Synthetically Lethal Interactions Classify Novel Genes in Postreplication Repair in *Saccharomyces cerevisiae*

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Abstract

Both prokaryotic and eukaryotic cells are equipped with DNA repair mechanisms to protect the integrity of their genome in case of DNA damage. In the eukaryotic organism *Saccharomyces cerevisiae*, *MMS2* encodes a ubiquitin-conjugating enzyme variant protein belonging to the *RAD6* repair pathway; *MMS2* functions in error-free postreplication repair (PRR), a subpathway parallel to *REV3* mutagenesis. A mutation in *MMS2* does not result in extreme sensitivity to DNA damaging agents; however, deletion of both subpathways of PRR results in a synergistic phenotype. By taking advantage of the synergism between error-free PRR and mutagenesis pathway mutations, a conditional synthetic lethal screen was used to identify novel genes genetically involved in PRR. A synthetic lethal screen was modified to use extremely low doses of MMS that would not affect the growth of single mutants, but would effectively kill the double mutants. Fifteen potential mutants were characterized, of which twelve were identified as known error-prone PRR genes. Characterization of mutations in strains SLM-9 and SLM-11, that are conditionally synthetically lethal with *mms2Δ,* revealed functions for both checkpoints and mating-type heterozygosity in regulating PRR. Cell cycle checkpoints monitor the integrity of the genome and ensure that cell cycle progression is deferred until chromosome damage is repaired. The checkpoint genes genetically interact with both the error-free and error-prone branches of PRR, potentially for delaying cell cycle progression to allow time for DNA repair, and for signaling the stage of the cell cycle and thus DNA content. Other potential monitors for DNA content are the **a**1 and α2 proteins encoded by the mating type genes

*MAT***a** and *MAT*α, respectively. Diploid cells heterozygous for mating type (**a**/α) show an increased resistance to UV damage and are more recombination-proficient than haploid cells. Haploid PRR mutants expressing both mating type genes show an increased resistance to DNA-damaging agents. This phenomenon is specific to PRR: it was not seen in excision repair-deficient and recombination-deficient mutants tested. The rescuing effect seen in PRR mutants heterozygous for mating type is likely the result of channeling lesions into a recombination repair pathway and away from the non-operational PRR pathway. Both checkpoint and mating type genes play a role in regulating PRR. Almost certainly these genes are required to monitor the cell cycle stage and DNA content to determine the best mechanism to repair the damaged DNA thus preventing genomic instability.

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List of Abbreviations

3MeA	3-methyladenine
4NQO	4-nitroquinoline-N-oxide
7MeG	7-methylguanine
Ade	adenine
Amp	ampicillin
AP	apurinic/apyrimidinic (abasic)
ARS	autonomously replicating sequence
BER	base excision repair
BIR	break induced replication
bp	base pair
CEN	centromere
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	double-strand break
dsDNA	double-strand DNA
EMS	ethyl methane sulfonate
FOA	5-fluoro-orotic acid
His	histidine
HJ	Holliday junction
HNPCC	hereditary nonpolyposis colon cancer
HR	homologous recombination
HU	hydroxyurea
IR	ionizing radiation
LB	Luria-Bertani medium
MGMT	O6-methyl-guanine DNA methyl transferase
MMR	mismatch repair
MMS	methyl methanesulfonate
MNNG	N-methyl N-nitro-N nitrosoguanidine
MOPS	3-(*N*-morpholino) propanesulfonic acid
mRNA	messenger RNA
MW	molecular weight
NBS	Nijmegen breakage syndrome
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
nt	nucleotide
O^6MeG	O^6-methylguanine
ORF	open reading frame
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI3K	phosphoinositide 3 kinase

PRR	postreplication repair
PSS	protruding single strands
RFC	replication factor C
RPA	replication protein A
SCE	sister chromatid exchange
SD	synthetic dextrose
SDSA	synthesis dependent strand annealing
ssDNA	single-strand
SSA	single strand annealing
SSB	single strand binding
SUMO	small ubiquitin-related modifier
TLS	translesion DNA synthesis
Trp	tryptophan
Ubc	ubiquitin conjugating enzyme
UV	ultraviolet
wt	wild type
XP	xeroderma pigmentosum
YCp	yeast centromere plasmid
YEp	yeast episome plasmid
YIp	yeast integration plasmid
YRp	yeast replicating plasmid
Δ	deletion mutation (null mutations)

Chapter One -- Introduction

1.1. DNA Damage

DNA is an essential carrier of genetic information in all living cells. With the understanding that DNA is an active chemical component of genetic material, the field of DNA repair gained widespread recognition and interest in the fundamental importance of genomic maintenance. The arrangement of the DNA double helix gave rise to the assumption that DNA is a highly stable molecule. However, the primary structure of DNA is dynamic and is subject to constant change from endogenous threats as well as those resulting from exposures to environmental chemicals and radiation. Since DNA is subject to continuous damage, the cell must have an arsenal of repair mechanisms allowing it to tolerate and respond to such harm. Failure of these processes can lead to genomic instability and severe diseases. Insight into enzymes responsible for the repair of DNA damage laid down the foundation for the involvement of DNA repair enzymes in human disease, as documented in many human hereditary diseases including xeroderma pigmentosum (XP) and hereditary non-polyposis colon cancer (HNPCC). The area of DNA repair has flourished in the last 50 years with significant discoveries having an enormous impact on the field of DNA repair in general.

1.2. Types of DNA Damage

The chemical structure of DNA allows changes to arise as consequences of errors introduced during replication, recombination, and repair itself. In addition, the inherent instability of specific chemical bonds that comprise the normal chemistry of nucleotides under physiological conditions, and the ability of the DNA to react easily with a variety of chemical compounds and physical agents contribute to the vast array of modifications that occur in the genetic material. DNA damage can be divided into two major classes, referred to as spontaneous and environmental damage.

1.2.1. Spontaneous DNA Damage

Cellular DNA is susceptible to accidental damage from a variety of endogenous products of cellular metabolism. The principal endogenous lesion, also known as spontaneous DNA lesions, are caused by deamination of cytosine to uracil, loss of purines to yield abasic sites, and reactive oxygen species that produce several types of lesions (Lindahl, 1993; Marnett and Burcham, 1993). Some of these endogenous adducts appear to be efficient premutagenic lesions, which raises questions about their contribution to human cancer. For example, replication errors introduced by DNA polymerases are a major mechanism of spontaneous mutagenesis. Fortunately, cells are equipped with exonucleolytic proofreading and mismatch repair (MMR) to minimize the introduction of mutations (Echols and Goodman, 1991; Kunkel and Alexander, 1986; Roberts and Kunkel, 1988).

Aerobically growing cells are exposed to active oxygen during normal metabolism. Generation of oxidated bases by reaction of DNA with intracellular oxidants is quantitatively the most important class of base modification occurring in

mammalian cells. It is estimated that oxidized adducts formed on a daily basis range from 10^4 to 10^6 per cell (Ames and Gold, 1991). Oxidation of DNA can result in damage to all four bases and deoxyribose. A major consequence of oxidative stress on DNA is the formation of 8-oxo-guanine, which base-pairs preferentially with adenine rather than cytosine. As a result, this lesion is pro-mutagenic during DNA replication, resulting in G:C to T:A transversion mutations (Kasai and Nishimura, 1984; Shibutani et al., 1991). Both *Escherichia coli* and mammalian cells are equipped with specific DNA glycosylases responsible for the removal of these lesions (Tchou et al., 1991).

Deamination occurs spontaneously in the cell as a result of the loss of the amino group from the nitrogenous base. Deamination of purines and pyrimidines contribute to the transition mutations that accumulate in cells by changing the coding properties of the base (Friedberg et al., 1995). Deamination of cytosine to uracil happens at a significant rate in the cell, but this lesion can be repaired by a specific repair process which detects uracil, a base not normally present in DNA (Friedberg et al., 1995; Lindahl, 1993). Deamination of methylcytosine to thymine can also occur in the cell. Methylcytosine occurs in the human genome at the sequence 5'CpG3', which is normally avoided in the coding regions of genes. This deamination can not be recognized by a DNA repair system and results in an increase in transition mutations (Friedberg et al., 1995).

The spontaneous loss of DNA bases to form apurinic-apyrimidinic (AP) sites occurs constantly in cells. AP sites are partly caused by the slow hydrolysis of the *N*-glycosyl bonds that attach these bases to the DNA (Lindahl and Andersson, 1972). AP sites are also formed by the enzymatic action of DNA-*N*-glycosylases that remove

damaged or mispaired bases (Sakumi and Sekiguchi, 1990). Apart from natural AP sites, various modified forms of oxidized AP sites are generated endogenously by free radical attack on the bases and deoxyribose sugar (von Sonntag, 1987). AP sites often block DNA synthesis by DNA polymerases resulting in a lethal lesion. However, occasionally DNA polymerases can bypass AP sites by incorporating the incorrect nucleotide into the synthesized DNA strand, thus resulting in a permanent genetic change (Goodman et al., 1994; Loeb et al., 1986).

A tautomeric shift is the spontaneous isomerization of a nitrogen base to an alternative hydrogen-bonding form, thus affecting the normal Watson-Crick base pairing (Friedberg et al., 1995). During replication, any base in the template strand existing in its rare tautomeric form can result in the misincorporation of a base in the daughter strand (Friedberg et al., 1995). If the misincorporated base of the daughter strand is not removed, the lesion will be fixed into a mutation.

1.2.2. DNA Polymerase Proofreading Prevents Copying Errors

Three different components contribute to the fidelity of DNA replication: the polymerization reaction itself, proofreading by a 3' → 5' exonuclease activity and postreplicational MMR (Friedberg et al., 1995). Replication fidelity has received a great deal of attention, specifically as to how DNA polymerases prevent misinsertion of a nucleotide. A base substitution results if misinsertion of a Watson-Crick nucleotide is followed by mismatch extension without proofreading. Depending on the polymerase, error rates for single-base substitutions due to proofreading-deficient DNA polymerases vary from 10^{-3} to $>10^{-6}$ (Kunkel and Bebenek, 2000).

