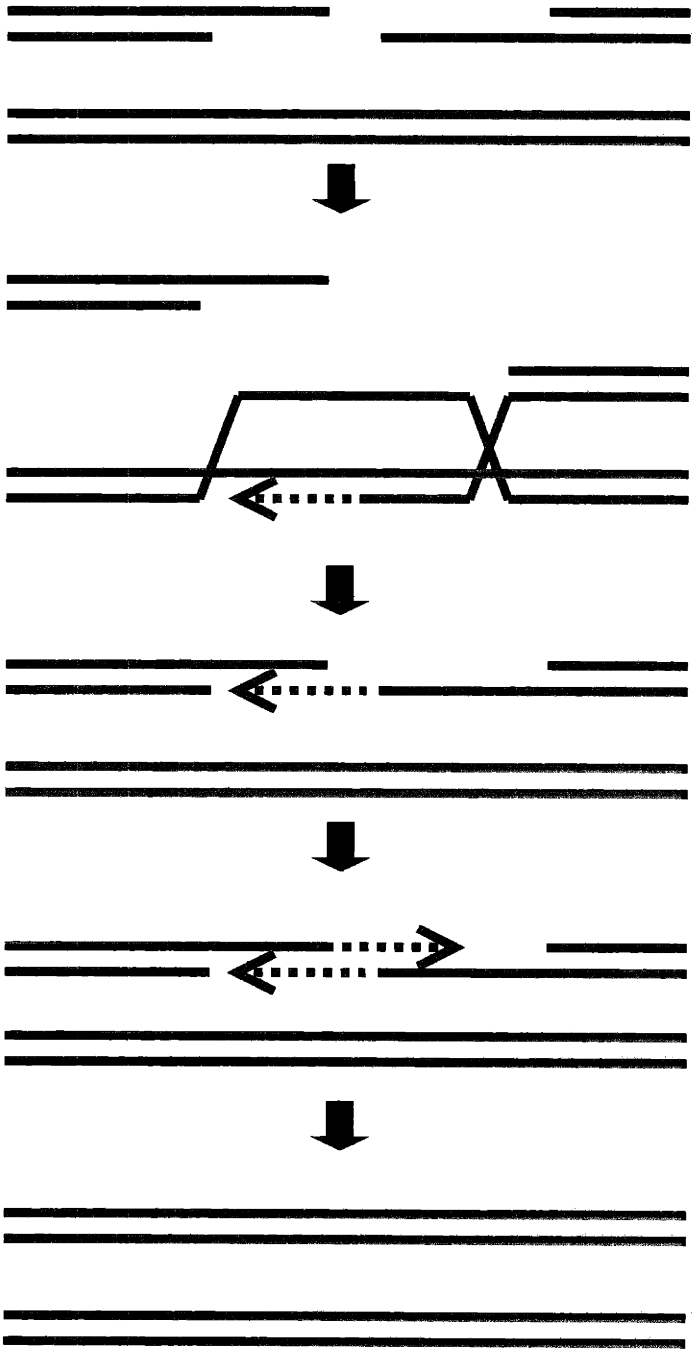

Figure 1-4. Repair of a double strand break by synthesis dependent strand annealing. Strand invasion and DNA synthesis are initiated by a single 3' overhang. Replication occurs in a D loop, and the newly synthesized strand is displaced from its template and anneals to the 3' overhang at the other end of the double strand break. Adapted from (1).

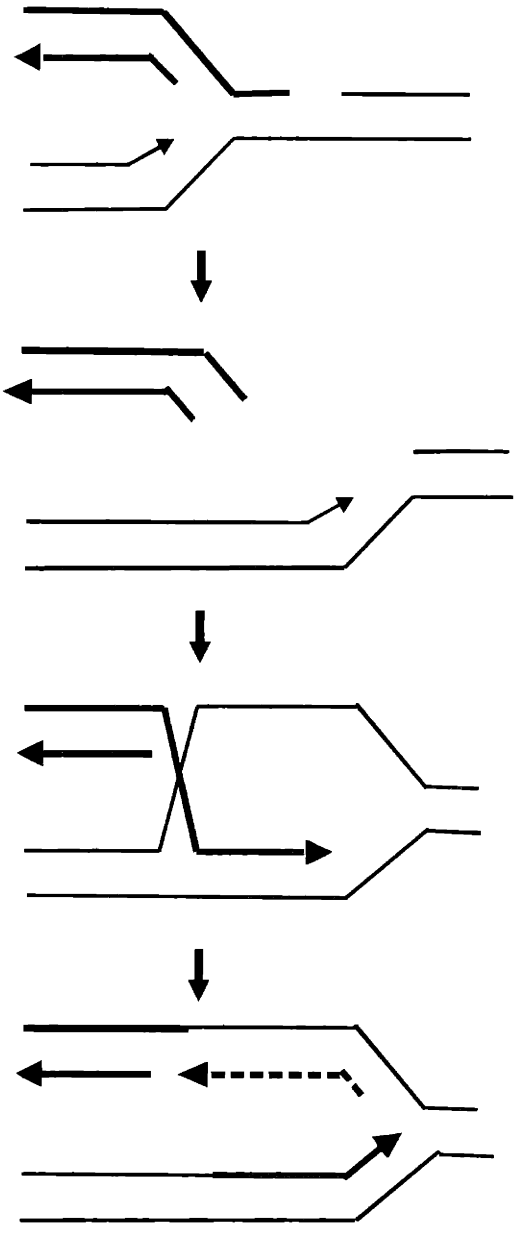

Figure 1-5. Model of break-induced replication. When the replication machinery encounters a gap in the template DNA, the replication fork collapses and a double strand end is created. Homology directed insertion of the 3' end and subsequent DNA synthesis results in formation of a Holliday junction and reconstitution of the replication fork. Cleavage of the Holliday junction completes BIR.

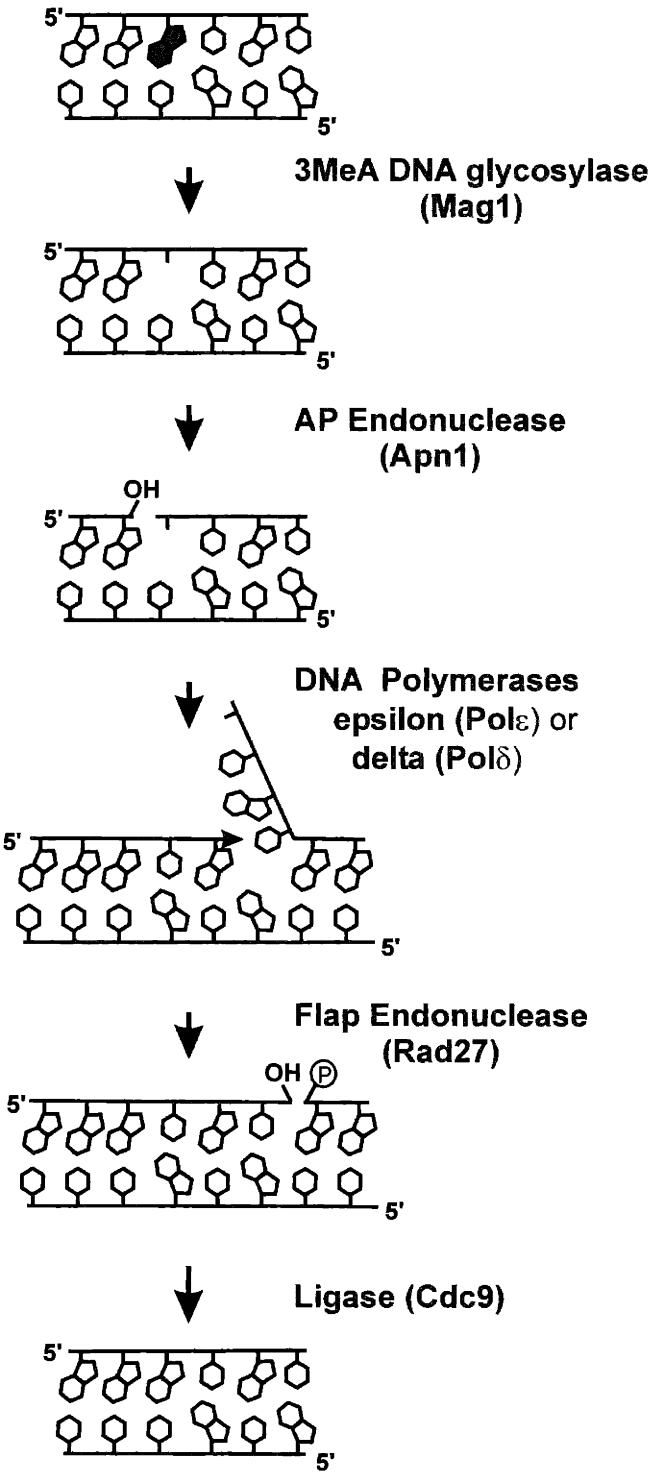

Figure 1-6. Repair of an alkylated base by base excision repair (BER) in *S. cerevisiae*. Shown above are the steps and components of BER. Adapted from (59) and (148).

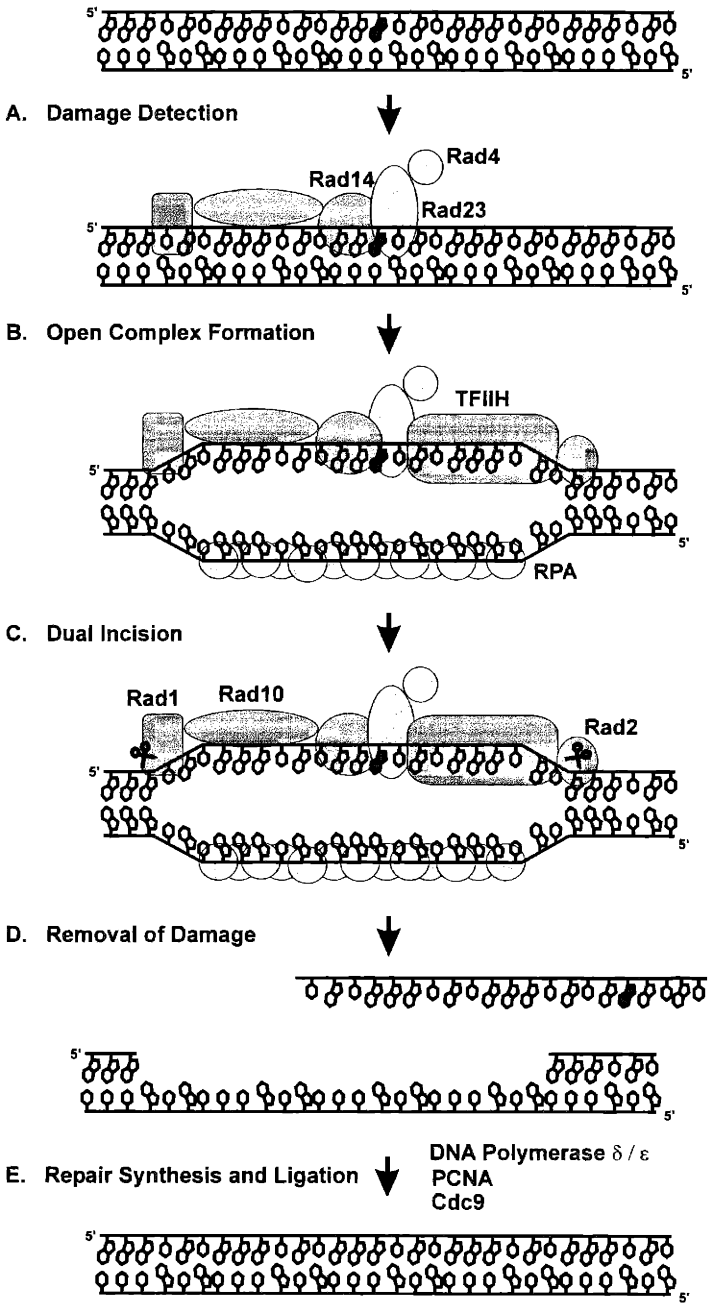

Figure 1-7. Model of nucleotide excision repair in *S. cerevisiae*. (A) Rad14 and Rad4-Rad23 recognize a DNA lesion. (B) TFIIH unwinds the DNA to create single strand regions upstream and downstream of the lesion. (C) Rad10-Rad1 and Rad2 incise the DNA 5' and 3' to the lesion, respectively. (D) A DNA fragment 25-30 nucleotides long is released resulting in a single strand gap. (E) The replication machinery fills the gap and ligase seals the nick. (A-E) NEFs are distinguished by color: NEF1, blue; NEF2, yellow; and NEF3, green. Note that NEF4, which is not required for incision and functions primarily in the repair of nontranscribed DNA, is not shown.

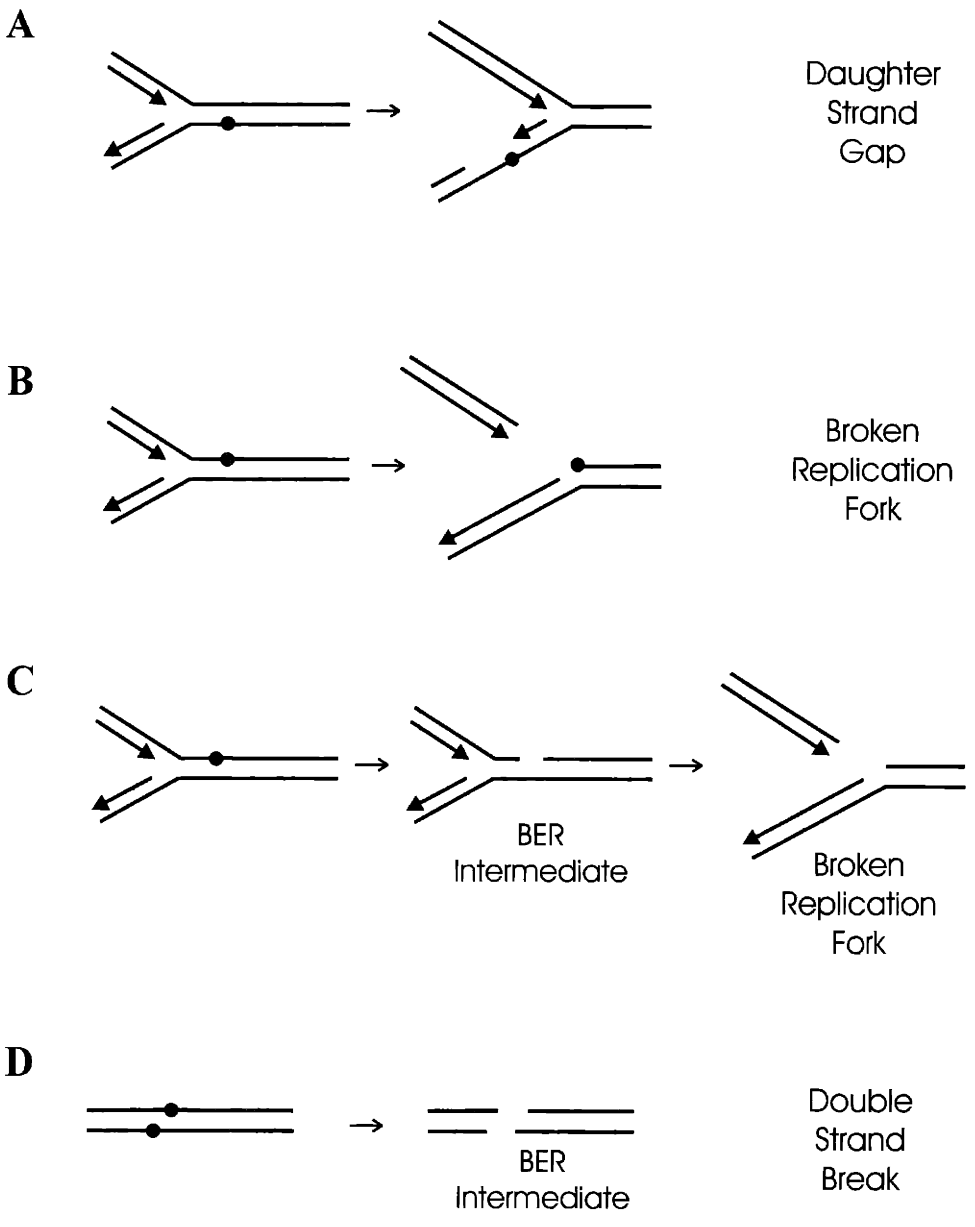

Figure 1-8. Possible mechanisms of alkylation induced recombination. (A) An unrepaired lesion blocks lagging strand synthesis and leads to the formation of a recombinogenic daughter strand gap. (B) Inhibition of leading strand synthesis results in replication fork breakdown and the creation of a double strand end. (C) Incomplete BER results in a nick that when encountered by the replication machinery, causes collapse of the replication fork and formation of a double strand end. (D) Incomplete repair of two lesions in close proximity gives rise to a double strand break.

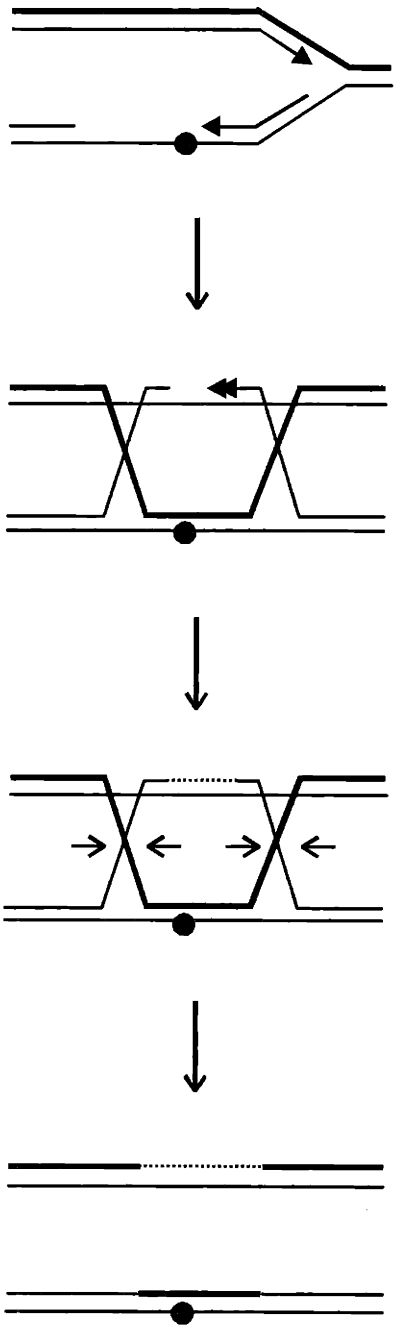

Figure 1-9. Homologous recombinational repair of daughter strand gaps. An unrepaired lesion can block lagging strand synthesis and lead to the formation of a daughter strand gap. Homology-directed invasion of the single strand ends results in formation of a double Holliday junction. Resolution of the Holliday junction completes restoration of sequence information. (It is not yet known whether or not cleavage of the single strand region in the daughter strand gap is a necessary step in daughter strand gap repair.)

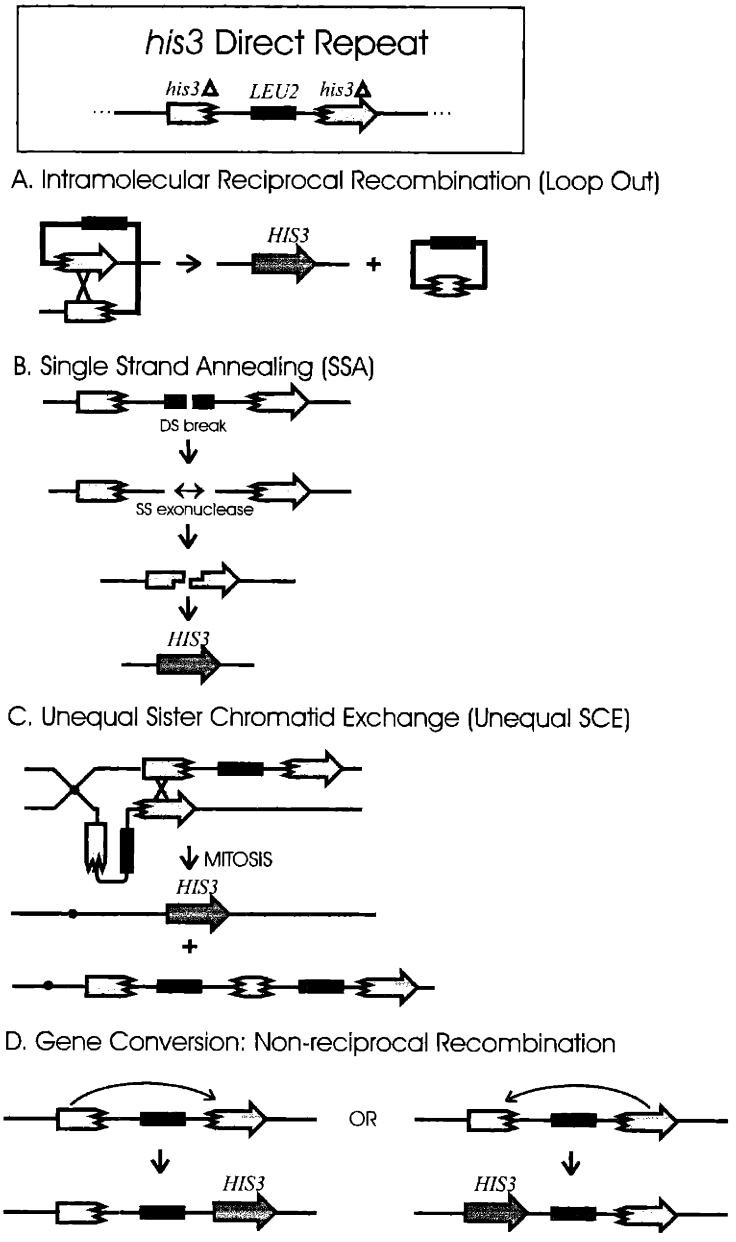

Figure 1-10. Possible mechanisms of *HIS3* reversion at a *his3* direct repeat. Two nonfunctional *his3* alleles, one truncated at the 3' end (*his3'Δ*) and the other truncated at the 5' end (*his5'Δ*), are arranged in a direct repeat. A *LEU2* cassette and pBR322 sequences are present between the *his3* alleles. (A) Intrachromatid exchange restores functional *HIS3* while excising intervening sequences. The resulting plasmid is maintained only if an origin of replication has been incorporated. (B) A double strand break occurs between the repeats and the intervening sequences are degraded to produce complementary strands that reanneal to restore full length *HIS3*. (C) Misalignment of *his3* alleles in a cross-over event between sister chromatids results in a *HIS3* recombinant that lacks *LEU2*. (D) Either *his3* allele can serve as donor or recipient in a gene conversion event that restores functional *HIS3* while retaining the *LEU2* cassette.

Chapter II

The *S. cerevisiae* Mag1 3-Methyladenine DNA Glycosylase Modulates Susceptibility to Homologous Recombination

Chapter 2

2.1 Abstract

DNA glycosylases, such as the Mag1 3-methyladenine (3MeA) DNA glycosylase, initiate the base excision repair (BER) pathway by removing damaged bases to create abasic (AP) sites that are subsequently repaired by downstream BER enzymes. Although unrepaired base damage may be mutagenic or recombinogenic, BER intermediates (*e.g.*, AP sites and strand breaks) may also be problematic. To investigate the molecular basis for methylation-induced homologous recombination events in *Saccharomyces cerevisiae*, spontaneous and methylation-induced recombination were studied in strains with varied *MAG1* expression levels. We show that cells lacking Mag1 have increased susceptibility to methylation-induced recombination, and that disruption of nucleotide excision repair (*rad4*) in *mag1* cells increases cellular susceptibility to these events. Furthermore, expression of *E. coli* Tag 3-methyladenine DNA glycosylase suppresses recombination events, providing strong evidence that unrepaired 3MeA lesions induce recombination. Disruption of *REV3* (required for Polymerase ζ) in *mag1 rad4* cells causes increased susceptibility to methylation-induced toxicity and recombination, suggesting that Polymerase ζ can replicate past 3MeAs. However, at subtoxic levels of methylation damage, disruption of *REV3* suppresses methylation-induced recombination, indicating that the effects of Polymerase ζ on recombination are highly dose-dependent. We also show that overproduction of Mag1 can increase the levels of spontaneous recombination, presumably due to increased levels of BER intermediates. However, additional *APN1* endonuclease expression or disruption of *REV3* does not affect *MAG1*-induced recombination, suggesting that downstream BER intermediates (*e.g.*, single strand breaks) are responsible for *MAG1*-induced recombination, rather

than uncleaved AP sites. Thus, too little Mag1 sensitizes cells to methylation-induced recombination, while too much Mag1 can put cells at risk of recombination induced by single strand breaks formed during BER.

2.2 Introduction

Base excision repair (BER) and nucleotide excision repair (NER) preserve sequence integrity by excising damaged nucleotide(s) (1-4). When excision repair fails to fully restore the DNA prior to DNA replication, processes involving homologous recombination can help cells tolerate DNA damage (5). For example, if a DNA polymerase is inhibited by a lesion, homologous recombination may facilitate substitution of an undamaged template for one that contains the lesion (Figure 2-1*A*). In addition, single strand breaks (and possibly unrepaired lesions that inhibit DNA polymerases) in the template DNA may lead to replication collapse, and there is mounting evidence that homologous recombinational repair restores collapsed replication forks (Figure 2-1*B*) (6-9). Processes that involve homologous recombination generally do not result in sequence changes. However, recombination between misaligned sister chromatids or between homologous chromosomes can lead to deletions and loss of heterozygosity (10). Although our understanding of the relationship between damaged bases and their resulting point mutational spectrum is rather sophisticated, much less is known about how changes to base structure lead to homologous recombination events.

Alkylating agents comprise one of the broadest classes of DNA damaging agents and these agents are present both in the environment and within normal cells (4,11). As a model for this class of damage, we have focused on methylation damage, one of the simplest forms of alkylation damage. Methylating agents

produce many different types of lesions with varied biological consequences (11,12). The most abundant methylation lesions are 7-methylguanine (7MeG, 60-80%), O^6-methylguanine (O^6MeG, 0.3-8%) and 3MeA (8-12%) (13). Although 7MeG is readily removed from the genome of mammalian cells ($t_{1/2}$ = ~20 h for mouse embryonic stem cells; (14)), the normal steady state level of 7MeG in mouse cells is >10,000 adducts/cell (15), which suggests that the genome is under constant assault by methylating agents. Among the different types of methylated bases, 3MeA and O^6MeG have some of the most serious biological consequences. While several studies have addressed the effects of O^6MeG on mitotic homologous recombination in eukaryotes (*e.g.*, (16-18)), much less is known about the recombinogenic potential of 3MeA.

3MeA is a toxic lesion that inhibits DNA polymerases *in vitro* (19). In *S. cerevisiae*, one of the main defenses against 3MeA is the Mag1 3MeA DNA glycosylase (20). Mag1 initiates BER by cleaving the glycosylic bond between 3MeA and the deoxyribose of the sugar phosphate backbone (Figure 2-1*C*). The resulting apurinic/apyrimidinic (AP) site is then cleaved 5' to the abasic sugar by the major AP endonuclease, Apn1, which is responsible for >95% of the AP endonuclease activity in *S. cerevisiae* (21). The 3' hydroxyl group created by Apn1 is then extended one or several nucleotides by DNA polymerase δ or ε (22,23). Finally, the abasic deoxyribose is removed by the Rad27 flap-endonuclease (24), or possibly by a deoxyribose phosphatase, to facilitate DNA ligation (1,25). *S. cerevisiae* deficient in 3MeA DNA glycosylase activity are very sensitive to the toxic effects of agents that create 3MeA (20), and the additional disruption of genes required for homologous recombination makes such cells even more sensitive to methylation damage (26), suggesting that both BER and homologous recombination help prevent methylation-induced toxicity.

NER deficient *S. cerevisiae* show very little sensitivity to methylation damage (27). However, *S. cerevisiae* lacking both Mag1 and NER are far more sensitive to the toxic effects of the methylating agent methyl methanesulfonate (MMS) than are cells lacking only Mag1, suggesting that NER can effectively remove potentially toxic lesions normally repaired by Mag1 (27,28). NER involves concomitant dual incisions on either side of the original damage, generating an oligonucleotide 25-30 bases long for removal (2). In *S. cerevisiae*, NER involves over a dozen proteins. Unlike BER, wherein components of the pathway are able to act independently of one another, most of the NER components are required to act in concert to cleave the DNA. As an example, although Rad4 and Rad23 interact to form a damage recognition complex (29,30), Rad4 is also absolutely required to be present in the NER complex in order for incision to take place (29,31).

Mag1 deficient cells are highly sensitive to the toxic effects of methylation damage (20). Paradoxically, expression of *MAG1* from the *GAL1* promoter also makes *S. cerevisiae* sensitive to methylating agents (32). Since 7MeG comprises the vast majority of methylation-induced lesions, high levels of Mag1 activity may overburden downstream BER enzymes by converting otherwise benign 7MeG lesions into cytotoxic abasic sites or other downstream BER intermediates (32,33).

Another mechanism for tolerating DNA lesions is translesion replication. Polymerase ζ (Pol ζ), formed from a complex of the Rev3 and Rev7 proteins, is a non-essential polymerase that facilitates DNA replication past AP sites and other DNA lesions known to inhibit DNA replication (34-38). Cells deficient in Pol ζ have an increased susceptibility to recombination caused by AP sites, presumably because inhibition of DNA replication induces homologous recombination (39). It is not yet known if Pol ζ plays an analogous role in the case of 3MeA lesions.

In this work, we have explored the ability of 3MeA and its downstream BER intermediates to induce homologous recombination in *S. cerevisiae*. In the studies presented here, we show that cells deficient in the ability to repair 3MeA lesions have an increased susceptibility to methylation-induced mitotic homologous recombination, which is consistent with a model wherein unrepaired 3MeA induces recombination. On the other hand, overexpression of *MAG1* increases cellular susceptibility both to methylation-induced recombination events and to spontaneous recombination events, suggesting that BER intermediates are potentially recombinogenic. The effects of Pol ζ on spontaneous and damage-induced homologous recombination were also investigated.

2.3 Materials and Methods

Media and Growth Conditions

S. cerevisiae strains were grown non-selectively in yeast extract-peptone supplemented with adenine (1% yeast extract-2% Bacto-peptone-2% dextrose-1.5% agar for plates-30 mg/ml adenine). Cells were grown in synthetic complete (SC) medium (40) lacking leucine during liquid culture (to prevent expansion of recombinant cells) and in SC medium lacking histidine to select for recombinants (controls were grown in SC medium). For gene induction experiments, medium was supplemented with 2% glucose, 2% raffinose, or 2% galactose. When galactose-inducible protein expression was required, cultures were expanded in media supplemented with raffinose, incubated in liquid media supplemented with galactose for 2.5 h, and plated on solid media supplemented with galactose. MMS was purchased from Sigma Chemical Co. (St. Louis, MO) and was added to molten media at 55°C at the indicated concentrations; cells were plated within 2.5

h after the media solidified. 5-Fluoroorotic acid (5-FOA) was purchased from Sigma Chemical Co. (St. Louis, MO) and plates were prepared according to standard protocols (41).

Strains

Table 2-1 lists yeast strains used in this study. Strains CHY7 and CHY34 were created from RSY6 and Y433, respectively (42), by one-step gene replacement of *APN1* with an *EcoRI/BamHI* deletion/disruption cassette released from pSCP108 (a kind gift of B. Demple) (21). Transformants that were *apn1::URA3* were identified by MMS sensitivity (data not shown). Strains CHY8 and CHY35 were constructed from RSY6 and Y433, respectively, using a *mag1*-deletion/disruption cassette generated by PCR amplification of the 1.6 kb gene blaster fragment of pUG6 (43). The primers contained 58-60 bp overhangs with homology to sequences flanking the *MAG1* coding sequence. Properly targeted $G418^r$ *mag1* clones were identified by sensitivity to MMS-induced toxicity (data not shown). To create CHY9 and CHY10, *RAD4* was disrupted using a *rad4*-deletion/disruption cassette generated by PCR amplification of the 2.0 kb gene blaster fragment of pMPY-ZAP (44). The primers contained 55-57 bp overhangs with a homology to the regions directly 5' and 3' to the *RAD4* sequence. Strains CHY36 and CHY37 were constructed using a 5.3 kb *Kpn*I *rad4*-deletion/disruption cassette released from pNKY-rad4 (for construction of pNKY-rad4, see below). All *rad4Δ::URA3* clones were confirmed both by PCR amplification of a fragment bridging the inserted and flanking sequences and by increased sensitivity to UV-induced toxicity (data not shown). The *REV3* gene was disrupted in MRY11, MRY12, MRY38, and MRY39 by one-step gene replacement with an 8 kb *REV3*-deletion/disruption cassette released from pYPG101 by *Kpn*I (pYPG101 was a gift of C. Lawrence). The *rev3Δ::URA3*

clones were confirmed both by PCR amplification of a fragment bridging inserted and flanking sequences (the appropriate sequence for the flanking primer was obtained by determining the sequence of 200 bases on the 5' end of the targeting cassette and obtaining sequences outside the targeting vector from The *Saccharomyces* Genome Database™). Proper disruption of *REV3* was also confirmed by increased sensitivity to the toxic effects of both UV (data not shown) and MMS. All *URA3* markers were recycled (45) by selection on 5-FOA (46) immediately following strain verification. Diploid strains were constructed by mating the MATa and MATα haploid strains and selecting on plates lacking lysine and tryptophan.