1.2.3. Exogenous DNA Damage

It is important to develop an understanding of how cells respond to levels of DNA damage that is significant in terms of human exposure to environmental DNA-damaging agents such as sunlight and carcinogens present in the diet. Environmental agents can cause damage to the structure of DNA, leading to genomic instability and cancer.

1.2.3.1. Physical DNA Damaging Agents

Radiation was the first mutagenic agent identified, with its effects on genes first reported in the 1920's (Mutscheller, 1925). Ionizing radiation (IR) is a naturally occurring source of physical damage to the DNA and to all cellular components (Friedberg et al., 1995). X- and gamma-rays are energetic enough to produce reactive ions when they come into contact with biological molecules. IR produces a range of damage to the cells primarily due to the production of free radicals and by direct action on the DNA (Friedberg et al., 1995). Lesion produced by IR include single- and double-strand breaks, damage to or loss of bases and crosslinking of DNA to itself or proteins (Friedberg et al., 1995).

Ultraviolet (UV) radiation is less energetic, but its wavelengths are preferentially absorbed by bases of DNA and by aromatic amino acids of proteins. The UV radiation spectrum is classified in terms of its wavelength: UV-A (320 nm – visible), UV-B (290 – 320 nm) and UV-C (180 – 290 nm) (Friedberg et al., 1995). The major lesions caused by UV radiation are cyclobutane pyrimidine dimers where

adjacent pyrimidines become covalently linked by the formation of a four-membered ring structure (Friedberg et al., 1995). These dimers, like bulky lesions from chemicals, block transcription and DNA replication and are lethal if left unrepaired (Friedberg et al., 1995). A second lesion, the pyrimidine-pyrimidone (6-4) photoproduct, occurs at a frequency several fold lower than that of pyrimidine dimers (Friedberg et al., 1995).

1.2.3.2. Chemical DNA Damaging Agents

The first report of the mutagenic action of a chemical was in 1942 by Charlotte Auerbach, who showed that nitrogen mustard, used in chemical warfare, could cause mutations in cells (Brookes, 1990). Since that time, many other mutagenic chemicals have been identified, many with an important stimulus from the field of cancer chemotherapy. Several of these substances have been identified as chemical carcinogens and have a broad range of structures with no obvious unifying features; however, they can be classified into two broad categories: direct-acting and indirect-acting. Direct-acting carcinogens are reactive electrophiles. By chemically reacting with nitrogen and oxygen atoms in DNA, these compounds modify certain nucleotides so as to distort the normal pattern of base pairing (Beranek, 1990). Indirect-acting carcinogens generally are unreactive, water-insoluble compounds. These carcinogens require metabolic activation carried out by enzymes that are normal body constituents. In animals, activation of indirect-acting carcinogens is often carried out by liver enzymes that normally function to detoxify noxious chemicals (Donnelly et al., 1998).

Alkylating agents have been a mainstay of chemotherapy for malignant diseases for decades. Alkylating agents form unstable positively charged ions that form covalent

bonds with nucleophilic sites on DNA bases (Saffhill et al., 1985). Interaction of alkylating agents with DNA occurs in a variety of ways, each of which having specific implications for the cell involved. Reactivity of alkylating agents with DNA is summarized by their Swain-Scott substrate constant (*s*). Alkylating agents with low *s* values have reduced reaction selectivity and are referred to as S_N1 compounds. Alternatively, alkylating agents with *s* values approaching 1 are referred to as S_N2 compounds (Beranek, 1990). The initial interaction is often monoadduct formation with specific atoms of specific DNA bases (Shulman, 1993). Ninety percent of interactions occur at the ring nitrogen N^7-guanine position, and less commonly at the ring nitrogens of other DNA bases (Shulman, 1993). Conversely, bifunctional alkylating agents can cause crosslinks. Interstrand crosslinking is an important mechanism for cell death or chromosome loss due to alkylating agents. If interstrand crosslinks are formed and not repaired they will prevent the dissociation of DNA strands required for successful DNA replication (Shulman, 1993). The type of lesion produced is of importance due to the cellular consequences, with certain lesions being mutagenic and others lethal. Major lesions of biological significance created by the alkylating agent methyl methanesulfonate (MMS) are 3-methyladenine (3MeA) and 7-methylguanine (7MeG). The 3MeA lesion causes replication stalling when encountered during S-phase of the cell cycle and results in lethality. Both 3MeA and 7MeG can result in secondary lethal lesions attributable to the conversion into abasic sites that can lead to strand breakage (Friedberg et al., 1995). The agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) produces a significant level of O^6-methylguanine (O^6MeG) that can base pair with thymine during replication, thus resulting in a mutagenic lesion.

As mentioned above, bifunctional alkylating agents can react with two different nucleophilic centers in DNA resulting in an interstrand DNA cross-link. Given that interstrand crosslinks prevent DNA strand separation and inhibit DNA replication and transcription, chemicals that produce crosslinks have been extensively used in cancer chemotherapy. Nitrogen and sulfur mustard, mitomycin and *cis*-platinum are examples of well-studied chemical agents for chemotherapeutic uses (Brendel and Ruhland, 1984).

1.3. Repair of DNA Damage

In addition to the proofreading activity of DNA polymerases that can correct miscopied bases during replication, cells have developed mechanisms for repairing DNA damaged by chemicals or radiation. If the DNA repair process was 100% effective, chemicals and radiation would not pose a threat to cellular DNA. However, not all lesions caused by environmental agents are repaired efficiently, and such lesions can lead to mutations that ultimately cause cancer. DNA repair can be defined in a general sense as a range of cellular responses associated with restoration of the genetic instructions provided by the normal primary DNA sequence. However, DNA damage may simply be tolerated, or abnormal base sequences can be generated for the sake of cell survival. Although a complete understanding of DNA repair is still deficient, extensive studies in prokaryotes and lower eukaryotes have vastly increased our understanding of DNA repair systems.

DNA-repair mechanisms have been studied most extensively in *E. coli*, using a combination of genetic and biochemical approaches. The repair mechanisms

discovered by these studies can be divided into three broad categories: MMR, excision repair and repair of double-strand DNA (dsDNA) breaks. MMR, which occurs immediately after DNA synthesis, uses the parental strand as a template to correct an erroneous nucleotide incorporated into the newly synthesized strand. Excision repair involves the removal of a damaged region by specialized nuclease systems followed by DNA synthesis to fill the gap. dsDNA breaks are repaired either by homologous recombination (HR) or by a non-homologous end-joining (NHEJ) process (Friedberg et al., 1995). In addition to the three main repair pathways, cells have the option of directly reversing the damage or tolerating the damage via an error-free or error-prone translesion DNA synthesis (TLS) mechanism (Friedberg et al., 1995). The basic mechanisms of DNA repair have been conserved throughout evolution.

1.3.1 Reversal of Damage

Most damage to DNA is repaired by removal of the damaged bases followed by resynthesis of the excised region. Some lesions in DNA, however, can be repaired by direct reversal of the damage, which may be a more efficient way of dealing with specific types of DNA damage that occur frequently. Although it might seem that direct reversal of damage would be the simplest way to correct the damage, in most cases the reverse reaction is not thermodynamically or kinetically favorable. In a few cases, the reaction is reversible and mechanisms have been developed to take advantage of this reversibility. Only a few types of DNA damage are repaired in this way, particularly pyrimidine dimers resulting from exposure to UV light and alkylated

guanine residues that have been modified by the addition of methyl or ethyl groups at the O^6 position of the purine ring (Friedberg et al., 1995).

In bacteria and yeast, photolyases can directly reverse DNA damage resulting from UV exposure (Sancar et al., 1996). Probably the first and most important discovery which initiated the DNA repair field was the process of photoreactivation. Hermann Muller demonstrated that mutations and hereditary changes can be caused by X-rays (Muller, 1927). This discovery suggested the ability to artificially induce mutations in genes thus manipulating an organism's genome. This concept was broadened to more potent mutagens, namely UV radiation. Shortly thereafter, the initial indication of DNA damage and repair occurred when it was discovered that living cells are able to recover from the lethal effects of UV radiation. However, it was not until the end of the 1940's when the independent observations of Albert Kelner and Renato Dulbecco suggested a possible DNA-repair mechanism (Dulbecco, 1949; Kelner, 1949). Since these observations preceded our understanding that the molecular basis of mutagenesis is rooted in alterations in DNA, neither Kelner or Dulbecco addressed the phenomenon of DNA repair directly (Kelner, 1948). The initial experiments by Kelner were an attempt to determine if bacteria, which did not normally produce antibiotics, could be stimulated to do so when mutagenized. His inability to reproduce consistent survival rates suggested he was observing a biological phenomenon associated with the recovery of cells from the effects of UV radiation. This phenomenon was reproducible with eukaryotic cells. The survival of the spores from the fungus *Streptomyces griseus* that had been irradiated with UV light had dramatic variability depending on storage conditions (Kelner, 1949). In determining a factor that caused this recovery, he

identified light as the essential agent. At the same time Kelner made this discovery in yeast, Renato Dulbecco accidentally discovered the increased survival of phage upon exposure to visible light in the presence of bacterial cells. Stan Rupert extended this research into an *in vitro* system and was able to document evidence that photoreactivation of DNA was an enzyme-catalyzed reaction (Cleaver, 2003).