Plasmids

Vectors pYES-Tag, pYES-MAG, and pYES-MGT (gifts of B. Glassner and L. Samson) have the sequences of 3MeA glycosylases (*E. coli Tag* and *S. cerevisiae MAG1*), and the *S. cerevisiae* repair methyltransferase (*MGT1*) cloned into pYES2.0 (Invitrogen) between *BamH*I and *Xho*I. For AP endonuclease overexpression, pYES-APN1 was constructed by subcloning the *APN1* coding sequence (PCR amplified from YEpAPN1, a gift of B. Demple (21) into pYES2.0 between *BamH*I and *Xho*I. Effective expression was confirmed by reversion of the MMS sensitivity of CHY7 (data not shown). Plasmid pYES-MAG-APN was constructed to coexpress *MAG1* under the galactose-inducible promoter and *APN1* under its own promoter. The *Bgl*II fragment of the YEpAPN1 plasmid, containing the *APN1* gene under its own promoter, was PCR-amplified and subcloned into the pYES-MAG vector at *Spe*I. pNKY-rad4 is a derivative of pNKY51 (gift of R. Bennett and B. Demple) which contains a *URA3* marker flanked by *Salmonella typhimurium hisG* sequences (45). To create pNKY-rad4,

0.5-0.7 kb PCR products corresponding to the regions flanking *RAD4* were directionally cloned 5' and 3' to the gene blaster fragment of pNKY51.

Cell Survival and Recombination Assays

Non-quantitative determination of MMS sensitivity of strains was evaluated by gradient plate analysis of stationary phase cells as described previously (47). Non-quantitative determination of UV sensitivity was similarly determined by gradient plate analysis of log phase cells.

Cells were grown to log phase (UV studies) or stationary phase (MMS studies and spontaneous recombination studies) in minimal media lacking leucine, serially diluted, and 20 μl aliquots were placed on solid minimal complete media and solid media lacking histidine. For studies of UV effects, cells were exposed to UV in a Stratalinker (Stratagene, Inc., La Jolla, CA) immediately after plating. For studies of MMS effects, strains were plated onto control and MMS containing plates in 20 μl aliquots (up to 36 aliquots per 9 cm^2 square dish). Plates were incubated at 30°C for 1-4 days. Colonies were counted under a 10X dissecting microscope once a standard average colony size was reached. A minimum of 10 colonies total were counted among at least 2 independent aliquots for each measurement. The frequency of *HIS3* recombinants was calculated as colonies/ml on media lacking histidine divided by colonies/ml on minimal complete media.

Nucleic Acid Techniques

Restriction endonucleases were from New England Biolabs (Beverly, MA). Taq polymerase was from Gibco BRL, while Advantage 2 polymerase was from Clontech (Palo Alto, CA). Taq polymerase was used for analytical PCR and Advantage 2 polymerase for PCR subcloning. Plasmid DNA and yeast chromosomal DNA were isolated and manipulated according to standard

procedures (41). Gene disruption cassettes and plasmid DNA were transformed into yeast by electroporation, or by lithium acetate transformation (41). Cell lysates for PCR from single colonies were prepared by lyticase disruption (2.3 μg/ml lyticase, 15 min, 30°C) followed by one freeze thaw cycle at –78°C. More than 25 sets of oligonucleotides (provided by Amitof Co., Allston, MA) were used to create vectors and confirm accurate gene targeting. Sequences are available upon request.

2.4 Results

Overexpression of *S. cerevisiae MAG1* sensitizes cells to both MMS-induced cell death and mitotic recombination.

MMS creates 7MeG (80-85%), 3MeA (9-12%), 3-methylguanine (0.3-0.7%), O^6MeG (0.3%), 7-methyladenine (1.8%), phosphotriesters (0.8%) as well as more minor lesions (13). Although MMS has been shown to induce homologous recombination in *S. cerevisiae* (48), the lesions responsible for this effect were not known. To explore the basis for methylation-induced mitotic homologous recombination, we created diploid strains of *S. cerevisiae* with varied repair and damage tolerance capacities in which recombination can be observed by reconstitution of two mutant copies of the *HIS3* gene. One mutant allele containing a deletion in the 3' end (*his3*-Δ3') precedes another mutant allele containing a 5' deletion (*his3*-Δ5'), while the entire open reading frame of the *HIS3* allele on the other homologue has been deleted (42) (Figure 2-2). The two nonfunctional *his3* alleles flank a *LEU2* cassette and pBR322 sequences. Note

that *LEU2* is lost in ~99% of homologous recombination events (42). Consequently, by growing cells in the absence of leucine, *HIS3* recombinants can be prevented from expanding in culture, thus minimizing variation in recombination frequency. It is noteworthy that diploid strains were used throughout these studies because the effect of Mag1 on MMS-induced recombination was not apparent in haploid cells. The underlying cause(s) for the observed differences between haploid and diploid *mag1* mutant cells are not yet known.

Uncleaved abasic sites inhibit DNA replication (49), and AP endonuclease deficient cells show increased levels of spontaneous homologous recombination, presumably due to replication inhibition caused by increased levels of uncleaved abasic sites (39). Glassner *et al.* have shown that expression of *MAG1* from the galactose-inducible *GAL1* promoter leads to increased abasic site-induced point mutations (32). Thus, we first set out to explore the possibility that overexpression of *MAG1* might induce mitotic homologous recombination by creating replication-blocking uncleaved AP sites. Wild type dipoid cells harboring the *his3* direct repeat and carrying the galactose-inducible pYES-MAG vector (32) were expanded in medium lacking leucine, incubated in the presence of galactose to induce *MAG1* expression, and subsequently plated on solid media containing varying concentrations of MMS. Consistent with previous studies (32), cells overexpressing *MAG1* have increased susceptibility to MMS-induced toxicity compared to their wild type counterparts expressing normal levels of Mag1 (Figure 2-3*A*). This effect is thought to be due to increased levels of potentially toxic BER intermediates (32). Here, we find that cells overexpressing Mag1 also have an increased susceptibility to MMS-induced homologous recombination

(Figure 2-3*B*). These data suggest that Mag1 creates BER intermediates that are more recombinogenic than the lesions being removed by Mag1.

Mag1 not only removes 3MeA, but it also takes out 7MeG, 7-methyladenine and even normal bases (20,50-52). We were therefore interested in the nature of the lesions that were being converted into recombinogenic intermediates by Mag1. To explore this question, we asked if overexpression of a glycosylase that has a more narrow substrate range than Mag1 has the same effect. *E. coli* Tag almost exclusively repairs 3MeA and does not act on 7MeG or normal bases (52,53). Unlike Mag1, overexpression of *tag* does not affect either MMS-induced toxicity or homologous recombination (Figure 2-3) (*Tag* expression, however, does rescue Mag1 deficient cells from MMS toxicity, see below). Thus, the increased levels of recombination observed in cells overexpressing *MAG1* does not appear to be caused by removal of 3MeA. Furthermore, the observation that expression of *tag* does not induce recombination suggests that there are not enough 3MeA lesions in the DNA to cause levels of BER intermediates to rise to toxic or recombinogenic levels. Alternatively, 3MeA itself may be recombinogenic, so that *tag* expression facilitates conversion of a recombinogenic lesion into a recombinogenic repair intermediate.

MAG1 overexpression induces spontaneous homologous recombination in *S. cerevisiae.*

Even in the absence of exogenous methylating agent, high levels of *MAG1* expression leads to a significant increase in mitotic homologous recombination (Figure 2-4*A*). Although the identity of the Mag1 substrates is not known, the ability of Mag1 to take out normal bases, albeit inefficiently, may contribute to this effect (52). Induced expression of *tag*, on the other hand, does not affect

spontaneous homologous recombination (Figure 2-4*A*). Therefore, removal of 3MeA by *MAG1* does not appear to be responsible for the observed increase in spontaneous recombination in cells overexpressing *MAG1*.

MAG1 overexpression may increase the steady state levels of any of several different BER intermediates, including uncleaved abasic sites, single strand breaks, and possibly flaps created if repair synthesis displaces one or more nucleotides (Figure 2-1*C*) (1). To explore the possibility that overexpression of *MAG1* induces homologous recombination by increasing the levels of uncleaved AP sites, we varied the levels of AP endonuclease activity in cells overexpressing *MAG1*. Although wild type and *apn1* mutant cells show similar levels of spontaneous recombination (Figure 2-4*B*), overexpression of *MAG1* in either wild type cells or *apn1* mutant cells has very different effects. Overexpression of *MAG1* in a wild type background causes an ~7 fold increase in the levels of spontaneous recombination, while overexpression of *MAG1* in an *apn1* mutant strain causes a dramatic ~20 fold increase in spontaneous recombination (Figure 2-4*B*). When *APN1* expression is restored, this increase is suppressed, suggesting that uncleaved abasic sites generated by *MAG1* can induce recombination.

Nevertheless, it remains possible that single strand breaks, rather than uncleaved abasic sites, underlie *MAG1*-induced recombination in wild type cells that harbor normal levels of *APN1*. Thus, *APN1* was coexpressed in cells that overexpress *MAG1*. Expression of additional *APN1* activity did not suppress *MAG1*-induced recombination in wild type cells, suggesting that uncleaved AP sites were not responsible for Mag1-induced recombination in cells harboring normal levels of *APN1* (Figure 2-4*B*). We conclude that in the absence of *apn1*, *MAG1* overexpression creates enough uncleaved AP sites to induce recombination, but that wild type levels of AP endonuclease activity may be sufficient to prevent uncleaved AP sites from inducing recombination.

Alternatively, *APN1* expression may be converting recombinogenic uncleaved AP sites into downstream BER intermediates that are comparably recombinogenic. To differentiate among these two possibilities, the effects of *MAG1* overexpression were determined under conditions where cells have increased susceptibility to recombination induced by uncleaved AP sites, as described below.

Disruption of Pol ζ in AP endonuclease deficient cells causes a dramatic increase in spontaneous homologous recombination events (39), which is consistent with a model wherein the ability of Pol ζ to bypass AP sites prevents such uncleaved AP sites from inducing mitotic homologous recombination. To address the question of whether or not uncleaved AP sites are responsible for *MAG1*-induced recombination, we disrupted *REV3*, which is essential for Pol ζ activity (34). Pol ζ does not affect *MAG1*-induced recombination (Figure 2-4*C*). Taken together, the results presented in Figure 2-4 are consistent with a model wherein uncleaved AP sites are not responsible for *MAG1*-induced recombination in cells that express normal levels of AP endonuclease activity. It is likely that the nicks and gaps that are the inevitable consequence of processing such AP sites are responsible for *MAG1*-induced recombination in cells expressing normal levels of AP endonuclease.

Unrepaired methylated bases cause intrachromosomal homologous recombination.

Although it is known that unrepaired UV-induced lesions induce recombination in yeast (54), little is known about potential of unrepaired methylated bases to induce recombination. Previous work shows that mouse cells deficient in 3MeA DNA glycosylase activity have increased susceptibility to 3MeA-induced sister chromatid exchanges (SCEs) (15,55), which is consistent

with the possibility that unrepaired 3MeA induces recombination. However, NER is still active in these mouse cells and it may be the case that NER intermediates, rather than unrepaired 3MeA, are the cause of the observed increase in SCEs. To explore the possibility that unrepaired 3MeA can induce homologous recombination in eukaryotic cells, we created cells deficient in both Mag1 and NER in which recombination can be assayed at the *his3* direct repeat (Table 2-1).

Before determining the frequency of methylation-induced recombination in such strains, it was first necessary to optimize the experimental approach. A standard approach for measuring MMS-induced homologous recombination in *S. cerevisiae* involves culturing cells for 17 h in liquid media that contains MMS and that lacks leucine (to prevent *HIS3* revertants that have lost the *LEU2* allele from expanding in culture) (48). After 17 h, the frequency of *HIS3* revertants/viable cell is determined by a colony forming assay. However, because MMS breaks down in water, the effective concentration of MMS is expected to decrease during the 17 h exposure time. Given that eukaryotic cells lacking 3MeA DNA glycosylase activity are more prone to MMS-induced cell cycle arrest than are wild type cells (15), it is possible that certain concentrations of MMS may be high enough to induce cell cycle arrest in repair deficient cells, while having no effect on the doubling time of wild type cells. Since the recombination frequency is dependent on the total number of viable cells, a delayed cell division would reduce the culture density so that it would appear that when compared to wild type cells, the repair deficient strain has an increased number of recombinants/survivors. Cells were therefore exposed to MMS or UV only after being plated, so that the number of recombination events is evaluated by colony number (rather than by the total number of *HIS3* cells), which is independent of doubling time.

Doubly BER and NER deficient diploid cells were created by gene disruption, plated at various dilutions on control and selective media, and exposed to UV light or MMS. As expected from previous studies of NER mutant cells (54), the *rad4* mutant cells have increased sensitivity to UV-induced killing and increased susceptibility to UV induced-homologous recombination (Figure 2-5,*A* and *B*). The additional disruption of *MAG1* expression in the *rad4* null cells has no effect, which is consistent with the inability of Mag1 to act on UV damage. When exposed to methylation damage, cells lacking *MAG1* expression have increased susceptibility to both methylation-induced toxicity and recombination (Figure 2-5,*C* and *D*). To determine if NER intermediates are causing recombination in the *mag1* mutant cells, we compared methylation-induced recombination in *mag1* and *mag1 rad4* double mutant cells. Disruption of NER (*rad4* mutants) did not suppress methylation-induced recombination in *mag1* cells, which suggests that NER intermediates are not causing recombination in cells lacking Mag1. On the contrary, *mag1 rad4* cells have increased susceptibility to methylation-induced recombination compared to *mag1* mutant cells (Figure 2-5*D*), which is consistent with a model wherein both Mag1 and NER prevent unrepaired methylated bases from inducing recombination.

It is noteworthy that recombination tends to rise at the same doses of MMS that cause significant toxicity (Figure 2-5,*C* and *D*). While it is often observed that the frequency of homologous recombination increases at lethal levels of DNA damaging agents, in nature, cells are often exposed to sublethal levels of damaging agents. We were therefore interested in the possibility that Mag1 might prevent methylation-induced recombination even at sublethal levels of exposure. Figure 2-6*A* and *B* show that cells lacking Mag1 have an increased susceptibility to methylation-induced recombination, even at doses of methylating agent that do not affect survival. This effect is even more apparent in cells lacking

both Mag1 and NER. We therefore conclude that Mag1 excises potentially recombinogenic lesions from the genome of eukaryotic cells.

Since methylated bases can be produced endogenously in eukaryotic cells at measurable levels (*e.g.*, (15)), we also examined whether or not Mag1 prevents spontaneous homologous recombination. Although cells lacking NER show a significant increase in susceptibility to spontaneous mitotic recombination (as has been shown previously (56)), disruption of *MAG1* expression has no effect on spontaneous mitotic recombination, either on its own or in combination with an NER deficiency (data not shown). These results indicate that the levels of spontaneous damage repaired by Mag1 are not sufficient to induce detectable changes in the frequency of homologous recombination. The basis for the increased spontaneous recombination in NER deficient cells is not yet known.

Unrepaired 3MeA is a primary cause of methylation-induced homologous recombination in excision repair-deficient cells.

From the studies of MMS-induced recombination presented above, we cannot conclude that a particular lesion is the primary cause of homologous recombination in Mag1 deficient cells, because MMS creates many different types of methylated bases, several of which can be repaired by Mag1.

E. coli Tag 3MeA DNA glycosylase is highly specific for 3MeA (53). To determine if unrepaired 3MeA can cause homologous recombination, cells were induced to express *tag* using the pYes-Tag vector (32). Expression of *tag* virtually eliminates MMS-induced recombination in *mag1 rad4* cells at both non-toxic (Figure 2-6, *C* and *D*) and toxic levels of exposure (Figure 2-6, *E* and *F*), providing very strong evidence that unrepaired 3MeA lesions are indeed recombinogenic. Consistent with these results, induced expression of the yeast O^6MeG repair methyltransferase had no effect on MMS-induced recombination in

the *mag1 rad4* cells (data not shown), showing that the NER deficiency does not make cells vulnerable to recombination induced by O^6MeG lesions.

Pol ζ modulates methylation-induced recombination in excision repair deficient cells.

Pol ζ suppresses AP site-induced mitotic homologous recombination, presumably by facilitating bypass of replication-blocking AP sites (39). To explore the possibility that Pol ζ plays an analogous role in modulating the effects of 3MeA lesions, *rev3* mutations were introduced into wild type and *mag1 rad4* strains (Table 2-1). Figure 2-7A clearly shows that Rev3 helps prevent MMS-induced toxicity in wild type cells, which is consistent with previous studies (26). Here we show that at toxic levels of exposure, *rev3* mutant cells also have increased susceptibility to methylation damage-induced homologous recombination (Figure 2-7*B*).

In cells lacking both Mag1 and NER, disruption of *REV3* causes a concomitant decrease in survival and increase in methylation-induced recombination (Figure 2-7, *A* and *B*). The deficiency in Pol ζ in the *mag1 rad4 rev3* cells causes significant toxicity at about half the concentration of MMS required to elicit a similar response in the *mag1 rad4* cells. Since AP sites are not being generated by Mag1 in *mag1* mutant strains, these results are consistent with a model wherein Pol ζ helps prevent the toxic and recombinogenic effects of 3MeA by facilitating translesion replication. Interestingly, disruption of *REV3* does not make *mag1 rad4* cells more sensitive to methylation-induced recombination at lower levels of exposure (Figure 2-7*C*). Indeed, the *mag1 rad4 rev3* cells show decreased susceptibility to methylation-induced recombination compared to their *mag1 rad4* counterparts when exposed to non-toxic doses of

MMS. Thus, the effects of Pol ζ status are highly dose-dependent. Possible explanations for these observation are discussed below.

2.5 Discussion

In this study, we have explored the genetic basis for methylation-induced mitotic homologous recombination and the interplay between BER, NER, replicative bypass, and homologous recombination. Although elegant studies exploiting meganucleases that introduce site-specific double strand breaks have made it abundantly clear that double strand breaks created in such a direct repeat are highly recombinogenic (*e.g.*, (57,58)), it is not yet clear how base damage causes homologous recombination. Here, we have explored how the BER pathway affects susceptibility of cells to homologous recombination. We have found that cells lacking Mag1 are highly sensitive to methylation-induced recombination and that disruption of NER sensitizes *mag1* mutant cells to methylation-induced recombination, which clearly indicates that NER intermediates are not causing recombination in *mag1* cells. Furthermore, expression of *E. coli tag* prevents methylation-induced recombination in the *mag1 rad4* cells, providing very strong evidence that unrepaired 3MeA lesions are responsible for the observed increase in recombination. Given that Mag1 is able to remove a broad spectrum of lesions, there has been a great deal of interest in identifying those Mag1 substrates that are the basis for the evolutionary conservation of Mag1 activity in nearly every species. The results presented here make a case for Mag1 serving to remove potentially recombinogenic 3MeA lesions. Although a deficiency in Mag1 has no detectable effect on spontaneous

recombination, it is possible that exposure to conditions that cause methylation damage may play a role in the evolutionary pressure to retain Mag1.

Although too little Mag1 is problematic, overproduction of Mag1 results in increased susceptibility to spontaneous homologous recombination. This may be due to the ability of uncleaved abasic sites to inhibit DNA replication (49), or it may be that other downstream BER intermediates, such as single strand breaks, are recombinogenic. Studies by Swanson *et al.* clearly show that Pol ζ plays an important role in suppressing recombination induced by uncleaved abasic sites in AP endonuclease deficient cells (39).

Here we show that while overexpression of *MAG1* induces spontaneous homologous recombination, neither disruption of *REV3* (which is required for Pol ζ-mediated translesion replication), nor increased levels of AP endonuclease activity have any effect on Mag1-induced recombination. Thus, although uncleaved AP sites can induce recombination, this induction is dependent upon the combined conditions of increased expression of a DNA glycosylase that is able to take out normal bases and simultaneous disruption of expression of the major AP endonuclease (shown here) or by the combined disruption of multiple enzymes able to cleave such AP sites (shown by Swanson, *et al.*)(39). We propose that even under conditions of imbalanced BER initiated by overexpression of *MAG1*, normal levels of AP endonucleases may be sufficient to prevent uncleaved abasic sites from playing a major role in inducing recombination in *S. cerevisiae*. Instead, base damage may become recombinogenic by its conversion into single strand breaks, which cannot be bypassed by translesion polymerases. Indeed, Galli and Schiestl have shown that single strand breaks site-specifically introduced into a direct repeat induce recombination in a replication-dependent fashion (59). In addition, it has been proposed that persistent single strand breaks, which are thought to be converted into recombinogenic double strand ends during

DNA replication (Figure 2-1), are responsible for the high levels of recombination observed in ligase mutants (60). Thus, we propose that downstream BER intermediates, such as single strand breaks, are most likely responsible for the observed effects of Mag1 overexpression on recombination.

In this study, we also explored the role of Pol ζ in modulating the effects of unrepaired methylation damage by disrupting *REV3* in cells lacking excision repair. When cells lacking Mag1 and NER are exposed to toxic levels of methylation damage, *REV3* prevents methylation-induced recombination. These results are consistent with a model wherein Pol ζ is able to replicate past 3MeA lesions. However, at lower levels of methylation damage, *REV3* expression does not offer any protection against recombination, and if anything, *mag1 rad4 rev3* cells are more resistant to methylation-induced recombination than are *mag1 rad4* cells. This apparent paradox can be explained by a model that takes into consideration the presence of multiple polymerases with varied ability to replicate past damaged bases (61-63). It is not yet clear how polymerases gain access to a damaged template, but it seems likely that eliminating one polymerase may make it possible for other polymerases to gain access. In the absence of Pol ζ, unrepaired 3MeA lesions may become accessible to other DNA polymerases, such as Polymerase η (64). At low levels of exposure, another polymerase may be more effective than Pol ζ at replicating past 3MeA, and thus be able to suppress homologous recombination. As the dose increases, this polymerase may become saturated, making the cells extremely sensitive to both 3MeA-induced toxicity and recombination. The results presented in this work clearly show that the effects of Rev3 are highly dose-dependent; further studies are necessary to elucidate the relationships among those polymerases that are able to replicate past damage.

An elegant study by Kadyk and Hartwell provides strong evidence that replication is required for unrepaired UV lesions to induce recombination (54).

Furthermore, studies by Galli and Schiestl show that MMS induced recombination is suppressed in cells arrested in G1 and that the frequency of UV-induced recombination increases with time in S phase (65). Several models have been proposed for mechanisms by which an encounter between a replication fork and a replication-blocking lesion causes recombination at a direct repeat, and these are briefly summarized here. If the lesion is in the lagging strand, a daughter strand gap would be created that is either itself recombinogenic (as shown in Figure 2-1A) or is converted into a recombinogenic double strand break (not shown). If the lesion is in the leading strand, and lagging strand synthesis continues, single stranded DNA in the leading strand may promote recombination with the sister chromatid. Alternatively, an encounter with a lesion in the leading strand template may lead to replication fork breakdown, creating a recombinogenic double strand end (66-68) (Figure 2-1*B*). Finally, studies by Lovett *et al.*, suggest that replication slippage can convert direct repeats into single copies in *E. coli* (69,70). However, there is no direct evidence that slippage can happen between repeats as long as those used in this study, particularly when there is an intervening ~2 kb cassette between the repeats. Furthermore, UV-induced recombination at this *his3* direct repeat is *RAD52* dependent (R. Schiestl, personal communication), which is consistent with models that involve homologous recombination rather than replication slippage. Taken together, the results of this work and previous studies are consistent with a model wherein unrepaired 3MeA lesions inhibit DNA replication, which leads to single stranded regions and/or double strand ends that are subject to recombinational repair.

Previous studies suggest that single strand annealing (SSA) is the predominant mechanism underlying recombination at the *his3* repeat (42). Single strand annealing involves a double strand break positioned between the repeats, followed by reannealing of the flanking sequences. Since MMS does not directly

create two-ended double strand breaks, we do not think it is likely that SSA is the predominant mechanism of recombination in our studies. Instead, we think it more likely that the repeats are recombined during replication fork reconstitution, as described above. It is noteworthy that SSA and replication fork reconstitution would be indistinguishable at a direct repeat such as the one used in these studies.

In this work, we have explored the role of Mag1 in modulating mitotic homologous recombination. Together, the Mag1 initiated BER pathway and the NER pathway normally help prevent 3MeA-induced recombination events, but a very high level of expression of Mag1 (which is able to remove normal bases) causes the accumulation of recombinogenic BER intermediates. Thus, in wild type cells exposed to high concentrations of methylation damage, both unrepaired 3MeA and BER intermediates are potentially recombinogenic. Indeed, neither expression of Tag nor expression of Apn1 (data not shown) significantly suppressed MMS-induced recombination in wild type cells, which indicates that ridding the cell of any residual unrepaired 3MeA or uncleaved AP sites only causes an increase in other recombinogenic downstream intermediate(s). Thus, it seems that the BER capacity of *S. cerevisiae* is optimal for tolerance of low levels of damage, and when the damage levels become too high, both unrepaired lesions and BER intermediates can become problematic to genomic stability. In conclusion, the benefit of removing a particular lesion must be weighed against the cost of creating BER intermediates. The optimal balance of these forces depends upon both the quality and the quantity of the lesions and the relative proportions of the enzymes in the BER pathway.

2.6 Acknowledgements

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Table 2-1

S. cerevisiae strains used to study the role of the Mag1 3MeA DNA glycosylase in modulating susceptibility to homologous recombination.

Strain	Genotype	Reference
Haploids		
RSY6	*MATa HIS3::pRS6 leu2-3,112 ura3-52 trp5-27 ade2-40 ilv1-92 arg4-3*	R. Schiestl (42)
CHY7	same as RSY6 except *apn1Δ::hisG*	This study
CHY8	same as RSY6 except *mag1Δ::kan^r*	This study
CHY9	same as RSY6 except *rad4Δ::hisG*	This study
MRY11	same as RSY6 except *rev3Δ::hisG*	This study
CHY10	same as RSY6 except *mag1Δ::kan^r rad4Δ::hisG*	This study
MRY12	same as RSY6 except *mag1Δ::kan^r rad4Δ::hisG rev3Δ::hisG*	This study
Y433	*MATα his3-Δ200 leu2-Δ98 ura3-52 ade2-101 lys2-801*	R. Schiestl (42)
CHY34	same as Y433 except *apn1Δ::hisG*	This study
CHY35	same as Y433 except *mag1Δ::kan^r*	This study
CHY36	same as Y433 except *rad4Δ::hisG*	This study
MRY38	same as Y433 except *rev3Δ::hisG*	This study
CHY37	same as Y433 except *mag1Δ::kan^r rad4Δ::hisG*	This study
MRY39	same as Y433 except *mag1Δ::kan^r rad4Δ::hisG rev3Δ::hisG*	This study
Diploids		
RS112	RSY6 X Y433	R. Schiestl (42)
CHY113	CHY7 X CHY34	This study
CHY114	CHY8 X CHY35	This study
CHY116	CHY9 X CHY36	This study
MRY113	MRY11 X MRY38	This study
CHY115	CHY10 X CHY37	This study
MRY114	MRY12 X MRY39	This study

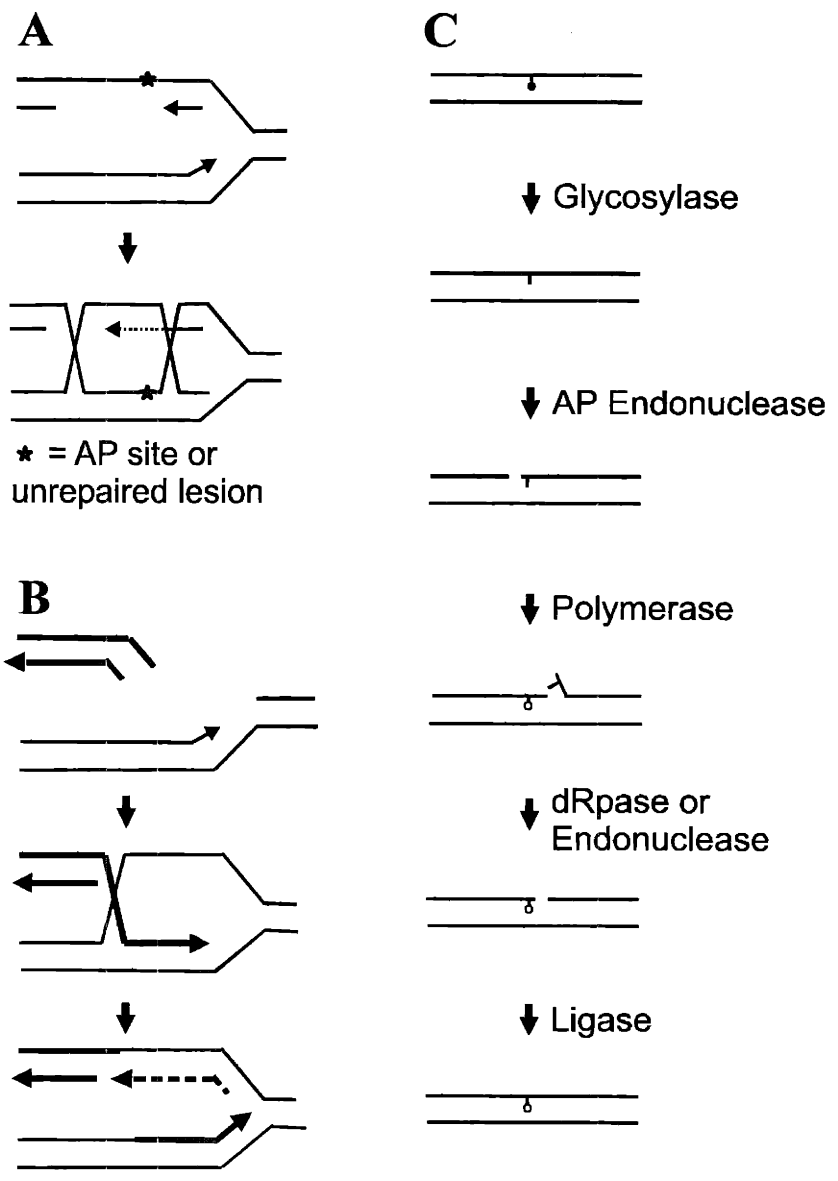

Figure 2-1. (A and B) Possible consequences of a replication fork encountering unrepaired lesions or BER intermediates. (A) Repair of a daughter strand gap via sister chromatid exchange (endonucleolytic cleavage of the single stranded DNA gap may initiate this process; not depicted here). (B) A single strand break, or possibly an unrepaired lesion, causes collapse of the replication fork, which can then be reinserted via homologous recombination. Note that endonucleolytic processing to create a single stranded 3' overhang precedes homology searching. Resolution of the resulting Holliday junction restores the replication fork in break-induced replication [Paques, 1999 #2736]. (C) Schematic representation of the BER pathway. In this version, only short-patch BER initiated by a monofunctional glycosylase that lacks AP lyase activity (*e.g.*, Mag1) is shown.

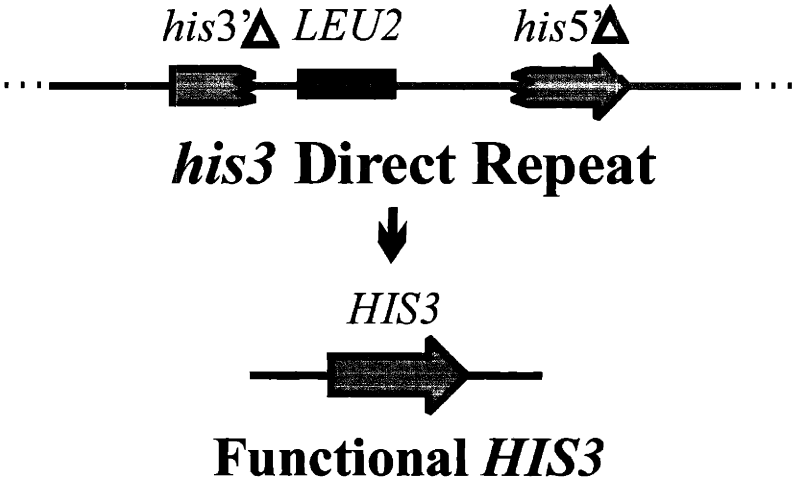

Figure 2-2. Schematic representation of the *his3* direct repeat used to detect recombination. Two nonfunctional *his3* alleles, one containing a deletion of the 3' end of the *HIS3* gene (*his3'*Δ) and the other containing a deletion of the promoter of the gene (*his5'*Δ), flank a *LEU2* cassette and pBR322 sequences. The two *his3* deletion alleles share approximately 400bp of homology, thus a homologous recombination event between the two mutant alleles can restore a functional *HIS3* gene.