A second direct repair enzyme is responsible for the phenomenon designated as the 'adaptive response to alkylation damage'. This phenomenon was noted when *E. coli* cells were continuously exposed to low doses of MNNG, resulting in the production of mutations for the first 60 min but not thereafter. When the cells were subsequently challenged with a higher dose of the alkylating agent there was a marked resistance to both the lethal and mutagenic effects of the chemical (Samson and Cairns, 1977). In humans, the O^6MeG DNA methyltransferase (MGMT) is important in the repair of alkylation damage. The alkyl group from the lesion is transferred to a cysteine residue in the active site of MGMT in an irreversible reaction (Hazra et al., 1997). In addition to O^6-MeG, MGMT repairs larger alkylation lesions, including O^6-ethylguanine, O^6-butylguanine and O^4-methylthymine, but with lower efficiency (Sancar, 1995).

1.3.2. Excision Repair

In attempts to understand photoreactivation, it was noted that a large amount of light-dependent survival occurs across a wide number of microbial species. As a result, researchers focused on determining the 'key factor' that caused the lethal effect upon UV exposure. This resulted in the identification of the *cis-syn* thymine dimer; the major

photoproduct of UV irradiation. Also, the possibility of the cell containing one repair system inspired the field in determining if other such repair mechanisms exist. It was soon discovered by Richard Setlow that in the absence of photoreactivation, certain radiation resistant strains of UV-irradiated *E. coli* cells were able to survive better than their wild-type counterparts (Setlow and Carrier, 1964). A search for the mechanism led to the discovery of nucleotide excision repair (NER). Excision repair relies on the redundant information in the DNA helix to remove a damaged base or nucleotide and replace it with a new base. This can occur through the use of the complementary strand as a template.

Although direct repair is an efficient way of dealing with particular types of DNA damage, the most pro-active repair process is likely base excision repair (BER). The BER pathway corrects DNA modifications that arise either spontaneously or from attack by reactive chemicals. Moreover, BER is often responsible for the repair of DNA base damage that causes only minor disturbances in the helical structure of DNA such as oxidized, alkylated, deaminated or absent bases. Overall, BER copes with inappropriate bases (mismatched or damaged) that arise from replication errors or via chemical modification; sites of base loss that are formed by enzyme-catalyzed, spontaneous or mutagen-induced base release; and strand breaks that are products of free radical attack of DNA (Krokan et al., 2000; Memisoglu and Samson, 2000).

BER involves the concerted effort of several repair proteins that recognize and excise specific DNA damage, eventually replacing the damaged moiety with a normal nucleotide and restoring the DNA back to its original state. Typically, the first step of BER involves the DNA glycosylases, which are required for the removal of an

inappropriate base from DNA. DNA glycosylases bind specifically to a target base and hydrolyze the cleavage of the N-glycosylic bond between the base and the deoxyribose moieties of the nucleotide residues. To complete the process, a DNA polymerase fills in the gap beginning at the exposed 3'OH, and DNA ligase seals the nick (Friedberg et al., 1995).

NER is required for the removal of a large variety of DNA lesions, particularly those that distort the DNA helix such as damage induced by UV light and DNA intra- and interstrand crosslinks. NER is characterized by the incision of the damaged DNA strand on both sides of the lesion removing the damaged oligonucleotide fragment. Genetic studies in yeast have been instrumental in defining the functions of NER genes involved in the different stages of the repair reaction and in revealing their roles in cellular processes. Mutations in the genes involved in the incision step of NER display a high sensitivity to UV light and other DNA damaging agents (Prakash et al., 1993), whereas mutations involved in downstream events result in a moderate degree of sensitivity to DNA damaging agents (Prakash and Prakash, 1979).

Defects in excision repair of UV-damaged DNA have been identified in human patients suffering from XP. Individuals with XP exhibit extreme skin sensitivity to sunlight and suffer from a high incidence of skin cancers. Seven XP complementation groups, A through G, have been identified and all display a deficiency in the incision step of excision repair (Cleaver and Kraemer, 1989; De Weerd-Kastelein et al., 1972; Vermeulen et al., 1991). The *ERCC1*, *ERCC2/XPD* (Lehmann et al., 1992) and *ERCC3/XPB* (Weeda et al., 1990) genes exhibit a high degree of homology to the yeast *RAD10*, *RAD3* and *RAD25* genes, respectively (Gulyas and Donahue, 1992; Park et al.,

1992; van Duin et al., 1986; Weber et al., 1990). The *XPA* gene is homologous to yeast *RAD14* (Bankmann et al., 1992), and the *XPC* gene is the homolog to yeast *RAD4* (Legerski and Peterson, 1992).

1.3.3. Mismatch Repair

The primary source of spontaneous alterations in DNA occurs through the generation of mismatched bases during DNA replication. Mismatched or unpaired bases in a DNA duplex can arise through several processes, including errors in DNA replication, genetic recombination, deamination of 5-methylcytosine to thymine and the action of chemical mutagens. Normally, in *E. coli* spontaneous mutations arise at a frequency of approximately one in every 10^{10} bases synthesized. Significantly, cells lacking a MMR system have a spontaneous mutation frequency approximately 1000 times greater than normal cells. Since mismatches are comprised of normal Watson-Crick bases, MMR systems rely on secondary signals within the helix to distinguish daughter and parental DNA strands. In *E. coli*, repair is directed by the state of adenine methylation of d(GATC) sequences (Modrich and Lahue, 1996). The nature of the strand signal has not been defined in eukaryotic organisms.

Our fundamental understanding of MMR comes from years of work performed on the methyl-directed MMR system in *E. coli*. The basic mechanism of MMR in all systems studied to date involves three steps: recognition of the mismatch, excision of the misincorporated base and DNA surrounding the mismatch, and finally repair synthesis to replace the excised DNA. Genes involved in MMR were originally isolated in *E. coli* strains displaying elevated levels of spontaneous mutations. These genes

include *mutS, mutL, mutH, uvrD* and *dam* (Grilley et al., 1989; Hickson et al., 1983; Marinus and Morris, 1975; Su and Modrich, 1986). MutS is a DNA-mismatch binding protein that can bind to a variety of mispaired bases and small, 1-5 bases, single-stranded loops (Marinus and Morris, 1975; Parker and Marinus, 1992; Su and Modrich, 1986). In a reaction requiring ATP hydrolysis, MutL, together with the MutS-mismatched DNA complex stimulate strand excision by MutH, opposite a *dam* methylated GATC parental DNA sequence, ensuring that the DNA excised is the newly replicated unmethylated daughter DNA (Grilley et al., 1990). In the excision step, ATP hydrolysis fuels the unwinding and degradation of single stranded (ssDNA) containing the mismatched base from the MutH nick site through the mismatched base. UvrD is the DNA helicase responsible for unwinding DNA in an ATP-dependent manner (Hickson et al., 1983).

Homologs of the bacterial MutS and MutL proteins have been identified in *S. cerevisiae*. MMR in yeast utilizes two MutL homologs, post meiotic segregation increased 1 (*PMS1*) and *mutL* homolog 1 (*MLH1*) (Kramer et al., 1989; Prolla et al., 1994). Mutations in either *MLH1* or *PMS1* results in elevated mutation rates, and genetic studies of *mlh1, pms1* and *mlh1 pms1* double mutation strains show identical phenotypes, which suggests that *MLH1* and *PMS1* are components of the same MMR pathway (Prolla et al., 1994). The yeast *mutS* homolog 2 (*MSH2*) gene shows similar phenotypes with disruption of *PMS1* and *MLH1* (Reenan and Kolodner, 1992). Two additional *S. cerevisiae* MMR homologs, *MSH3* and *MSH6*, display a weak mutator phenotype, but strains deleted for both *MSH3* and *MSH6* have a mutation rate similar to that observed in *msh2* strains (Marsischky et al., 1996).

1.3.4. DNA Damage Tolerance

Not all DNA damage is or can be removed immediately, and some damage may persist in the cell for an extended period of time. Therefore, all organisms need to deal with the problems that arise when a moving replication fork encounters damage in the template strand. Obviously the best way to deal with this damage is to repair it by one of the mechanisms discussed above. In some cases the damage may not be repairable, or the advancing replication fork may already have unwound the parental strands, thus preventing excision mechanisms from using the complementary strand as a template for repair. Replication of mildly damaged DNA in diploids can be advantageous to the cell as it provides a sister chromatid that can be used as a template for repair by homologous recombination.

In eukaryotes, DNA replication initiates at multiple sites and it may be able to resume downstream of a lesion, leaving a "gap" of single-stranded unreplicated DNA. The gap is potentially just as dangerous if not more so than the lesion if the cell divides. The cell has devised mechanisms to repair the gap by recombination with either the homologous chromosome or the sister chromatid to yield two intact daughter molecules, one of which still contains the lesion.

1.3.5. Repair of Strand Breaks

Double-strand breaks (DSBs) and single-strand gaps in damaged DNA are the most dangerous lesions for a cell. If left unrepaired such damage can result in cell cycle arrest, cell death, and if repaired incorrectly, the damage may result in the loss of

genetic information or the accumulation of mutations. DSBs can arise in cells by exposure to IR and other types of DNA-damaging agents or through mechanical stress. In addition, cellular processes such as DNA replication result in DSBs by replicating across nicked chromosomes or by the active processing of stalled replication forks by specific enzymes (Michel, 2000). Cells are equipped with a recombination repair mechanism; an important process involved in repairing DNA damage, ensuring that the correct information will be restored. There are two main pathways that compete for the repair of DSBs, HR and NHEJ.

HR is a universal process important in generating genetic diversity and in ensuring accurate chromosome segregation during meiosis. HR can be divided into conservative and non-conservative recombination. Both types of HR are dependent on Rad52 and Rad51 (Paques and Haber, 1999; Shinohara and Ogawa, 1995). Conservative HR is an accurate reconstitution of broken chromosomes by copying sequence information from the sister chromatid during mitosis or the homologous chromosome during meiosis. HR can include both reciprocal and nonreciprocal information exchange, referred to as crossing over and gene conversion, respectively. Crossing over results in a precise reciprocal exchange that does not alter the content of the genome, but does result in a rearrangement of genetic linkage patterns. Conversely, gene conversion involves information transfer from a donor locus to a recipient locus, and this may result in a net gain or loss of functional information. Conservative HR has been separated into three pathways including DSB repair, synthesis dependent strand annealing (SDSA), and break induced replication (BIR; (Pfeiffer et al., 2000)). Non-conservative HR is carried out by the single stranded annealing (SSA) pathway. This

pathway is used when a break occurs between two flanking homologous regions. The homologous regions come together resulting in a deletion of one copy of the homologous sequence as well as the intervening sequence (Haber, 1999).