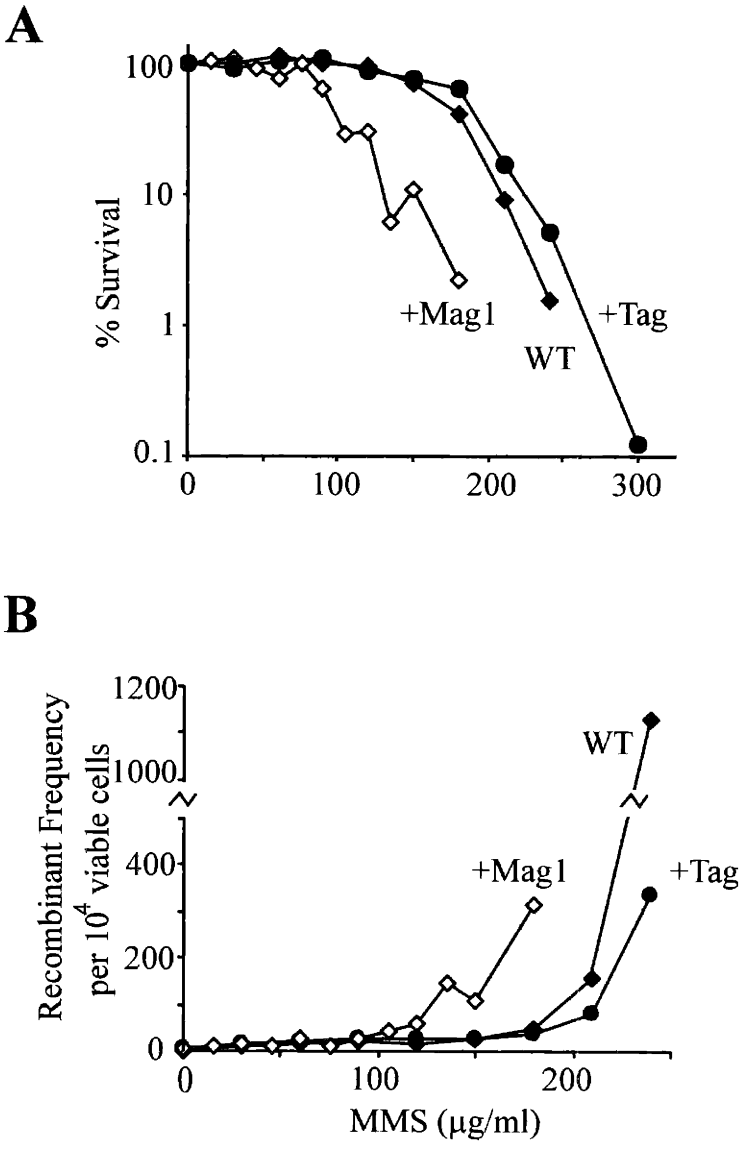

Figure 2-3. Effects of *MAG1* overexpression on MMS-induced cell death and recombination. RS112 diploid yeast were induced to express *S. cerevisiae MAG1* 3MeA DNA glycosylase (open diamonds) or *E. coli tag* 3MeA DNA glycosylase (closed circles) by culturing stationary phase cells in galactose containing media for 2.5 h prior to exposure to MMS on solid media containing galactose. Wild type cells carry the pYES control vector (WT; closed diamonds). Percent survival was calculated from a colony forming assay and recombination frequency is plotted as the number of *HIS3* revertant colonies per viable cell. Representative data from two independent experiments are shown.

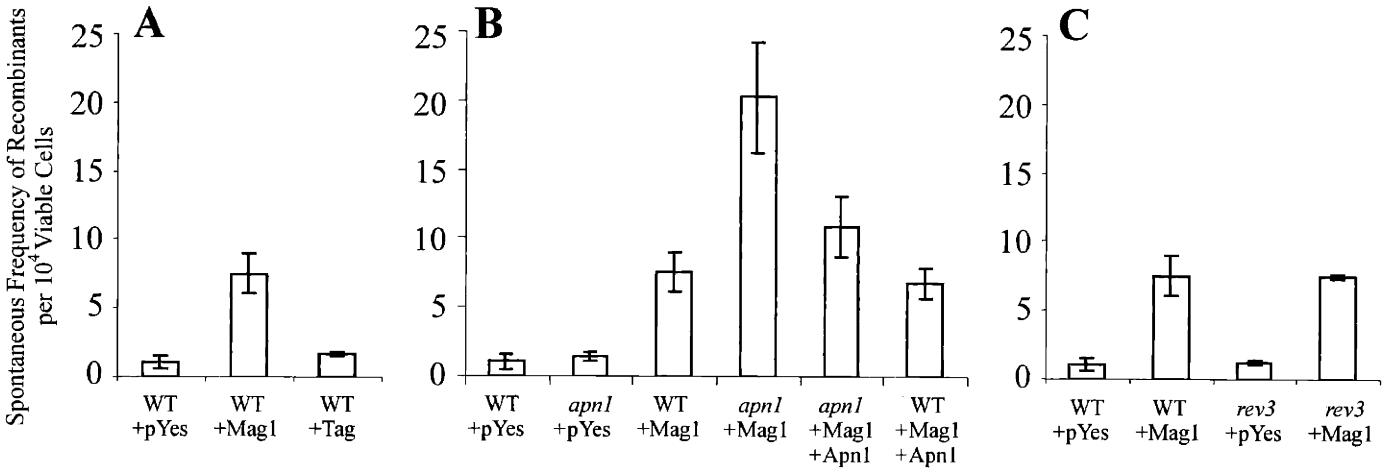

Figure 2-4. Frequency of spontaneous recombination in yeast with modified capacities for BER. Diploid strains carry the indicated mutations and express *S. cerevisiae MAG1* or *E. coli tag* from a galactose inducible promoter. When coexpressed with *MAG1, S. cerevisiae APN1* is under its own promoter. Approximately 1000 cells were expanded to stationary phase in 3-5 independent cultures (media lacking leucine was used to suppress expansion of recombinant cells). Stationary phase cells were cultured under inducing conditions for 2.5 h and subsequently diluted onto galactose plates to determine viable cell number and spontaneous recombination frequency. Consistent results were observed in at least three independent experiments. The error bars indicate one standard deviation where each independent culture is weighted equally. (A) Comparison of the effects of overexpression of *tag* and *MAG1*. (B) The effects of varied expression of *MAG1* and *APN1*. (C) The effects of *REV3* status on spontaneous recombination and *MAG1*-induced recombination. The same data for wild type cells (WT and WT +pYes) and wild type cells induced to express *MAG1* (WT +Mag1) are shown in A, B, and C to facilitate comparisons.

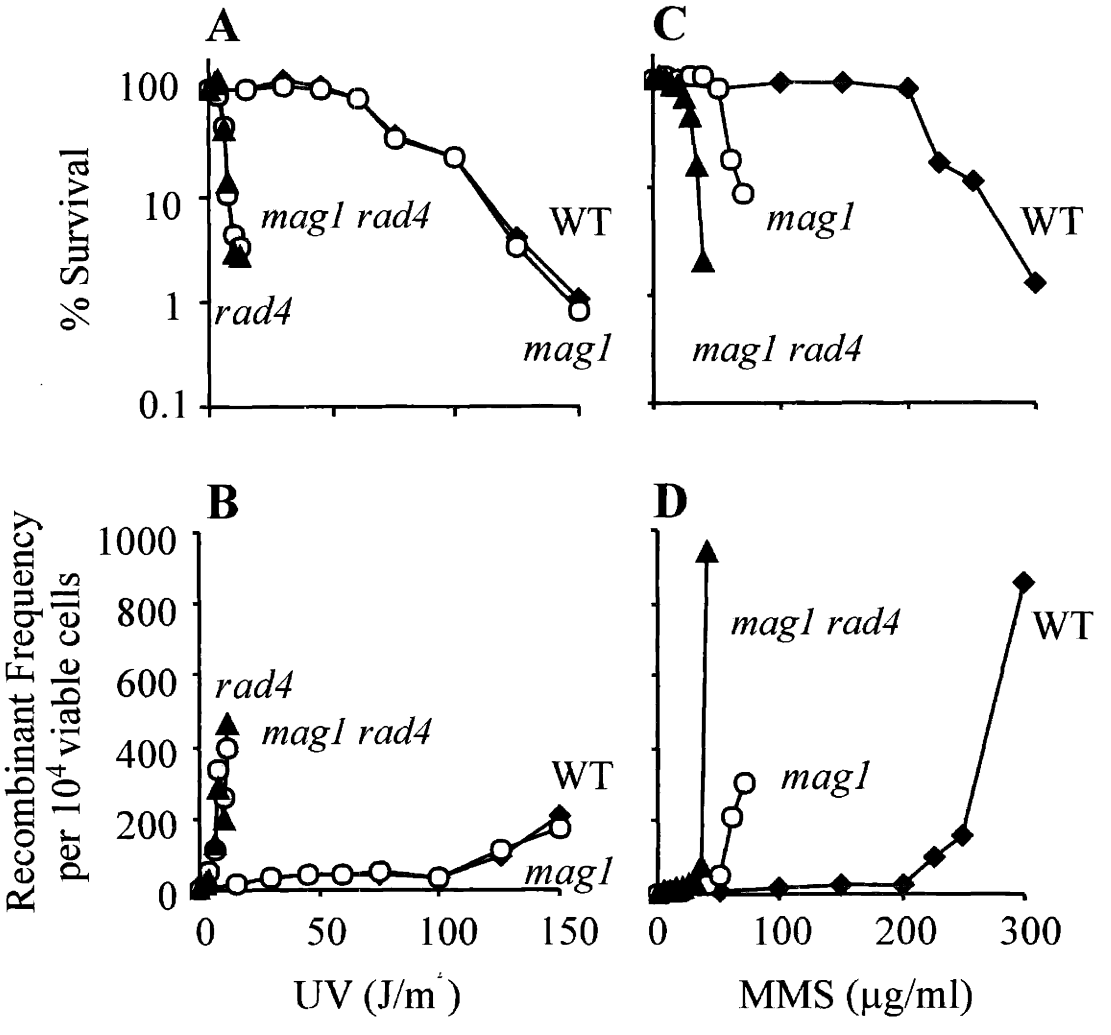

Figure 2-5. UV and MMS-induced cell death and recombination in 3MeA DNA glycosylase (*mag1*) and/or NER (*rad4*) mutant yeast. Diploid cells deficient in Mag1 activity (*mag1*) and/or nucleotide excision repair (*rad4*) were exposed to either UV (A and B) or MMS (C and D) and their sensitivity to DNA damage-induced killing and recombination were compared to wild type cells. The strains are wild type (WT; closed diamonds), *mag1* (open circles), *rad4* (shaded circles), *mag1 rad4* (closed triangles). For UV experiments, log phase cells were plated at various dilutions and exposed to the indicated doses of UV. For MMS experiments, cells were grown to stationary phase and plated on solid media containing the indicated concentrations of MMS. Experiments were repeated a minimum of three times and the data presented here show a representative curve.

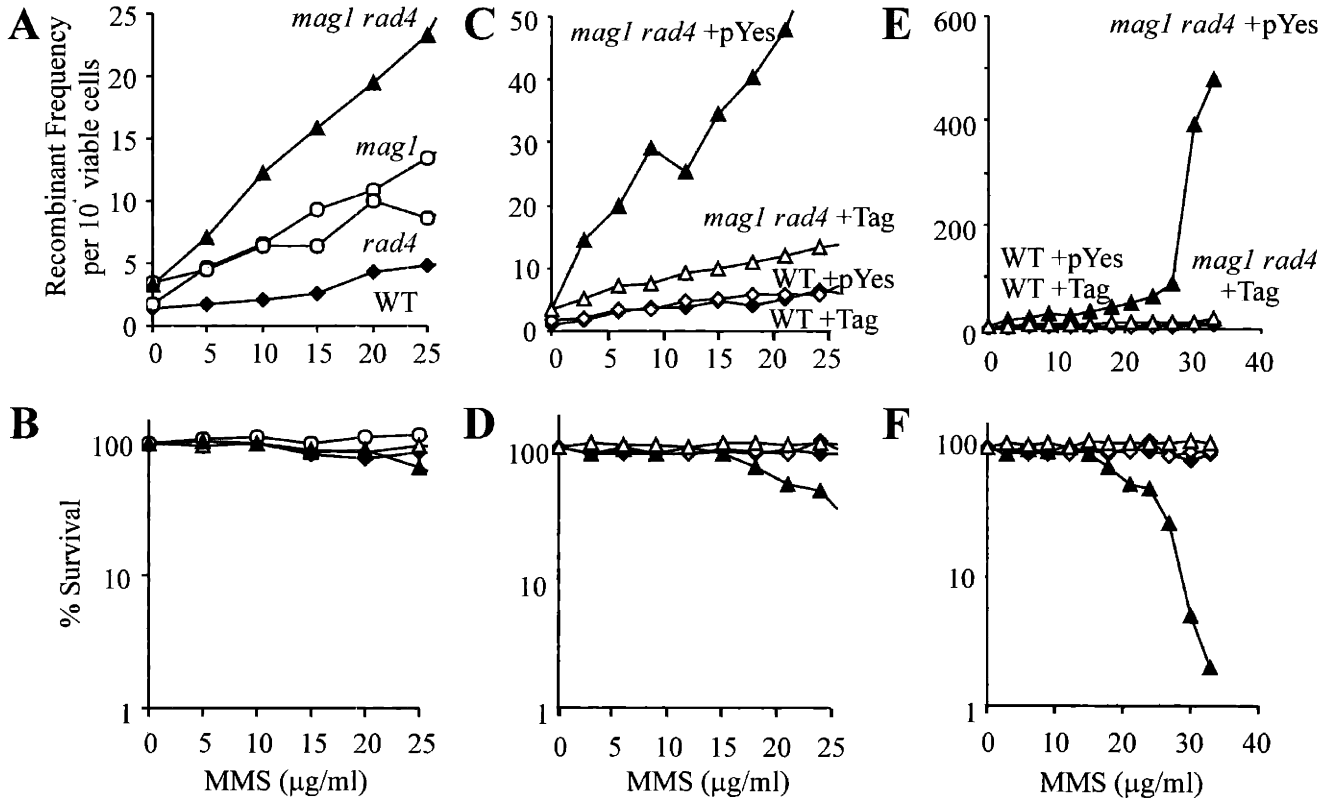

Figure 2-6. Effects of deficiencies in 3MeA DNA glycosylase activity (*mag1*) and/or NER (*rad4*) and expression of *E. coli tag* 3MeA DNA glycosylase on MMS-induced recombination and toxicity. (A) The frequency of *HIS3* recombinants is shown relative to the number of viable cells for wild type cells (WT; closed diamonds) and the following repair deficient strains: *rad4* (shaded circles), *mag1* (open circles), and *mag1 rad4* (closed triangles). (B) At the indicated doses of MMS, there is essentially 100% survival for all genotypes. (C-F) Tag suppresses both MMS-induced toxicity and recombination in *mag1 rad1* cells exposed to sublethal (C and D) and lethal (E and F) levels of MMS. The strains are: wild type *S. cerevisiae* carrying the pYes control vector (solid diamonds); wild type cells expressing *tag* (shaded diamonds); *mag1 rad4* carrying pYes (solid triangles); *mag1 rad4* expressing *tag* (open triangles). Stationary phase diploid cells were plated on solid media containing the indicated concentrations of MMS. For *tag* induction conditions, see "Materials and Methods." Experiments were repeated a minimum of three times and the data presented are a representative curve.

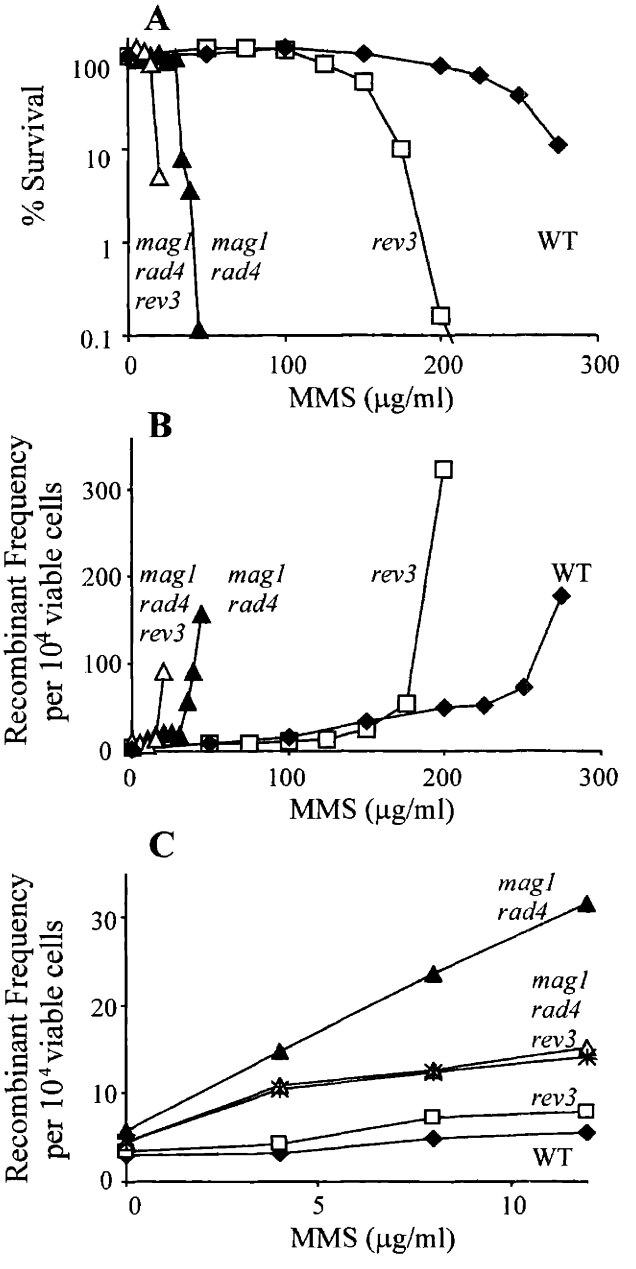

Figure 2-7. Modulation of methylation-induced toxicity and homologous recombination by Pol ζ. Diploid *S. cerevisiae* carrying the indicated mutations were grown to stationary phase and various dilutions were plated onto either SC media or media lacking histidine. The strains are wild type (WT; closed diamonds), *rev3* (open squares), *mag1 rad4* (closed triangles), *mag1 rad4 rev3* (open triangles and asterisks). (A and B) The effects of Mag1, Rad4, and Rev3 on MMS-induced toxicity and *HIS3* reversion. This experiment was repeated two times, and a representative curve is shown. (C) MMS-induced *HIS3* reversion frequencies at non-toxic doses. This experiment was repeated three times and a representative curve is shown.