1.3.5.1. Mechanisms of Homologous DSB Repair

The initial model of DSB repair assumed that DSBs were resected on both strands to create large gaps flanked by short regions of ssDNA that could invade a homologous template and initiate DNA repair (Resnick, 1976; Szostak et al., 1983). In *E. coli*, the RecA protein forms a nucleoprotein filament on the DNA, and carries out a homology search followed by strand-exchange reactions (Friedberg et al., 1995; Kowalczykowski et al., 1994). The RecBCD complex is involved in formation of a 3'-OH ssDNA tail at the end of DSBs by unwinding the duplex DNA from DSB ends and degrading the DNA from its 5'-end (Kowalczykowski et al., 1994). After pairing with the homologous DNA, Holliday junctions (HJ) are processed by RuvA, RuvB, RuvC and RecG (Kowalczykowski et al., 1994).

In *S. cerevisiae*, DSBs are processed to generate 3'-OH ssDNA tails at the DSB ends. The ssDNA can then invade a DNA duplex in a homology-dependent manner. Information is restored to the daughter strand by copying a template, which anneals with a single-stranded tail on the other side of the DSB. This process produces a Holliday (branched) structure that, depending on the direction of resolution, results in two progeny with or without a cross-over event (Shinohara and Ogawa, 1995).

1.3.5.2. DSB Repair Model

Molecular models were designed to account for the association of gene conversion with crossovers. The initial model of Szostak et al. assumed that DSBs were resected on both strands to create large gaps flanked by rather short regions of single-strand DNA that could invade a homologous template and initiate DNA repair (Sun et al., 1991; Szostak et al., 1983). The 3' ends of both meiotic and mitotic DSBs are not resected while the 5' ends of the DNA are resected back over long distances. The 3' ends are available to invade an intact homologous template in a manner similar to RecA. The 3' ends of the invading stands can then act as primers for the initiation of new DNA synthesis. This process results in the formation of two HJs. Cleavage of the HJs in the same way will result in gene conversion without crossing over, but if the noncrossover strands of one HJ are cleaved while the crossover strand on the second are cleaved a crossover will occur (Figure 1.1) (Paques and Haber, 1999).

1.3.5.3. Gene Conversion

Gene conversion is defined as a nonreciprocal transfer of genetic information from one molecule to its homolog. Gene conversion occurs between two alleles of a gene but can include many contiguous genes, including the entire distal part of a chromosome arm. Initially, gene conversion was defined in meiosis to explain the non-Mendelian segregation of alleles. Mortimer and Fogel (1969) documented several key characteristics of gene conversions, including the idea that gene conversion exhibited polarity, whereby the likelihood that a nearby marker would be co-converted along with a specific gene decreased with the distance between the markers. A second important

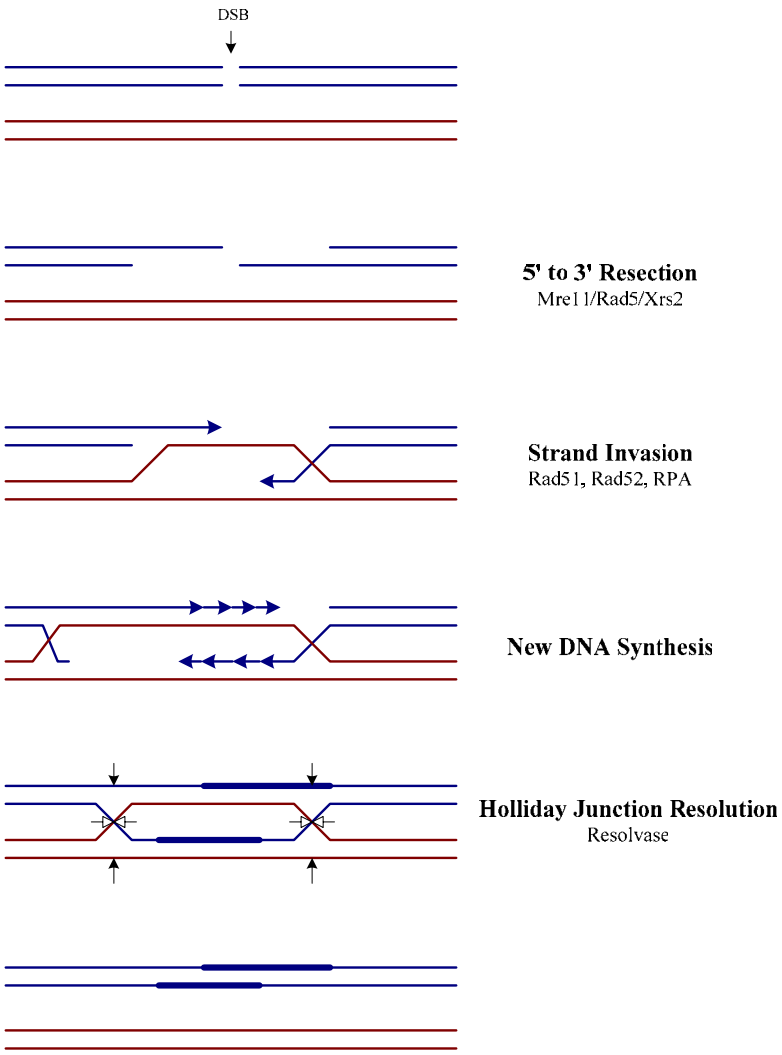

Figure 1.1. Double-strand break repair model of recombination. One of the two recombining DNA duplexes sustains a DSB. The 5' ends of the broken duplex are exonucleolytically degraded to expose single-stranded tails with 3' termini. One of the tails invades an uncut homologous duplex, resulting in the displacement of a D loop. DNA synthesis primed by the second 3' end completes repair. Branch migration results in the formation of two Holliday junctions. If both Holliday junctions are resolved in the same direction, then the parental configuration of flanking markers is preserved. Resolution of the two Holliday junctions in opposite directions results in a reciprocal crossover between markers that flank the region of strand exchange (not shown) (Adapted from Szostak et al. 1983).

observation was that gene conversions were associated with crossing over (Fogel and Hurst, 1967; Fogel et al., 1981; Mortimer and Fogel, 1969). Gene conversions are divided into two different families: DSB repair model of Szostak et al. and SDSA.

1.3.5.4. The Synthesis-Dependent Strand Annealing Model

It has been shown that many mitotic gene conversion events are not associated with crossovers (Gloor et al., 1991; Johnson and Jasin, 2000; Nassif et al., 1994). These findings led to the alternative model, SDSA. The basic feature of this model is that the newly synthesized DNA strands are displaced from the template and returned to the broken molecule, allowing the two newly synthesized strands to anneal to each other. This could occur through one of two possible mechanisms; topoisomerases or helicases actively dismantle the replication structure (McGill et al., 1989; Thaler et al., 1987), or the replication 'bubble' remains small and the newly synthesized strand is continuously unwound from its template (Formosa and Alberts, 1986). In either case, DNA synthesis is conservative instead of semiconservation as in the DSB repair model. This suggests that the two ends of DSBs behave independently from each other during the homology search, and a stable heteroduplex between ssDNA tails and templates cannot be formed. This model also suggests the absence of a Holliday structure as a recombination intermediate, which can explain the production of gene conversion without crossing-over seen in some types of recombination (Nassif et al., 1994).

1.3.5.5. Break-Induced Replication

BIR is a nonreciprocal recombination-dependent replication process involved in the repair of a broken chromosome. BIR involves a one-ended recombination event, either due to only one free DNA end or because only one of the two ends of the DSB succeeds in strand invasion of a homologous sequence. In *S. cerevisiae*, Esposito (Esposito, 1978; Golin and Esposito, 1984) reported examples of mitotic recombination in which there was a nonreciprocal recombination event that extended hundreds of kilobases down a chromosome arm. As a result, one daughter cell was identical to the parent diploid, in that it was still heterozygous for markers extended along the chromosome arm whereas the other cell was homozygous for all these alleles. Further studies of BIR using a site-specific DSB created by the HO endonuclease showed the DSB was repaired by a recombination event leading to the formation of a 30-kb nonreciprocal translocation in a Rad52 dependent manner (Bosco and Haber, 1998).

Yeast telomere maintenance in the absence of the telomerase enzyme appears to employ BIR. Chromosome ends slowly shorten in the absence of telomerase, in part because they fail to be replicated to the very end. Depending on the initial length of the telomere and the size of a telomere repeat required to "cap" the chromosome, the cell can proliferate for many generations. In *S. cerevisiae*, cells deficient in telomerase can still survive, but require the function of the *RAD52* gene (Lundblad and Blackburn, 1993). Studies revealed two distinct *RAD52*-dependent telomere maintenance pathways. Deletion of the *RAD51, RAD54, RAD55* and *RAD57* genes result in an accelerated loss of viability, similar to *rad52* cells. In contrast, deletion of *RAD50, MRE11* and *XRS2* caused a slower rate of senescence. Conversely, deletion of both *rad51* and *rad50* eliminates survivors (Le et al., 1999). Thus, telomere maintenance in

the absence of telomerase appears to adhere to the same genetic system as for the repair of a single DSB created in a diploid.

1.3.5.6. Single Strand Annealing

DSBs occurring between two flanking homologous regions are repaired in an efficient SSA mechanism, which results in a deletion containing a single copy of the repeated sequence. SSA relies on the resection of the ends of the DSB by an exonuclease, thus producing long single-stranded tails in which complementary strands of the duplicated sequence are exposed and can reanneal (Paques and Haber, 1999).

1.3.5.7. Genes Involved in Recombination Repair

In *S. cerevisiae*, mutants in the *RAD52* epistatic genes are sensitive to γ-irradiation but not UV, and they are defective in mitotic recombination. The *RAD52* epistasis group can be subdivided into at least five major groups including *RAD52*, the *RAD51* family, the *RAD59* family, the *MRE11* family, and *RAD53* (Paques and Haber, 1999). *RAD53* is not directly involved in DSB repair and will not be discussed here. The main proteins involved in yeast homologous exchange are divided into strand transfer proteins or nucleolytic strand resection proteins. The strand transfer proteins include Rad51, Rad52, Rad54, Rad55 and Rad57. The Rad50-Mre11-Xrs2 complex is required for the resection of the DSB ends (Chen and Kolodner, 1999).

The *RAD52* gene is required for nearly all HR events, but is not required for the *RAD52*-independent pathway of recombination found in diploids undergoing spontaneous recombination between heteroallelic markers (Haber and Hearn, 1985) or

when one of two homologs is cut by HO endonuclease (Malkova et al., 1996). A *rad52* mutant has a hyporecombination phenotype and a spontaneous mutator phenotype (Tran et al., 1995), with most of the events corresponding to deletions between short direct repeats.

The *RAD51* family includes *RAD51*, *RAD54*, *RAD55* and *RAD57*. Deletions in this group of genes has a less severe effect on mitotic HR as compared to *rad52* mutants (Rattray and Symington, 1994). Analysis of the DNA intermediates has shown that mutants accumulate unrepaired DSBs with single-stranded tails longer than those of the wild type (Shinohara et al., 1992; Sugawara et al., 1995). These gene products seem to function in a step subsequent to the processing of DSB ends (Shinohara and Ogawa, 1995).

The *RAD59* gene was isolated by searching for mutants preventing *rad51*-independent events (Bai and Symington, 1996). Rad59 shares some homology to Rad52, but *RAD59*-mediated events still require *RAD52* (Bai and Symington, 1996). Both *rad51* and *rad59* mutants display modest defects in spontaneous recombination between chromosomal inverted repeats, but the *rad51 rad59* double mutant is as defective as the *rad52* mutants (Bai and Symington, 1996). In addition, *rad59* mutants have defects in recombination between inverted repeats but not the ratio of associated crossover events. In contrast, these events are preferentially increased in a *rad51* mutant (Rattray and Symington, 1994; Rattray and Symington, 1995).

Several studies have shown that *MRE11* functions in meiosis, DNA repair, recombination (Ajimura et al., 1993; Johzuka and Ogawa, 1995) and telomere maintenance (Nugent et al., 1998). Mre11 has been shown to complex with Rad50 and

Xrs2 (Johzuka and Ogawa, 1995; Usui et al., 1998). In humans and yeast the main activity of Mre11 in DNA repair includes an Mn^{2+}-dependent 3'-to-5' dsDNA exonuclease activity and a ssDNA endonuclease activity (Furuse et al., 1998; Moreau et al., 1999; Paull and Gellert, 1998; Usui et al., 1998). Mre11 also has a 3' to 5' exonuclease activity on ssDNA in yeast (Usui et al., 1998).

1.3.5.8. Non-homologous End-joining

S. cerevisiae predominantly uses a HR system to repair DSBs, whereas mammalian cells mainly use homology independent mechanisms to repair DSBs. NHEJ acts directly on the ends of DSBs and results in ligation of compatible ends or non-complementary ends resulting in mutagenic potential (Pfeiffer, 1998). Efficient NHEJ is dependent on the presence of Ku70-Ku80 heterodimers and the ability of the cell to disable the Rad52-dependent HR pathway (Figure 1.2). Ku70 and Ku80 bind the ends of broken DNA and may function by protecting the ends from degradation and thus enhancing the accuracy of NHEJ (Feldmann et al., 2000; Liang and Jasin, 1996). In contrast, the error-prone NHEJ pathway is independent of Ku70 and Ku80 (Boulton and Jackson, 1996; Feldmann et al., 2000). In the absence of Ku, two alternative NHEJ pathways can repair DSBs. One pathway produces blunt ends by filling in 5' protruding single strands (PSS) and degradation of 3' PSS. The alternative Ku-independent pathway creates small deletions at sites of microhomology. The factors involved in the error prone Ku-independent pathways are presently unknown. However, it is hypothesized that the Rad52 protein might bind the DNA ends and promotes a SSA mechanism (Feldmann et al., 2000).

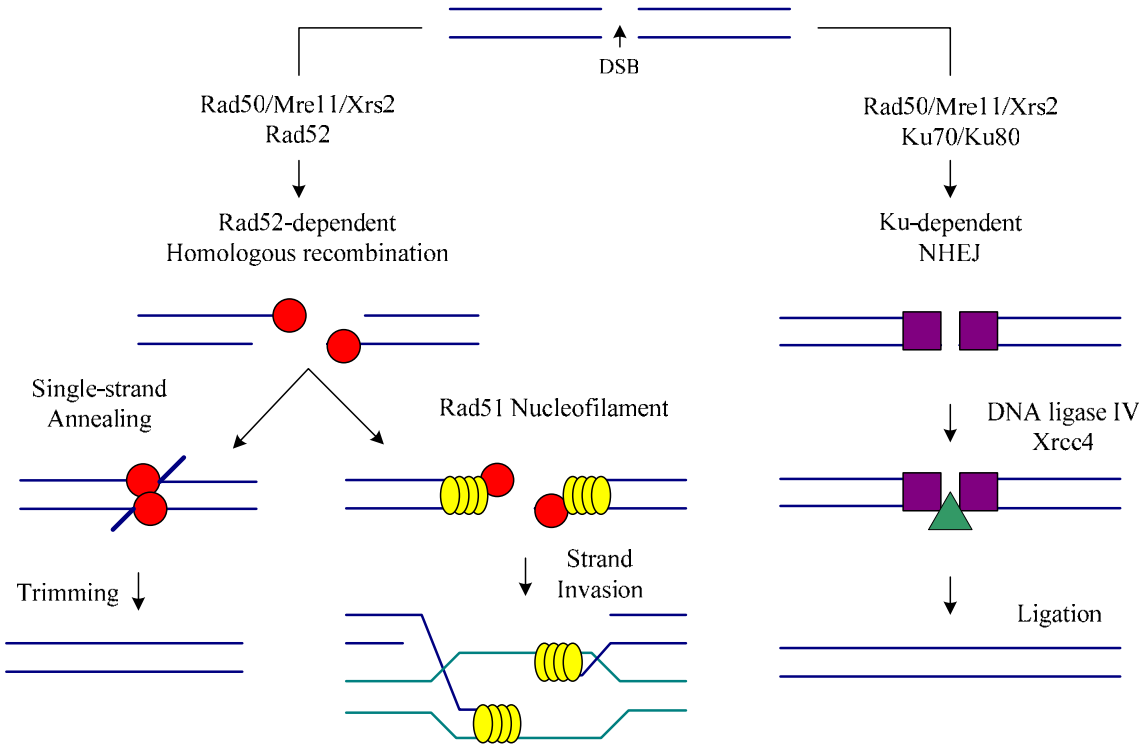

Figure 1.2. Comparison of DSB rejoining in yeast and mammalian cells. Yeast cells use predominately a Rad52-dependent homologous recombination reaction. The prevalent pathway used in mammalian cells is a Rad52-independent Ku-dependent non-homologous end-joining mechanism.

1.4. Postreplication Repair in Prokaryotes

When *E. coli* cells are irradiated with UV, the molecular weight of their native chromosomal DNA is not reduced; however, when DNA from irradiated cells is treated with alkali, the denatured DNA has a lower molecular weight compared to unirradiated control cells. This indicates the presence of single-strand interruptions throughout the irradiated chromosome. Most of these interruptions are due to the ongoing excision repair of pyrimidines dimers, since in excision repair-deficient mutants the molecular weight of the irradiated chromosome is almost identical to the intact chromosome. Nevertheless, not all replication-blocking lesions are a product of DNA damage. In addition to replication-blocking adducts, abasic sites and DNA strand breaks are induced by a variety of cellular influences such as topological stress, aberrant DNA structures, availability of nucleotide pools and tightly bound protein complexes, which can also lead to replication fork arrest. Stalled replication forks are responsible for the generation of ssDNA gaps or DSBs, which can be lethal lesions. For example, the persistence of DNA lesions such as pyrimidine dimers in the template strand blocks the movement of the DNA replication machinery and results in a gap in the daughter strand. This gap could bring about the release of a free duplex DNA end, which would be a substrate for recombination enzymes, thus promoting inappropriate recombination. Whatever its source, a replicative block has to be repaired or bypassed so replication can be resumed. DNA repair systems and replication fork bypass mechanisms enable organisms to deal with the problems that arise when moving replication forks encounter unsuitable DNA templates or DNA damage.

To fill in a daughter-strand gap, a stalled replisome can be modified to allow it to carry out TLS. Experiments of Rupp, Howard-Flanders and colleagues (1971) uncovered a feature of daughter-strand gap repair in *E. coli* where the filling in of the gaps was accompanied by the formation of a hybrid DNA complex between the newly synthesized and template DNA. The number of such strand exchanges between sister duplexes roughly coincided with the number of UV lesions in the template DNA, indicating that daughter-strand gap repair is accompanied by strand exchange and recombinational repair (Rupp et al., 1971). This conclusion was in line with the finding that daughter-strand gaps are not repaired in *recA* mutants deficient in HR (Ganesan, 1974; Smith and Meun, 1970).