Chapter III

Spontaneous Mitotic Homologous Recombination at an *EYFP* Direct Repeat in Transgenic Mice

Chapter 3

3.1 Abstract

A transgenic mouse has been created that provides a powerful tool for revealing genetic and environmental factors that modulate mitotic homologous recombination. The fluorescent yellow direct repeat (FYDR) mice described here carry two different copies of expression cassettes for truncated coding sequences of the enhanced yellow fluorescent protein (EYFP), arranged in tandem. Homologous recombination between these repeated elements can restore full length *EYFP* coding sequence to yield a fluorescent phenotype, and the resulting fluorescent recombinant cells are rapidly quantifiable by flow cytometry. Analysis of genomic DNA from recombined FYDR cells shows that this mouse model detects gene conversions, and based upon the arrangement of the integrated recombination substrate, unequal sister chromatid exchanges and repair of collapsed replication forks are also expected to reconstitute *EYFP* coding sequence. The rate of spontaneous recombination in primary fibroblasts derived from adult ear tissue is 1.3 ± 0.1 per 10^6 cell divisions. Interestingly, the rate is ~10 fold greater in fibroblasts derived from embryonic tissue. We observe an ~15 fold increase in the frequency of recombinant cells in cultures of ear fibroblasts when exposed to mitomycin C, which is consistent with the ability of interstrand crosslinks to induce homologous recombination. In addition to studies of recombination in cultured primary cells, the frequency of recombinant cells present in skin was also measured by direct analysis of disaggregated cells. Thus, the FYDR mouse model can be used for studies of mitotic homologous recombination, both *in vitro* and *in vivo*.

3.2 Introduction

Human cells incur approximately 10^6 base lesions per day (1), many of which inhibit DNA replication and/or induce DNA strand breaks. Homology directed repair provides an important strategy for preventing toxicity caused by such DNA lesions. More specifically, when the replication machinery stalls, recombination between sister chromatids can replace the damaged template with an undamaged copy (2). In addition, should a replication fork collapse to form a double strand break (*e.g.*, due to an encounter with a single strand break in the template DNA), the fork can be repaired by homology-directed reinsertion of the broken end (3-5). Thus, the frequency of recombination reflects the levels of certain types of DNA damage.

Repair and lesion avoidance pathways that involve homology-searching are integral to DNA replication (3-7). It is estimated that ~10 double strand breaks are formed each time the mammalian genome is replicated (3), and proteins that are essential for homologous recombination (*e.g.*, Rad51) are also essential for life (8-10). Most of the time, sequences are aligned perfectly and flanking sequences are not exchanged. However, misalignments may result in deletions, and exchanges between homologous chromosomes may lead to loss of heterozygosity, events that are known to promote cancer (11-14).

A useful approach to studying recombination is to engineer two mutant expression cassettes in a direct repeat and assay for restoration of wild type gene expression in cultured cells (2,15,16). For example, using direct repeat substrates, it has been shown that homologous recombination repairs 30-50% of double strand breaks in mammalian cells (17). Furthermore, direct-repeats have been used to demonstrate that

many tumor suppressor genes, including *BRCA1*, *BRCA2*, *ATM*, *WRN*, *MSH2*, and *NBS1* modulate spontaneous mitotic homologous recombination in mammalian cells (18-23). Despite their proven efficacy, few animal models have been created that exploit direct repeat substrates (24-26) and none are suitable for the detection of mitotic homologous recombination events in somatic tissues of mature animals. Consequently, little is known about the relative susceptibility of different cell types to homologous recombination, or how DNA repair and tumor suppressor genes influence homologous recombination in mammals (27).

Here, we describe the fluorescent yellow direct repeat (FYDR) mouse model in which cells become fluorescent following homologous recombination at a direct repeat. The spontaneous frequency and rate of homologous recombination in primary cells from these mice can be determined using flow cytometry. Interestingly, we found that the rate of spontaneous recombination is ~10 fold greater in embryonic fibroblasts compared to fibroblasts from ear tissue. Furthermore, recombinant cells within skin can be quantified without culturing cells *ex vivo*. Thus, the FYDR mice provide an effective means for studying homologous recombination in primary somatic cells, both *in vitro* and *in vivo*.

3.3 Materials and Methods

Enzymes, Oligonucleotides, Plasmids

Restriction enzymes were from New England Biolabs (Beverly, MA). Advantage II polymerase (Clontech, Inc.) was used for preparative PCR. Oligonucleotides were from Amitof, Inc. (Allston, MA). Vectors used in these studies were pEYFP-N1 (Clontech,

Inc.), pCX-EGFP (Gift of M. Okabe (28) and pBluescript (Stratagene). Primer sequences are available upon request.

Construction of the Recombination Substrate

Truncated coding sequences (lacking either 96 bp from the 5' end or 42 bp from the 3' end) were PCR amplified from pEYFP-N1 using primers carrying synthetic *Apo*I sites. *Apo*I digested PCR products were subcloned into *EcoR*1 digested pCX-EGFP (28) (coding regions were sequenced). In pCX-5'*eyfp*, *Sal*I was converted to *Not*I, and an adaptor containing *Sal*I-*Xho*I-*Hind*III-*Not*I was inserted between *BamH*I sites (*BamH*I sites were not conserved). The 3'*eyfp* cassette was released using *SalI/Hind*III and subcloned into *Xho*I/*Hind*III digested pCX-5'*eyfp* to create a direct repeat.

Creation of Transgenic Mice

The direct repeat *Not*I fragment was purified using QiaexII (Qiagen). Pronuclear injection into C57BL/6 fertilized eggs was performed by the Brigham and Women's Transgenics Facility (Boston, MA).

Isolation of Ear Fibroblasts and Ventral Skin Cells

Cells were isolated essentially as previously described (29). Briefly, washed skin was minced and incubated in dissolution media containing collagenase D and dispase neutral protease. After 45 min at 37°C, two volumes of fibroblast media was added (DMEM; 15% fetal bovine serum; penicillin [100 units/ml], streptomycin [100 μg/ml]; 5 μg/ml Fungizone [Gibco/BRL]). After 24 h at 37°C and 5% CO_2, cells were triturated,

filtered (70 μm; Falcon) and either analyzed by flow cytometry (disaggregated skin cells) or seeded into dishes (primary fibroblasts).

Isolation and Transformation of Primary Mouse Embryo Fibroblasts (MEFs)

Day 14.5 embryos were isolated, minced and resuspended in 15 ml trypsin/EDTA. After 25 min at 37°C with agitation, cells were pelleted, resuspended in fibroblast media (see above), and seeded in 55 cm^2 plates. To transform cells, primary MEFs were transfected with a vector to express SV40 large T antigen, as previously described (30).

Flow Cytometry

Pelleted cells were resuspended in OptiMEM (Gibco/BRL) and passed through a 70 um filter (Falcon) prior to analysis using a Becton Dickenson FACScan flow cytometer (excitation 488 nm, argon laser) or sorting using a MoFlo cytometer (Cytomation, Inc.; excitation 488 nm, argon laser; emission 580/30). Live cells were gated using forward and side scatter.

Calculation of Spontaneous Recombination Frequency and Rate in Primary Fibroblasts

~10^4 cells were seeded into ~24 independent cultures. For frequency studies, cultures were harvested once the density reached 10^6 cells/dish. Cultures in which the frequency of recombination indicated the presence of a fluorescent cell in the initial

population were excluded from analysis. The method of p_0 was used to calculate the rate and the mutation rate per cell division was determined as previously described (31,32).

Southern Analysis

^{32}P labeled *EYFP* coding sequence probes were prepared by random priming (NEBlot; New England Biolabs, Beverly, MA) and blots were processed using standard procedures (33). Blots were analyzed on a Storm 840 Molecular Dynamics Phosphorimager.

Subcloning Genomic DNA from FYDR Mice

Genomic DNA from FYDR mice was digested with *BamHI* and cloned into a ZAP Express λ library following manufacturers instructions (Stratagene). The library was screened with *EYFP* coding sequences.

Quantification of DNA Damage-Induced Recombination

Primary ear fibroblasts derived from multiple FYDR mice were pooled and 3 x 10^6 cells were seeded onto 148 cm^2 dishes. After 24 h, triplicate samples were exposed to the indicated doses of mitomycin C in DMEM for 1 h. After 72 h (~3.25 population doublings in control cells), samples were analyzed by flow cytometry. Population growth, relative to untreated control cells, was determined by counting the number of viable cells/dish.

3.4 Results

Creation of FYDR mice.

To create a substrate for detecting recombinant cells in transgenic mice, essential sequences (34) were deleted from either end of the enhanced yellow fluorescent protein (*EYFP*) coding sequence to create 5'*eyfp* and 3'*eyfp*. These truncated coding sequence expression cassettes are flanked upstream and downstream by identical promoter, intron and polyadenylation signal sequences. The flanking sequences were selected based on their proven efficacy for high level expression in most mouse tissues (28). A direct repeat was created by subcloning 5'*eyfp* and 3'*eyfp* in tandem, and the resulting 6 kb *Not*I fragment (Fig. 1*A*) was injected into mouse pronuclei to create transgenic mice. PCR analysis using primers that anneal to *EYFP* coding sequences revealed that two out of fifteen candidate transgenic mice carried at least a portion of the injected DNA (data identifying one candidate is shown in lane 7 of Fig. 1*B*).

Quantification of recombinant cells is possible only if the injected DNA is integrated into a locus that is expressed and that undergoes spontaneous recombination at a detectable frequency. To determine if mice that were positive by PCR had integrated the DNA at such a locus, we used flow cytometry to test for the presence of recombinant fluorescent cells within cultures of primary ear fibroblasts (Fig. 2*A*). When the relative fluorescence intensity per cell is plotted, normal unstained fibroblasts show a range of natural fluorescence intensities. As a positive control, we analyzed fluorescent cells from a mouse that expresses *EGFP* (28). Given that EGFP and EYFP are indistinguishable under these conditions (data not shown), we delineated the R2 region to capture almost all of the *EGFP* expressing cells. Fluorescent cells were detected both by flow cytometry

(Fig. 2*A*, right) and fluorescence microscopy (Fig. 2*B*) in cultures derived from one of the candidates. This founder was crossed with wild type C57Bl/6 mice to establish the FYDR mouse line. The integrated DNA is inherited in a Mendelian fashion (51% of offspring carry the integrated DNA; $n = 129$), suggesting that the integrated DNA does not significantly affect viability.

Genomic arrangement of the recombination substrate in FYDR mice.

To learn about the mechanisms of homologous recombination that can yield full length *EYFP* sequence in FYDR mice, the locus of integration and arrangement of the integrated DNA were determined as follows.

The number of integration sites was assessed by Southern analysis of FYDR genomic DNA. As there are no *EcoR*I sites within the injected substrate, the presence of a single *EcoR*I fragment indicates that there is a single integration site (Fig. 3*A*). To determine if multiple copies of the substrate had been integrated, a *BamH*I digest was Southern blotted. Since the injected *Not*I fragment has a single *BamH*I site downstream of the 3'*eyfp* coding sequences (Fig. 1*A*), a single *BamH*I band is expected if only one copy of the 6 kb *Not*I fragment is present. The presence of 7.4 kb and 2.6 kb fragments (Fig. 3*A*) indicates that more than one copy of the injected DNA had integrated into the mouse genome.

To further characterize the integrated DNA, we created a λ library from *BamH*I digested FYDR genomic DNA. Cloning and sequencing the 2.6 kb *BamH*I fragment revealed that it contains 473 bp of genomic DNA and part of a 3'*eyfp* cassette (Figs. 3*A*-3*B*). Using this genomic sequence to query the NCBI mouse genome database, a perfect

match was obtained to a single site on mouse chromosome 1. PCR using primers that anneal to published flanking sequences confirmed the site of integration (data not shown). There are no known genes at this site, so we do not anticipate that the integrated DNA will have significant biological effects.

Extensive Southern, PCR and sequencing analysis (data not shown) suggests that the arrangement of the integrated DNA is as shown in Fig. 3*D*. An example of an experiment that tests this arrangement is shown in Figure 3*C*. FYDR genomic DNA was digested with *Xba*I alone and in combination with either *Hind*III or *BamH*I. A 3 kb *Xba*I fragment was reduced to either 1.3 kb or 0.7 kb by these digestions, which is consistent with the arrangement shown in Figure 3*D*. We therefore conclude that the FYDR mice carry a degraded 3'*eyfp* cassette (missing the promoter and the first 250 bp of the intron) followed by a complete 5'*eyfp* cassette and a downstream degraded 3'*eyfp* cassette (lacking coding sequence and the entire polyadenylation signal).

Given that the downstream 3'*eyfp* cassette lacks essential coding sequences for both the amino terminus (engineered deletion) and the carboxy terminus (degraded during integration), recombination between the 5'*eyfp* cassette and this downstream 3'*eyfp* fragment cannot give rise to full length *EYFP* sequence. Thus, recombination between the upstream 3'*eyfp* and the central 5'*eyfp* is responsible for reconstitution of full length *EYFP* coding sequence and the observed expression of *EYFP*.

Mechanisms of homologous recombination in FYDR cells.

FYDR MEFs were immortalized with SV40 large T antigen to facilitate clonal expansion of isolated cells. Yellow fluorescent cells were then isolated by fluorescence

activated cell sorting and expanded in culture. PCR using primers that only amplify full length *EYFP* sequence (Fig. 4*A*) revealed that full length coding sequence is only present in fluorescent clones, and not in the non-fluorescent parent population (Fig. 4*B*). We therefore conclude that the yellow fluorescent phenotype is caused by restoration of full length coding sequence by homologous recombination events that join essential 5' coding sequences (uniquely present in 5'*eyfp*) with essential 3' coding sequences (uniquely present in the upstream 3'*eyfp* cassette).

To explore the mechanism of homologous recombination, DNA from fluorescent clones was analyzed by PCR and Southern, and an example is presented in Figure 5. PCR using primers that anneal to sequences flanking the coding region yields products of distinct lengths for the full length *EYFP* and each of the truncated copies (3'*eyfp* and 5'*eyfp*) (Fig. 5*A*). Interestingly, these primers yielded products for only *EYFP* and 3'*eyfp* when DNA from fluorescent clone Y1 was used as a template (Fig. 5*B*, lane 1). This result is consistent with a non-reciprocal gene conversion event, in which the upstream 3'*eyfp* has donated sequences to 5'*eyfp*. In such an event, the *BamH*I site in the 3'*eyfp* cassette is likely to be copied along with coding sequences (Fig. 5*C*). Indeed, Southern analysis shows the presence of a *BamH*I site at the expected location within the genome of clone Y1 (Fig. 5*D*, lane 2). We therefore conclude that clone Y1 underwent a gene conversion event. Molecular characterization of additional clones is in progress.

Quantification of fluorescent recombinant cells within FYDR mouse tissues and cells.

The use of fluorescence as an indicator of recombination enables direct analysis of cells from a mouse. In 16 different samples of disaggregated skin tissue, the recombinant cell frequency ranges from zero to 188 fluorescent cells per million viable cells (Fig. 6*A*, black bars). Cultures of primary untransformed ear fibroblasts were expanded from 31 different samples of ear tissue and were similarly analyzed (Fig. 6*A*, white bars). The observed variations in recombinant cell frequency are consistent with fluorescent cells giving rise to daughter cells during clonal expansion, either *in vitro* (ear cells) or *in vivo* (ventral skin cells). Overall, the frequency of recombinant cells was similar *in vitro* and *in vivo*.

Frequency and rate of homologous recombination in primary fibroblasts.

Having a mouse model makes it possible to compare recombination susceptibility among cells from different stages of development. We measured the rate of recombination (using the p_0 method [(31,32)]) and the frequency of recombinant cells in primary untransformed fibroblasts from embryos (MEFs) and from ear tissue. Interestingly, we found both the frequency and the rate of recombination to be 8-10 fold higher in embryonic fibroblasts compared to fibroblasts from adult animals (Table I).

Damage-induced recombination in primary FYDR fibroblasts.

Primary FYDR ear fibroblasts were exposed to a potent DNA crosslinking agent, MMC. At doses of MMC that cause between 33 and 90% reduction in relative growth, the frequency of fluorescent cells rises dramatically (Fig. 6*B*). An increase in recombination frequency was also observed in primary MEFs exposed to MMC (data not shown). Thus, primary untransformed cells from FYDR mice can be used to study damage-induced homologous recombination.

3.5 Discussion

Despite the importance of homology directed repair, both as a fundamental process during mitosis and as a risk factor in neoplasia, very few mouse models have been developed for the purpose of studying homologous recombination in mammals. The lack of such models has not only hampered studies of recombination *in vivo*, but has also prevented the application of the most basic tool of geneticists, namely the analysis of offspring from cross breeding. Further, the link between DNA damage and recombination indicates that such a mouse model could enable studies of environmentally induced DNA damage in animals. Here, we have described a new mouse model that provides an effective means for studying recombination, both *in vitro* and *in vivo*.