In *E. coli*, daughter-strand gaps are repaired by the RecF recombinational repair pathway (Horii and Clark, 1973; Rothman et al., 1975). In addition to RecA and RecF, other important players include RecO, RecR, RuvA, RuvB and RuvC proteins. Mutants in some of these genes are deficient in daughter-strand gap repair, but mutants in other genes fill in gaps efficiently and are proposed to work at a later stage of the repair reaction. A combination of genetic and biochemical evidence strongly suggests that RecF, RecO and RecR (RecFOR) proteins participate in a common step of DNA recombination and repair. The RecFOR complex is responsible for directing the loading of RecA protein specifically onto gapped DNA that is coated with single strand binding (SSB) protein, thereby accelerating DNA strand exchange (Morimatsu and Kowalczykowski, 2003). This RecFOR complex is also required to stabilize and maintain replication forks arrested by UV-induced DNA damage. In the absence of RecF, the nascent lagging strand of the arrested replication fork is extensively degraded

by the RecQ helicase and RecJ nuclease (Chow and Courcelle, 2004), suggesting that RecFOR acts at a common point during the recovery process and potentially acts in loading RecA filaments to maintain the replication fork structure after the arrest of replication by UV-induced DNA damage.

1.4.1. SOS Response in *E. coli*

When a cell suffers extensive DNA damage over a short time its repair systems are saturated. The cell runs into the danger of extensively replicating unrepaired lesions, thereby perpetuating mutations. In such situations, both bacterial and eukaryotic cells use inducible repair systems in an attempt to repair the lesions in a rapid manner.

In *E. coli*, one such inducible system for dealing with extensive DNA damage is by increasing the expression of more than 30 genes in the SOS repair response (Fernandez De Henestrosa et al., 2000). Because this system generates many errors in the DNA as it repairs lesions it is referred to as error-prone. The errors induced by the SOS system are at the site of lesions, suggesting that the mechanism of repair is insertion of nucleotides in place of the damaged ones in the DNA. This inducible system is used only as a last resort when error-free mechanisms of repair cannot cope with the damage.

The DNA damage tolerance activity in *E. coli* is induced in response to regions of single-stranded genomic DNA (Chaudhury and Smith, 1985). Two SOS-dependent mechanisms of DNA damage tolerance exist in *E. coli* to tolerate single-strand gaps in the DNA; a mutagenic translesional replicative bypass and an error-free

recombinational bypass. Both mechanisms of the DNA damage tolerance in *E. coli* require the RecA protein. RecA binds to ssDNA to form a nucleoprotein filament, thus promoting base pairing between the ssDNA-RecA nucleoprotein filament and a homologous DNA duplex (Roca and Cox, 1997).

The SOS response is induced and regulated by the LexA and RecA proteins. In an uninduced *E. coli* cell, the LexA protein acts as a repressor for more than 30 genes, including the *recA* and *lexA* genes. When the genome of an *E. coli* cell is damaged or DNA replication is inhibited, the SOS response signal is generated. The RecA protein binds regions of ssDNA, assumed to be the intracellular signal for SOS, and converts it to an activated form. LexA then diffuses to the activated RecA protein and interacts with the nucleoprotein complex resulting in the proteolytic cleavage of LexA and inactivating LexA as a repressor (Koch and Woodgate, 1998).

1.4.2. Recombination-mediated Replication Fork Restart

Recombination repair at stalled replication forks has been extensively studied in bacteria, which has greatly facilitated our understanding of recombination and replication in all organisms. Recombination is vital for the repair of damaged replication forks, as it reduces the risk of mutagenesis by bypass polymerases. Recombination-dependent replication has been documented in cases such as bacterial conjugation and transduction, as well as in eukaryotic cells during meiosis, DSB repair, telomere maintenance and nonmutagenic replication fork repair. The first gene known to be involved in recombination was identified by Clark and Margulies and was designated *recA* (Clark and Margulies, 1965). Genetic analysis of *recA* mutants

revealed sensitivity to both UV irradiation and X-rays (Clark, 1996; Howard-Flanders and Theriot, 1966), establishing a link between recombination and DNA repair. Further studies on the RecA protein showed an involvement in promoting DNA strand exchanges, autocatalytic cleavage of certain repressors to induce the bacterial SOS response, and a role in mutagenic TLS (Tang et al., 1999). Early work in bacteria led to a proposal that HR is utilized to repair broken replication forks after encountering strand breaks (Hanawalt, 1966; Skalka, 1974), and that the RecBC enzyme is employed in the processing of the resulting DSBs (Skalka, 1974). The resolution of a stalled replication fork has been postulated to occur through a four-stranded DNA HJ, which has been observed directly by electron microscopy (Higgins et al., 1976; Sogo et al., 2002; Viguera et al., 2000). HJs are formed by the unwinding of the template strands and winding of the nascent strands at stalled replication forks and are resolved by cleavage of the branch point of the junction by a helicase-endonuclease complex, such as RuvABC in *E. coli* (Seigneur et al., 1998; Zerbib et al., 1998), or they are processed in a non-cleavage manner (Flores et al., 2001; Gregg et al., 2002; McGlynn and Lloyd, 2001) (Figure 1.3).

1.5. Post-replication Repair in *Saccharomyces cerevisiae*

1.5.1. Recombination Repair in DNA Damage Tolerance

1.5.1.1. Recombination-mediated Replication Fork Restart in Eukaryotes

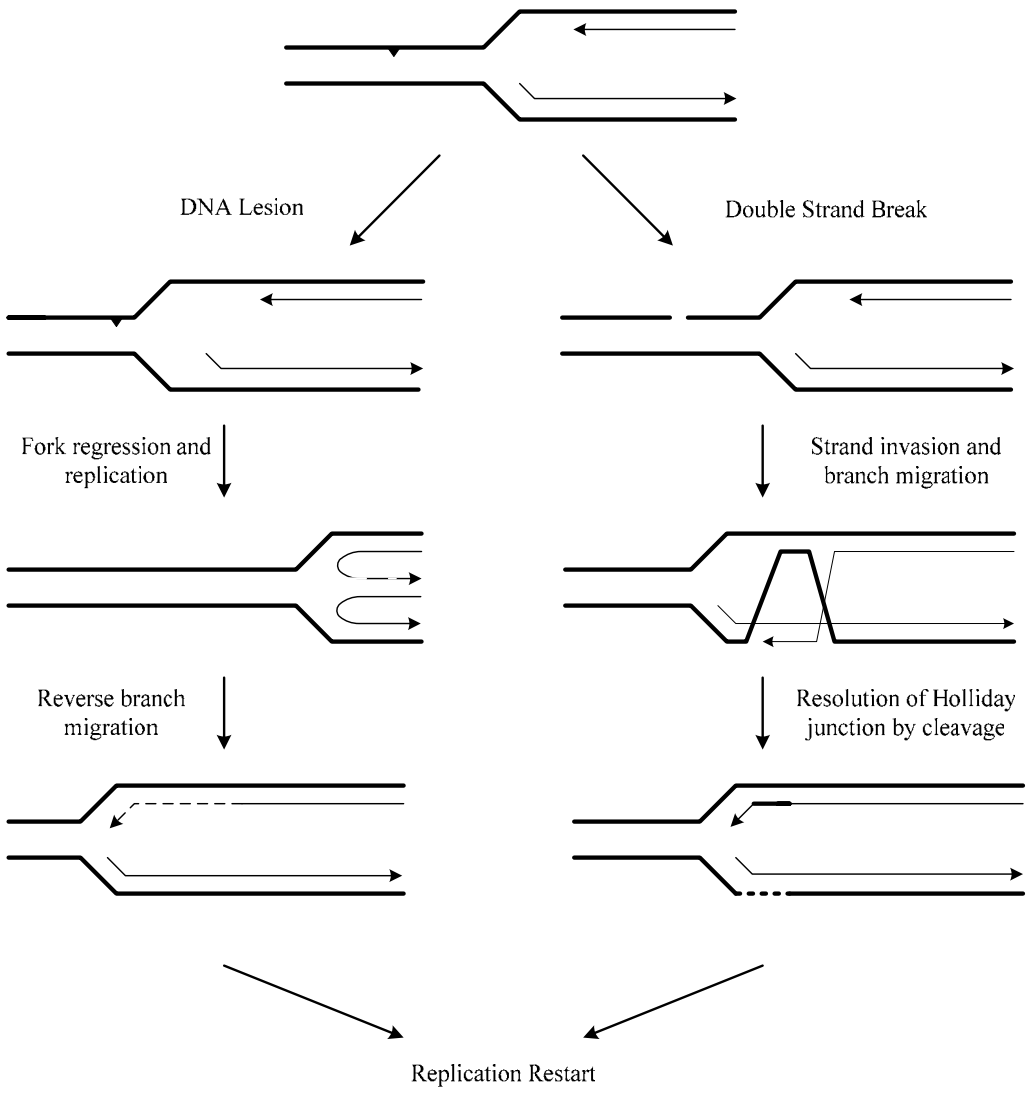

Figure 1.3. Potential mechanisms of Replication fork restart. A pathway involving fork regression is shown (left side), and a double-strand break repair path is shown for the repair of a fork collapsed at the site of a DNA strand break (right side). Arrowheads on DNA strands denote 3' ends. If a DNA lesion (other than a strand break) is responsible for halting the progress of a replication fork, note that replication fork repair does not entail repair of the lesion itself. Instead, the recombination and replication steps set up the lesion for repair by providing an undamaged complementary DNA strand (Adapted from Cox, 2001).

In contrast with the recombination events for replication repair, a 'gap-filling' model has also been proposed to account for lesion bypass during DNA replication in mammalian cells (Lehmann, 1972). This model focused on the idea that synthesis of one nascent strand continued beyond replication-blocking lesions on the opposite template strand until a new initiation point was reached at which replication would continue on both strands. The resulting ssDNA gaps would be repaired at a later time. However, recent evidence would argue for a recombination-mediated replication restart in eukaryotes. Firstly, a documented elevation in recombination in UV-irradiated *S. cerevisiae* cells has been reported. UV irradiation inhibits DNA replication in both prokaryotes and eukaryotes by inducing photoproduct formation, which blocks replication fork progression and reduces ssDNA fragment size (Lehmann, 1972; Rupp and Howard-Flanders, 1968). Incubation of NER^- cells following UV irradiation converts the small fragments into high molecular weight DNA in a process referred to as 'post-replication repair' (PRR). This allows for bypass of the lesion without removing the damage. The frequency of gene conversion and intergenic mitotic recombination increased in surviving cells upon induction by UV light; however, cells exposed to UV light appeared to have normal meiotic divisions and sporulation when compared to the controls (Snow, 1968). Secondly, results obtained from studies on mammalian cells suggest a regression of the stalled replication fork that allows recombination enzymes to repair the damaged DNA. This hypothesis is primarily based on the observation of DNA damage-induced Holliday structures using electron microscopy (Higgins et al., 1976). Thirdly, eukaryotic cells show defective PRR activity in *rad6*, *rad18* and *rad52* mutants (Prakash, 1981). Mutations in *RAD6* cause

similar phenotypes to those of *recA* mutants including sensitivity to UV, IR and DNA alkylating agents (Cox and Parry, 1968; Game and Mortimer, 1974; Lawrence et al., 1974; Prakash, 1974), and *RAD6* is required for radiation mutagenesis (Lawrence and Christensen, 1976; Lawrence et al., 1974). However, while the *rad52* mutant is defective in spontaneous and radiation-induced homologous mitotic recombination (Malone and Esposito, 1980; Prakash et al., 1980; Resnick, 1975), meiotic recombination (Game et al., 1980; Prakash et al., 1980) and UV-induced sister chromatid recombination in haploids (Prakash and Taillon-Miller, 1981), *rad6* mutants are proficient in the above processes (Montelone et al., 1981). A clue to the different roles of *RAD6* and *RAD52* in recombination may be found in the observed UV-induced genetic recombination in *rad6* mutants that occurs at times other than S phase (Fabre, 1978), while *rad52* mutants are defective in UV-induced recombination at all cell cycle phases (Prakash, 1981). This suggests a direct role for *RAD52* in recombination repair and the involvement of *RAD6* in only subset(s) of recombination activities or a regulatory role. Finally, yeast nucleotide excision repair deficient (*rad1*) cells tolerate replication-blocking lesions by utilizing a sister chromatid exchange (SCE) dependent process (Paulovich et al., 1998). Hence, *rad1* mutants are capable of replicating damaged DNA after exposure to low dose UV irradiation (James et al., 1978), suggesting a DNA damage tolerance mechanism in *rad1* cells. Daughter strand gaps formed by UV photoproducts present during replication are repaired in *rad1 rev3* mutants (Prakash, 1981), but these cells are defective in UV-induced mutagenesis (Morrison et al., 1989). These results suggest that the majority of gaps are filled by an alternative error-free mechanism in the absence of *REV3*. Conversely, *rad1 rad52*

double mutants are defective in the induction of SCE (Kadyk and Hartwell, 1993) as well as the repair of daughter strand gaps (Prakash, 1981), indicating that daughter strand gap repair is dependent on a functional recombination pathway.

1.5.1.2. Structure-specific Endonucleases in Eukaryotes

Although the above data collectively suggest a recombination mechanism, there is no direct evidence in eukaryotes that stalled replication forks are repaired through the formation and cleavage of HJ. However, it has been recently reported that a HJ-specific helicase-endonuclease activity exists in eukaryotic cells, and that this activity may be analogous to that of the *E. coli* RuvABC activity (Boddy et al., 2001; Chen et al., 2001; Kaliraman et al., 2001). The Mus81 protein was identified by its interactions with Cds1 (Boddy et al., 2000) and Rad54 (Interthal and Heyer, 2000) in *Schizosaccharomyces pombe* and *S. cerevisiae*, respectively. Mus81 shares homology with subunits of structure-specific endonucleases, Rad1-XPF, which act in NER (Boddy et al., 2000; Interthal and Heyer, 2000). Genetic analysis demonstrated that budding and fission yeasts carrying a mutation in *MUS81* are sensitive to DNA damaging agents such as MMS and UV (Boddy et al., 2000; Interthal and Heyer, 2000). However, major differences in biochemical properties between the budding and fission yeast are revealed. Initial work completed in the fission yeast, *S. pombe*, showed that a partially purified Mus81 complex can cleave synthetic HJs *in vitro* and that this enzyme facilitates the survival of *S. pombe* cells after UV irradiation (Boddy et al., 2001). Assays using an X12 HJ model, which has a 12-bp branch-migratable core, were efficiently cleaved by the *S. pombe* Mus81-Eme1 complex (Boddy et al., 2001). This

same cleavage has not been established with the *S. cerevisiae* Mus81-Mms4 complex. Additionally, a bacterial HJ resolvase, RusA, can partially rescue DNA-helicase-deficient *rqh1*$^{-}$ cells from UV sensitivity. Conversely, RusA was unable to reduce the hyper-recombination phenotype of *rqh1*$^{-}$. This led to the hypothesis that HJs are accumulated under Rqh1-depleted conditions, thus suggesting a possible role for Mus81-Eme1 in cleaving HJs (Doe et al., 2000). However, further investigation into the substrate specificity of Mus81-Eme1 and Mus81-Mms4 showed that these enzymes have the same substrate specificity. Both complexes cleave at unwound replication forks with a single-stranded tail, but show little or no activity on normal replication forks (Whitby et al., 2003). This is further supported by data showing Mus81-Mms4 cleaves at a nick 5 nucleotides 5' of the flap, which is determined by the 5' end of the DNA strand at the flap junction (Bastin-Shanower et al., 2003). Conversely the homologous endonuclease Rad1-Rad10 complex determines the cleavage by the branch point (Bastin-Shanower et al., 2003). While Mus81 is required for cleavage at specific branched molecules it is yet to be determined whether it is a true HJ resolvase. Although Mus81 may not function as a HJ resolvase it almost certainly has a role in the repair or stabilization of stalled replication forks. Firstly, *mus81* is synthetically lethal with deletion of the *SGS1* helicase gene (Mullen et al., 2001), implicating a role at stalled replication forks. Secondly, Mus81 was shown to interact with the protein kinase Cds1 (Boddy et al., 2000), also suggesting that the Mus81 endonuclease complex may act at stalled replication forks. Lastly, cells compromised in certain DNA polymerases (e.g. Polα and Polδ) rely on Mus81 to grow (Boddy et al., 2000). This data suggests that the Mus81-Eme1-Mms4 complex may be recruited to stalled

replication forks by checkpoint proteins; at this point the endonuclease activity would be able to process the stalled fork.

Several models have been proposed regarding replication fork restart and repair, which range from gaps leading to breakage or collapse of the replication fork to regression of a stalled fork due to a block in the template DNA (see (Kuzminov, 2001) for a review). However, most of the current models are based on observations made in prokaryotic organisms, which mainly rely on recombination to repair stalled replication forks (Berdichevsky et al., 2002). Data from eukaryotic cells suggest that both repair and lesion bypass mechanisms are available with error-free bypass being the predominate mechanism (Baynton et al., 1998; Eckardt-Schupp and Klaus, 1999). This is consistent with the hypothesis that DNA lesions may be tolerated by both recombinogenic and mutagenic pathways by preferentially channeling lesions into one pathway over the other (Aboussekhra et al., 1992; Heude and Fabre, 1993; Rong et al., 1991; Schiestl et al., 1990).

1.5.2. Error-free and Error-prone Replication Fork Bypass

Consistent with the above hypothesis, extensive studies in budding yeast have demonstrated that replication fork bypass can be achieved by channeling lesions into the *RAD6*-dependent DNA damage tolerance pathway. This channeling prevents the inappropriate resolving of stalled replication forks, and instead bypasses the damage, either by TLS, resulting in mutagenic or error-prone bypass, or by a damage avoidance mechanism that is deemed error-free. The ubiquitin-conjugating enzymes are an essential part of the PRR pathway. The process of ubiquitination requires the activity of

four different enzymes: a ubiquitin activating enzyme (Uba or E1), a ubiquitin conjugating enzyme (Ubc or E2), a ubiquitin protein ligase (Ubl or E3) and an E4 enzyme (Hershko and Ciechanover, 1998; Koegl et al., 1999). The E1 enzyme activates Ub by binding the ubiquitin molecules to the E1 active site in an ATP-dependent manner. The ubiquitin conjugating enzyme is required to accept the activated Ub onto a cysteine residue in its active site, and the Ub is attached by the E3 to the target protein as polyubiquitin chains, predominately linked through a Lys48 residue (Chau et al., 1989). Ubiquitin-conjugating enzyme variant (UEV) proteins resemble ubiquitin-conjugating enzymes but lack the active-site residue and have been genetically implicated in error-free PRR. The Rad6-Rad18 ubiquitin-conjugating enzyme complex is required for PRR (Bailly et al., 1994; Prakash, 1981). The *RAD6* gene encodes a Ubc (Ubc2) (Jentsch et al., 1987), whose active site cysteine residue is essential for all of its known functions (Sung et al., 1990). The *rad6* mutants are extremely sensitive to a variety of DNA damaging agents, defective in UV-induced mutagenesis and sporulation, and exhibit severe growth deficiencies (Prakash et al., 1993). However, unlike *rad6*, *rad18* mutants do not display either defective sporulation or growth retardation (Jones et al., 1988; Lawrence, 1982). Rad18 displays a ssDNA-binding activity (Bailly et al., 1994) allowing for the Rad6-Rad18 complex to bind the single-stranded regions that result from stalled DNA polymerases. It is speculated that the E2 activity of Rad6 could mediate the ubiquitination of the stalled replication machinery. The *MMS2*-encoded UEV protein was classified as a member of the error-free *RAD6* pathway (Broomfield et al., 1998; Xiao et al., 1999), and it has been shown to form a stable complex with a second E2, Ubc13; this complex is capable of assembling an