The direct repeat recombination substrate present in the FYDR mice allows detection of gene conversion events (Fig. 5), one of the most common mechanisms of recombination in mammalian cells (35,36). In addition, the arrangement of the integrated

DNA is compatible with detection of other types of recombination events, such as unequal sister chromatid exchanges and repair of collapsed replication forks. Single strand annealing, however, is not a mechanism that can restore *EYFP* expression, since annealing of a double strand break located between the upstream 3'*eyfp* and the 5'*eyfp* cassettes will result in an expression cassette lacking coding sequence from both the 5' and the 3' ends. Although it is theoretically possible that strand slippage could restore *EYFP* coding sequence (37,38), recombination at long direct repeats (similar to integrated DNA in the FYDR mice), has been shown to be both Rad51 and Rad52-dependent in eukaryotic cells (39), indicating that these events involve homology searching. Taken together, the arrangement of the integrated DNA within the FYDR mice allows for detection of mitotic homologous recombination and thus provides a simple and powerful approach for measuring recombination frequency in normal untransformed primary cells.

We measured the rates of recombination in primary ear fibroblasts and in embryonic fibroblasts and found the rates to be 1.3 ± 0.1 and 13.6 ± 1.5 per 10^6 cell divisions, respectively. Recombination rates reported for comparable direct repeat substrates in transformed mammalian cell lines are highly variable, but are generally between ~1 and ~7 per 10^6 cell divisions (15,40-44). Thus, despite fundamental differences in methodology (*i.e.*, fluorescence versus drug resistant phenotypes), the observed rates of recombination in fibroblasts of FYDR mice encompass the majority of previously reported rates.

Interestingly, embryonic fibroblasts appear to have a 10 fold higher rate of spontaneous recombination compared to fibroblasts cultured from ear tissue. Although it is formally possible that this difference could be due to variations in *EYFP* expression, it

appears more likely to reflect a difference in recombination rate, since the average fluorescence intensity per recombinant cell was essentially identical in embryonic and ear fibroblasts (data not shown). A possible cause for these apparent differences in recombination rates may be that embryonic cells have a greater propensity for homologous recombination (45), possibly due to differences in regulation of this pathway. Clearly, additional studies are necessary to reveal the underlying biological basis for the observed differences in recombination rates for embryonic versus adult cells.

Two mouse models, p^{un} and *APRT* +/-, have previously been used in studies of homologous recombination in somatic cells. The p^{un} mice carry a natural duplication in a gene that controls pigmentation and have been employed in studies of damage-induced recombination (*e.g.*, refs. 46-48). An important difference between p^{un} and FYDR mice is that in p^{un} mice, only recombination events that arise during development can be detected, whereas recombination events that occur both during development as well as in adult tissues can be detected in FYDR mice. Additionally, recombination can be detected in FYDR MEFs, making it relatively easy to determine how specific genes influence recombination. For example, whereas over 400 p^{un} mice were needed to determine effects of *WRN* on spontaneous homologous recombination (49), an analogous study using FYDR MEFs would require only about 10 mice.

APRT +/- mice provide a valuable system for studies of loss of heterozygosity caused by mitotic homologous recombination. However, loss of heterozygosity can also be caused by other mechanisms, including point mutations, deletions, aneuploidy, and mitotic homologous recombination (29,50-52). Specific identification of clones in which mitotic homologous recombination occurred required a combination of PCR, G-banding,

and chromosome painting (29). In contrast, only mitotic homologous recombination can restore *EYFP* coding sequences in FYDR mice, which thus provide a more direct approach for studying mitotic homologous recombination.

In addition to studies of recombination in cultured cells, the FYDR mice permit the detection of recombination events that occur *in vivo*. Using flow cytometry, we directly quantified fluorescent recombinant cells within disaggregated skin tissue. However, we did not detect any fluorescent cells in FYDR spleen, kidney and bone marrow, which may be due to lower levels of *EYFP* expression, or a lower frequency of recombinant cells within these tissues. We anticipate that ongoing studies will reveal whether there are cell type specific differences in expression and/or frequencies of recombination at this locus.

Direct repeat recombination substrates offer an effective approach for elucidating the effects of specific genes on recombination susceptibility. However, such studies are hampered by the fact that the locus of integration and the arrangement of the integrated DNA drastically influence the rate of recombination (42, 53-55). To overcome these problems, researchers have targeted homologous recombination substrates to specific loci (*e.g.*, refs. 18, 21 and 56). However, it remains technically difficult to simultaneously alter expression of multiple genes while keeping a recombination substrate constant. The FYDR mice offer a simple means for overcoming this barrier. For example, FYDR mice are currently being crossed with mice that carry mutations in specific DNA repair genes. These mice can then be further crossed with mice that carry mutations in additional genes of interest, such p53. Without a mouse model, it would not be feasible to create primary somatic cells that carry mutations in multiple genes of interest, while maintaining an

identical substrate for detecting recombination. Furthermore, the detection of recombination events in animals may have great utility as a measure of DNA damage caused by environmental factors, such as ionizing radiation or potential carcinogens. In conclusion, the FYDR mice can be used to study homologous recombination *in vivo*, and to provide primary cells for studies of recombination *in vitro*, thus providing valuable approaches for revealing genetic and environmental factors that affect genome stability in mammals.

3.6 Acknowledgements

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These studies would not have been possible without the pCX-EGFP construct and *EGFP* mice so graciously provided by Dr. M Okabe. The author is also grateful to Drs.

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Table 3-1. Frequencies and rates of homologous recombination in primary fibroblast cultures from FYDR mice.

			Frequency			Rate
		n	(mean x 10^{-6})		*n*	(per cell div. x 10^{-6})
Ear	Exp .1	17	4.7	Exp. 1	17	1.2
Fibroblasts	Exp. 2	22	3.9	Exp. 2	22	1.4
	Exp. 3	20	3.5	Exp. 3	20	1.4
	Ave. $\pm$ SD		4.0 $\pm$ 0.6*	Ave. Rate $\pm$ SD		1.3 $\pm$ 0.1*
Embryo	Exp. 1	16	22.0	Exp. 1	19	14.8
Fibroblasts	Exp. 2	9	32.0	Exp. 2	21	14.1
	Exp. 3	17	50.0	Exp. 3	23	11.9
	Ave. $\pm$ SD		34.7 $\pm$ 14.2*	Ave. Rate $\pm$ SD		13.6 $\pm$ 1.5*

Averages plus and minus one standard deviation (SD) are shown.
*Values for embryonic fibroblasts are significantly higher than those of ear fibroblasts (Students T-test; $p < 0.05$).

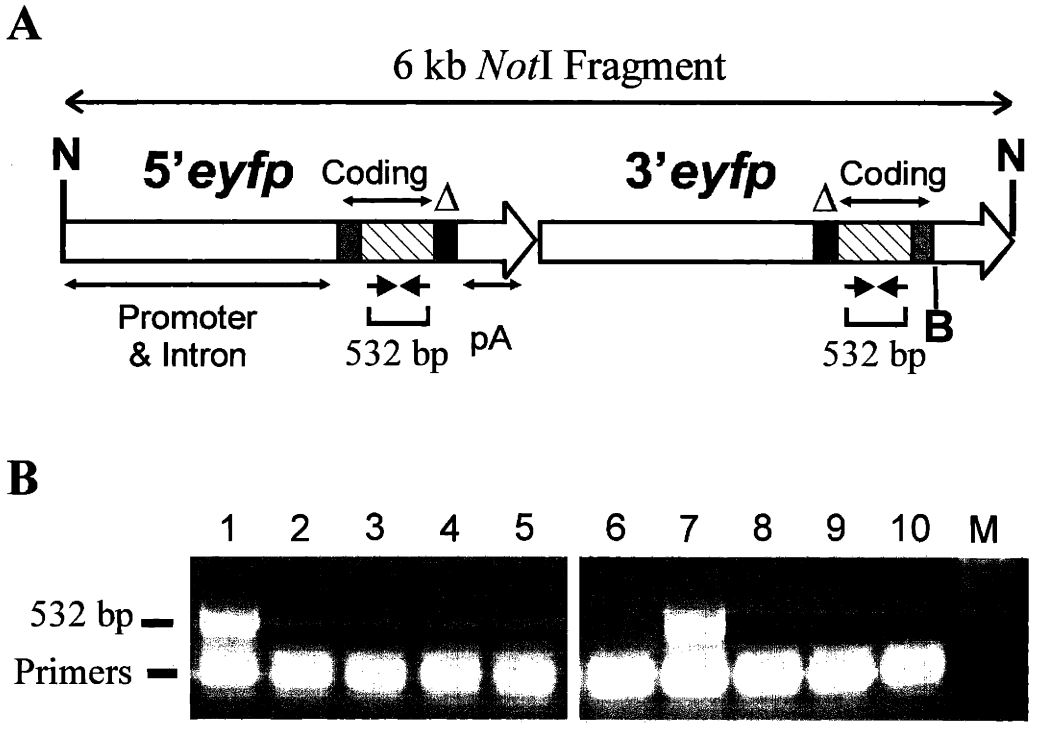

Figure 3-1. Recombination substrate and PCR screen of candidate transgenic mice showing an example of one transgenic mouse. (A) Sequences in 5'*eyfp* and 3'*eyfp* are represented by two large arrows with emphasis on the coding sequences (hatched), deleted regions (black) and sequences unique to each cassette (gray). Promoter, intron and polyadenylation signal (pA) are indicated. Expression is driven by the chicken β-actin promoter and cytomegalovirus enhancer. Restriction sites are *BamH*I (B) and *Not*I (N). (B) PCR analysis of candidate mice using primers that anneal to coding sequence, as shown in *A*. Template DNA was from mouse cells expressing *EGFP* (lane 1), wild type mouse cells (lanes 2-3), and a subset of candidate mice (lanes 4-10). M indicates size standards.

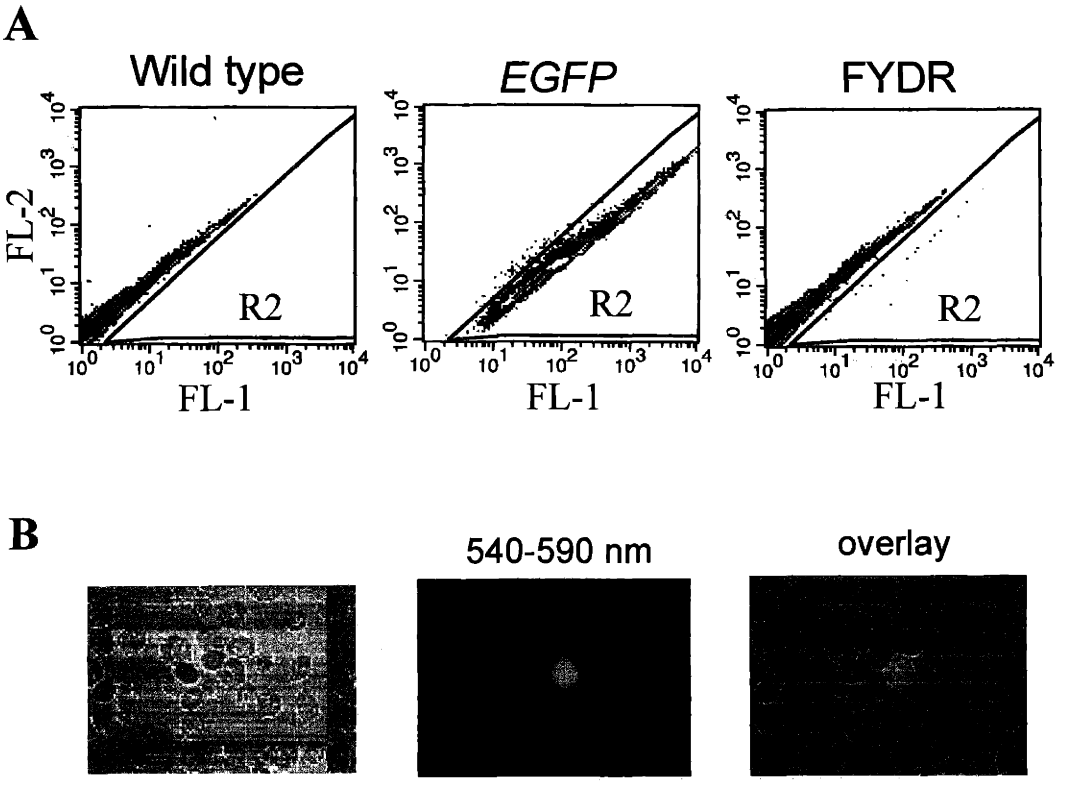

Figure 3-2. Analysis of cells from the FYDR founder mouse. (A) Flow cytometry results for ear fibroblasts derived from a wild type mouse, an *EGFP* expressing mouse (28), and the FYDR founder mouse. Relative fluorescence intensity for 515-545 nm (FL-1) versus 562-588 nm (FL-2). (B) Image of ear fibroblasts from the FYDR founder mouse. A fluorescent cell is shown under a 540-590 nm filter (center).

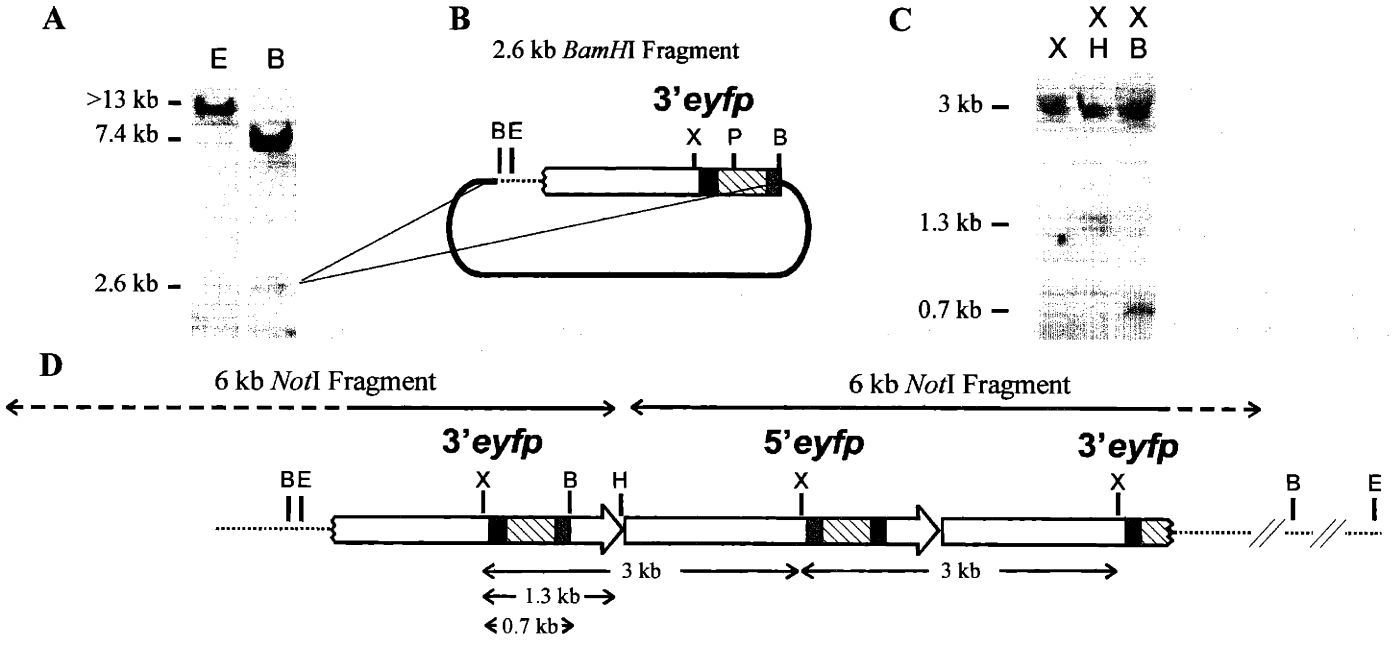

Figure 3-3. Arrangement of the integrated DNA within FYDR mice. (A) Southern analysis of FYDR genomic DNA probed with *EYFP* coding sequence. (B) Map of the 2.6 kb *BamHI* fragment subcloned into pBluescript (thick line). (C) Southern analysis of FYDR genomic DNA probed with *EYFP* coding sequences. (D) Diagram of the DNA integrated within the FYDR mice. Expression cassettes are indicated by large arrows, as in Figure 1*A*. Dotted lines indicate genomic DNA. Dashed portions of the upper arrows represent degraded sequences. (A-D) Restriction sites are *BamHI* (B), *EcoRI* (E), *Xba*I (X) and *Hind*III (H).

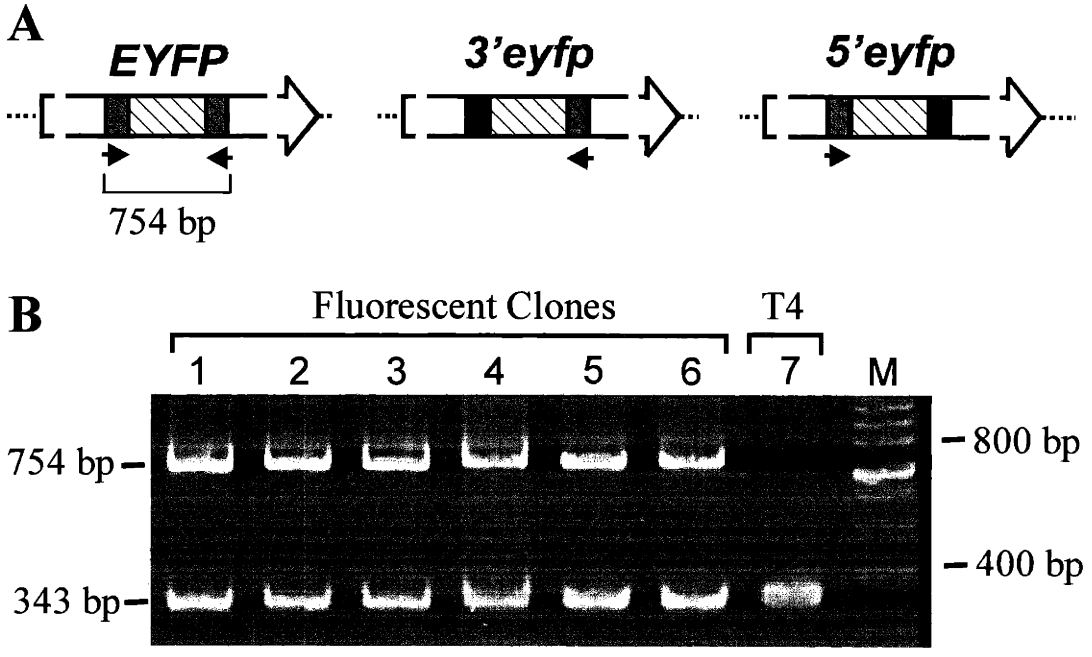

Figure 3-4. PCR analysis of recombinant yellow fluorescent clones. (A) Primers that bind sequences unique to either 3'*eyfp* or 5'*eyfp* yield a 754 bp product from full length *EYFP* only (large arrows are as in Fig. 1*A*, except shorter, as indicated by gaps). (B) PCR analysis of transformed cells (T4, lane 7) and yellow fluorescent clones (lanes 1-6) using primers that hybridize to full length *EYFP* (indicated in *A*) and primers that amplify a portion of the *Aag* 3-methyladenine DNA glycosylase gene (loading control, 343 bp). M indicates size standards.

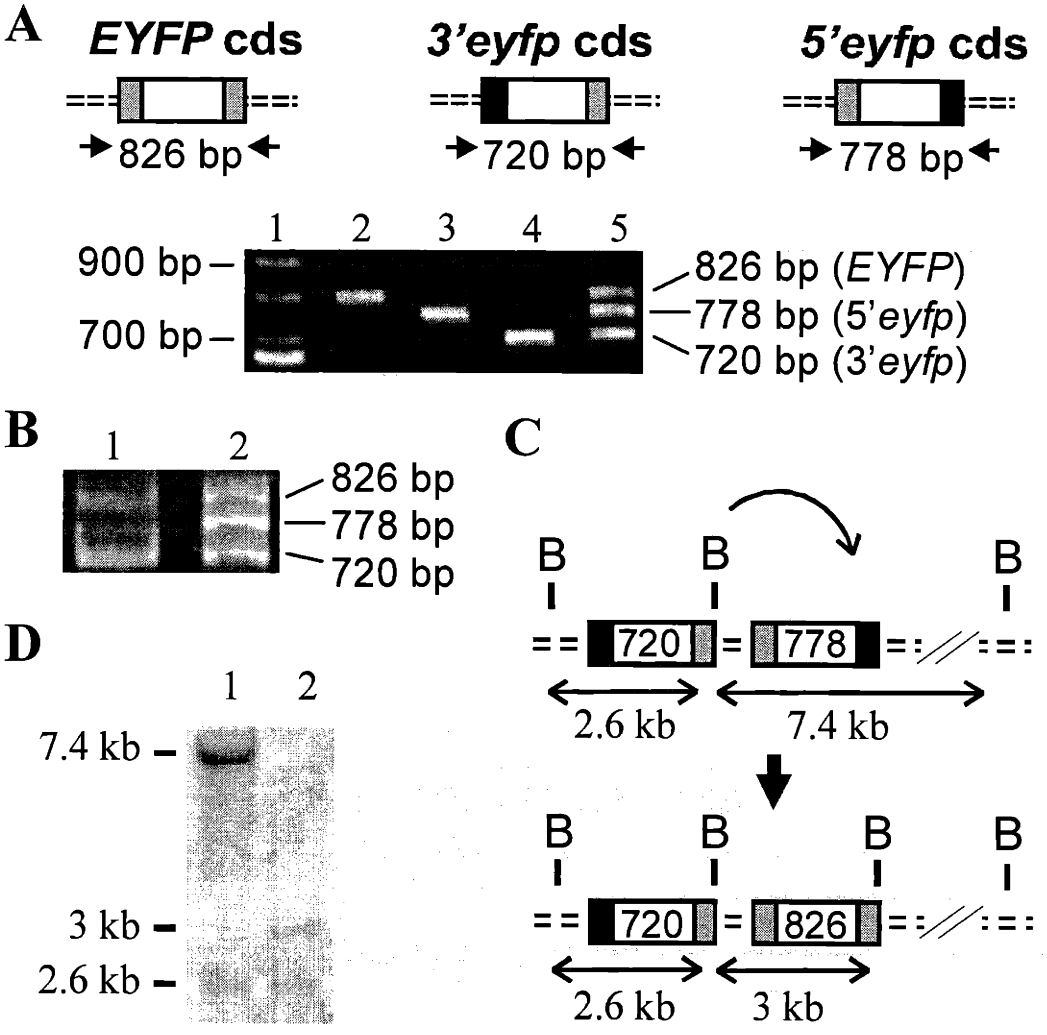

Figure 3-5. Analysis of the recombination mechanism in a recombinant clone. Diagrams depict coding sequences (cds) only. Common sequences are in white, essential terminal coding sequences in gray, and deleted sequences in black. (A) Primers that amplify coding sequences yield the indicated products (top). PCR of template DNA from plasmids carrying *EYFP* (lane 2), 5'*eyfp* (lane 3), and 3'*eyfp* (lane 4). Lane 1 shows size markers and lane 5 shows a mixture of PCR products from lanes 2-4. (B) PCR results from yellow fluorescent clone Y1 (lane 1) and a mixture of control PCR products (lane 2). (C) Schematic diagram of gene conversion. B, *Bam*HI (D) *BamH*I digested DNA from the nonfluorescent parent population (lane 1) and fluorescent clone Y1 (lane 2), probed with *EYFP* coding sequences. Note that the degraded downstream 3'*eyfp* cassette (Fig. 3*D*) cannot participate in events that reconstitute *EYFP*, cannot be amplified using the primers indicated in *A*, does not yield a detectable signal by Southern, and thus is not depicted in *C*.

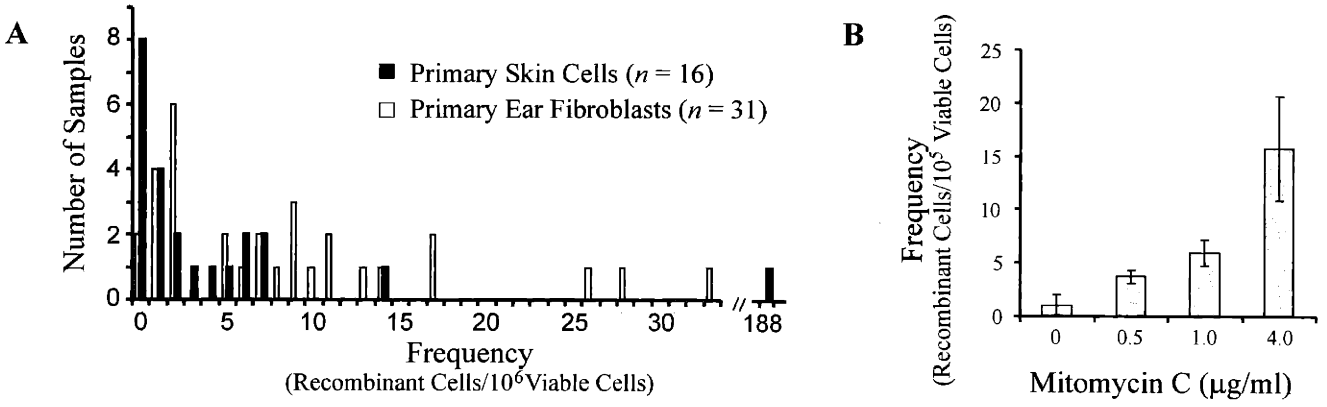

Figure 3-6. Recombination in FYDR skin cells and ear fibroblasts. (A) Spontaneous frequency of recombinant cells per 10^6 viable cells. (B) Mitomycin C-induced recombination in cultured ear fibroblasts. A representative histogram from one of three independent experiments is shown. 95% confidence intervals are indicated.

Chapter IV

Conclusions and Future Work

Given the importance of homologous recombination, both as a fundamental process during DNA replication and as a risk factor in neoplasia, it is critical that we learn more about the genetic and environmental factors that make cells susceptible to homologous recombination. The ultimate goal of our research is to elucidate the underlying causes and mechanisms of DNA damage-induced recombination, and to determine the role of DNA repair enzymes in modulating homologous recombination in yeast, mammalian cells and mice.

In this work, we have investigated the molecular basis for methylation-induced recombination in *S. cerevisiae*. Specifically, we have examined how BER, NER and replicative bypass of methyl lesions and/or their repair intermediates affect susceptibility of cells to homologous recombination. We show that while Mag1 initiated BER and NER normally help prevent 3MeA-induced recombination events, a very high level of expression of *Mag1* can saturate downstream BER enzymes and result in the accumulation of recombinogenic BER intermediates. Our results indicate that both unrepaired lesions and BER intermediates contribute to methylation-induced recombination is *S. cerevisiae*. In addition, they suggest that the ability of a DNA glycosylase to modulate recombination depends upon the quantity and relative recombinogenicity of the lesions being removed as well as the relative proportions of downstream enzymes in the BER pathway.

Analogous studies to explore the genetic and environmental factors that modulate susceptibility to homologous recombination in mammals have been hindered by the lack of effective tools for measuring recombination events *in vivo*. In this work, we have described novel transgenic mice that allow for the detection of homologous

recombination events in primary somatic cells, both *in vitro* and *in vivo*. The FYDR mouse is the first mouse model engineered to exploit direct repeats to detect mitotic homologous recombination events in the somatic tissue of mature animals.

Studies utilizing the FYDR mice have shown that fluorescence detection is a highly effective means for quantifying homologous recombination events in mice. Indeed, a fluorescence based recombination system offers several advantages over substrates that rely on drug resistance to select for recombinants. For example, using *APRT*+/- mice, cells that have undergone LOH at the *APRT* gene, possibly due to homologous recombination, are scored by selection with 8-azaadenine. Only those cells that can be extracted from mice and grown in culture are assayable using this system. In addition, detection of LOH events by 8-azaadenine resistance is relatively slow and laborious. It takes at least one week for resistant cells to form colonies and those colonies must then be stained and manually counted. Furthermore, extensive Southern, PCR and cytogenic analyses need to be performed to identify the cause of the LOH event as mitotic homologous recombination. In contrast, fluorescence detection by flow cytometry is rapid, automated and quantitative. FACS technology allows for the analysis of millions of cells within minutes and for the isolation of parental and/or recombinant cells. In addition, since fluorescence detection does not require cell culture, recombination events that occur *in vivo* can be quantified in any tissue that can be disaggregated into a single cell suspension. Ultimately, a fluorescence based detection system, such as the FYDR mouse, gives us the potential to capture recombination events *in situ*. Currently, a two-photon microscope screening system developed in the laboratory of Peter So (M.I.T.) is

being optimized for the detection of such fluorescent recombinant cells within intact FYDR tissues.

In this work, we have shown that FYDR mice provide a powerful tool for studying recombination in primary cells. This is the first such system to allow direct measurement of mitotic recombination in primary cells. The ability to monitor homologous recombination in primary cells is essential for exploring the genetic and environmental factors that modulate recombination in normal somatic cells. Since primary cells undergo a limited number of divisions before they senesce, without a mouse, it had been essentially impossible to create primary cells that carry an identical, stably integrated recombination substrate in every cell. Thus, the FYDR mouse is extremely valuable in that it can provide a constant source of primary cells for studies of recombination *in vitro*.

In addition, by crossing the FYDR mice with mice deficient in specific genes of interest, it becomes possible to create primary somatic cells that carry mutations in multiple genes. Without a mouse model, it would not be feasible to simultaneously inactivate gene expression in multiple genes while keeping a recombination substrate constant. Currently, FYDR mice are being crossed with mice deficient in DNA repair genes and p53. For example, crosses between FYDR mice and mice lacking the Aag 3-methyladenine DNA glycosylase, which is the mouse homolog of *S. cerevisiae* Mag1, will allow us to study the effects of alkylated bases, specifically 3MeA, on recombination susceptibility in primary mammalian cells. Further crosses of these mice (*i.e. Aag-/-*, FYDR) with mice deficient in other repair enzymes, such as the Mgmt O^6MeG

methyltransferase, will allow us to explore how multiple repair enzymes modulate homologous recombination in primary cells.

The ability to detect DNA damage-induced recombination in primary cells from FYDR mice to lends itself to many practical applications. Indeed, many chemotherapeutics currently used in the treatment of cancer are DNA damaging agents and it has been hypothesized that DNA damage caused by these agents may be one cause of secondary tumors in cancer patients. The availability of a relatively rapid system for analyzing the recombinogenic effects of newly developed cancer treatments could potentially be used to develop drugs with a lower potential of contributing to the development of secondary tumors. Additionally, the FYDR mouse could be used to evaluate the risks of exposure to environmental factors that may contribute to tumor formation.

Appendix

Using Embryonic Stem Cell Technologies to Generate a Transgenic Mouse for Detecting Recombination *in vivo*

A.1 Introduction

A major goal of this thesis was to develop tools that will allow us to better understand the underlying molecular basis for spontaneous and DNA damage-induced recombination in mammals. To assure success, we used two different approaches to create transgenic mice that carry recombination substrates for fluorescence detection of mitotic recombination. Chapter III describes how microinjection of the recombination substrate led to the creation of FYDR mice. Not described herein is an alternative approach in which mouse embryonic stem cells were injected into blastocysts to create chimeric mice that carry fluorescent recombination substrates. This work is described in detail in Molly Stitt's Masters thesis, entitled "Fluorescent Detection of Recombination in Mammalian Cells and Mice". Since the author of this thesis also contributed to these studies, the results and conclusions are summarized below.

One advantage to using a fluorescent signal to monitor recombination events in mice is that it gives us the potential to detect recombination events *in situ*. Our collaborators in the M.I.T. Department of Mechanical Engineering, Professor Peter So and Ki Hean Kim, have developed a two-photon microscope screening system that can detect fluorescent cells within intact mouse tissues. Ultimately, it is anticipated that optimization of this system will allow us to quantify rare recombination events in multiple mouse tissues and determine the particular cell type(s) that have undergone recombination.

A.2 Results

Mouse embryonic stem (ES) cells were engineered to contain two different mutant copies of the sequences required for expression of the enhanced yellow fluorescent protein (EYFP) arranged in a direct repeat. Mitotic recombination between these truncated *eyfp* sequences restores expression of *EYFP*. Rare fluorescent ES cells can be quantified and isolated by flow cytometry, and these cells contain DNA that has undergone a homologous recombination event.

An ES cell clone that carries a single copy of the *EYFP* direct repeat recombination substrate was injected into mouse blastocytes to generate chimeric mice. Spontaneous recombination events can be detected in primary embryonic fibroblasts (MEFs) and adult ear fibroblasts derived from these mice at frequencies similar to what was observed in ES cells (between ~1/20,000 and 1/100,000). In addition, 3D images of recombinant MEFs grown in dishes have been captured using quantitative scanning two-photon microscopy. Both the mouse ES cells and the mouse fibroblasts are similarly susceptible to mitomycin C induced recombination.

In total, seven highly chimeric mice were generated and bred to establish mouse lines. Of the seven, one chimera gave rise to three agouti offspring, indicating that the ES cells contributed to the germline of this mouse. Unfortunately, none of the agouti pups carried the *EYFP* recombination substrate, which suggests that the recombination substrate may have integrated at an essential locus.

In order to generate an *EYFP* control mouse, yellow recombinant ES cells were isolated by fluorescence activated cell sorting (FACS) and clonally expanded. Two

independent yellow clones were injected into mouse blastocysts, resulting in the generation of three chimeric mice. Two of these chimeras were bred and a single agouti pup was born. Once again, this pup did not carry the recombination substrate, consistent with the substrate integrating at an essential locus.

For more details on these experiments, see the Masters Thesis of Molly Stitt.

A.3 Conclusions

Many of these studies preceded the generation of the FYDR mice and as such were quite significant. For example, these studies demonstrated that fluorescence detection by flow cytometry is an effective means for quantifying mitotic homologous recombination events in mouse cells. In addition, they showed that ES cells could be engineered to carry recombination substrates and subsequently injected into blastocysts to generate transgenic animals. Indeed, current studies are underway to use this same approach to site-specifically integrate a recombination substrate at the *ROSA26* locus.