alternate type of Lys63 multiubiquitin chain (Hofmann and Pickart, 1999). Rad5, a chromatin-associated RING finger protein with E3 activity, may be responsible for the recruitment of the Mms2-Ubc13 complex to the Rad6-Rad18 complex (Ulrich, 2003; Ulrich and Jentsch, 2000). Until recently the target of the ubiquitin modification has remained elusive. It was recently reported (Hoege et al., 2002) that proliferating cell nuclear antigen (PCNA), a DNA-polymerase sliding clamp involved in DNA replication and repair, is a substrate for modification by ubiquitination and small ubiquitin-related modifier (SUMO) conjugation. SUMO conjugation of PCNA is regulated by the cell cycle and precedes the rise of PCNA during S phase. Conversely, mono- and multi-ubiquitination of PCNA occurs only after treatment with a sublethal dose of DNA-damaging agents, and ubiquitination targets the same conserved lysine residue (Lys164) of PCNA used for SUMO conjugation (Hoege et al., 2002). Mono-ubiquitination of PCNA is dependent on *RAD6* and *RAD18*, while *UBC13*, *MMS2* and *RAD5* are required to attach additional ubiquitins to the conjugate (Hoege et al., 2002). Conjugation of PCNA by ubiquitin or SUMO differentially affects resistance to DNA damage, indicative of a regulatory role of PCNA modification, which supports a notion that PCNA is involved in error-free PRR (Torres-Ramos et al., 1996; Xiao et al., 2000), probably in the process of replication restart and/or a template switch (Broomfield et al., 2001) (Figure 1.4.).

1.5.2.1. Error-prone Replication Fork Bypass

Whatever its source, a replication block has to be repaired or bypassed, and replication must restart. How do cells bypass or clear such obstacles? The major

alternative bypass pathway, TLS, can be either non-mutagenic or mutagenic, depending on the type of damage and on the repertoire of translesion polymerases available to the cell. Several observations suggest ubiquitination of members involved in the *RAD6*-dependent DNA repair pathway elicits a bypass response to a stalled replication fork. Firstly, Polζ, encoded by *REV3* and *REV7*, is a non-essential mutagenic polymerase capable of replicating over damaged regions of DNA with low processivity (Nelson et al., 1996). Secondly, epistatic analysis with *rev3* and *mms2* places *POL32,* encoding a non-essential subunit of Polδ (Gerik et al., 1998), in the error-prone branch of the *RAD6*-dependent pathway; however, *pol32* suppresses UV-induced mutagenesis but variably affects spontaneous mutagenesis (Huang et al., 2000)M. Hanna and W. Xiao, unpublished results). Lastly, Pol32, interacts with both Pol30 (Gerik et al., 1998) and Srs2 (Huang et al., 2000). These observations suggest that Pol32 may act as a coupling factor between replication and TLS by coordinating between Srs2 and Pol30/PCNA at the site of damage (Broomfield and Xiao, 2002). One may further speculate that the mono-ubiquitinated form of PCNA could be sequestered by Pol32, resulting in the recruitment of mutagenic polymerases (Figure 1.4).

1.5.2.2. Error-free Replication Fork Bypass

Error-free replication through UV-induced cyclobutane pyrimidine dimers is thought to be carried out by the *RAD30*-encoded DNA polymerase η under the control of *RAD6* (McDonald et al., 1997). Polη replicates through thymine dimers with the efficiency and accuracy comparable to undamaged thymines (Lee et al., 1999). In

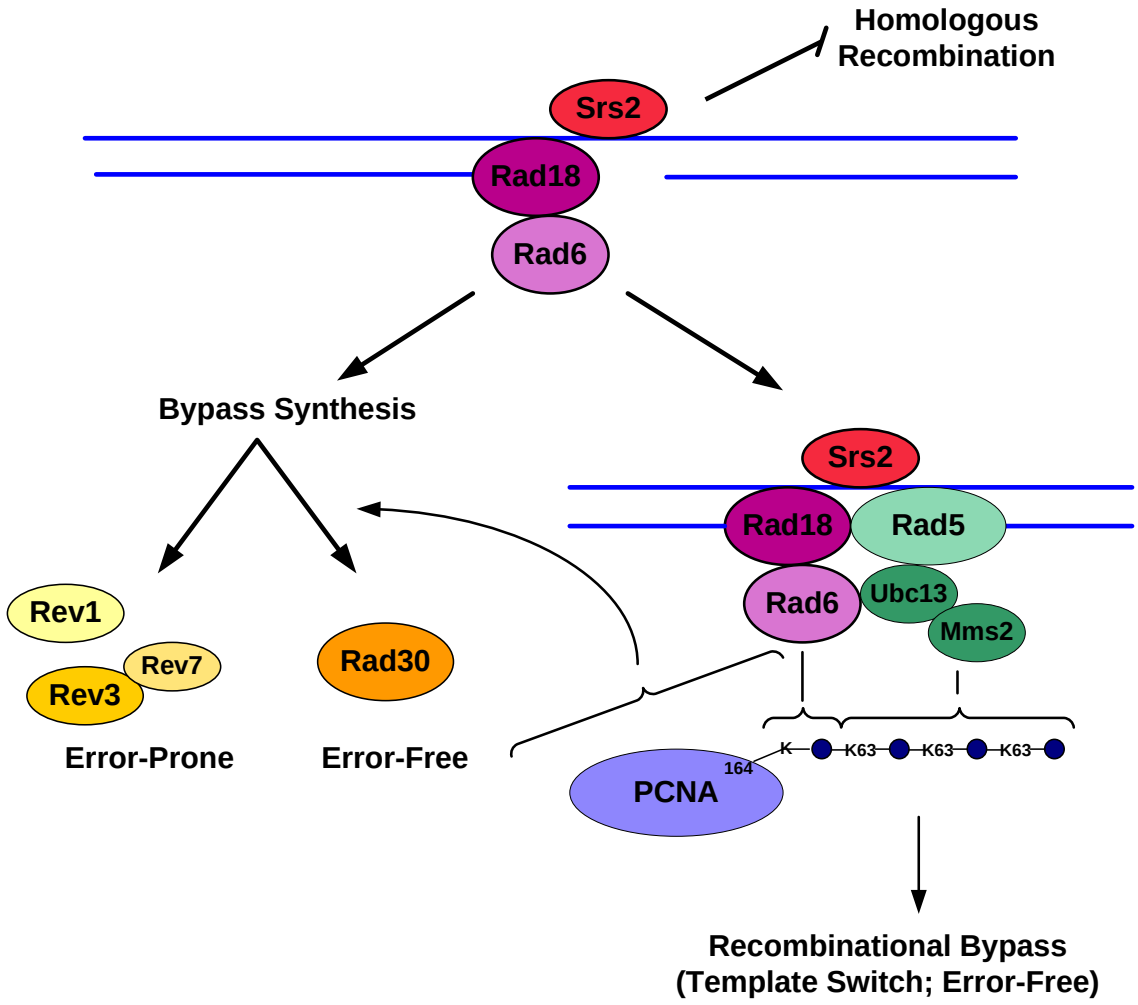

Figure 1.4. Proposed model for the *RAD6*-dependent processing of blocked replication forks. Ub conjugation of PCNA targets the conserved lysine residue (Lys164). Mono-ubiquitination of PCNA is dependent on *RAD6* and *RAD18*, while *RAD5*, *MMS2* and *UBC13* are required to attach additional ubiquitin to the conjugate. Poly-ubiquitination of PCNA shuttles the repair into an error-free damage avoidance via a template switching or a SCE mechanism. Mono-ubiquitination of PCNA will shuttle the lesion into a translesion synthesis via Polζ or Polη. (Adapted from Roswell Park Cancer Institute)

addition to thymine dimers, UV also induces the formation of cyclobutane dimers and (6-4) photoproducts at T-C and C-C sites (Lippke et al., 1981). Although these lesions are unstable and difficult to study, genetic analysis has shown an increased incidence of UV-induced mutations at T-C and C-C sites (Washington et al., 1999; Yu et al., 2001); it is not known if Polη will bypass these lesions with high fidelity. Overall Polη is regarded as a low-fidelity enzyme with a misincorporation frequency of ~10^{-2} to 10^{-3} (Washington et al., 1999). Furthermore, bypass polymerases would not be able to manage with non-informative lesions such as abasic sites. Hence, it is conceivable that an error-free bypass polymerase must have limited substrate specificity and an authentic error-free PRR must utilize the homologous template.

Recombination is vital for the repair of damaged replication forks to reduce the risk of mutagenesis by bypass polymerases. The mechanism of eukaryotic recombinational bypass has not yet been elucidated. Error-free PRR may rely on an undamaged homologous template brought into the stalled replication fork through an HR process. Several observations support a recombination repair bypass hypothesis. Firstly, the extreme UV and MMS sensitivities of *rad6* and *rad18* are suppressed by *srs2* only in the presence of a functional recombination repair pathway (Schiestl et al., 1990). The *srs2* mutation also suppresses the slow growth defect of *rad6* mutants to a limited extent, but not the UV mutagenesis and sporulation defects (Schiestl et al., 1990). Secondly, *srs2* cells display moderate UV and MMS sensitivity which can be suppressed by *rad51* (Aboussekhra et al., 1992; Chanet et al., 1996). The UV sensitivity of diploids homozygous for a genomic deletion of *SRS2* is suppressed by semidominant heterozygous mutations in *RAD51*. These results suggest that the Srs2

helicase is involved in the metabolism of single-strand gaps to prepare a substrate for lesion bypass or damage avoidance. The absence of Srs2 allows access of recombination proteins to these gaps to initiate recombination repair (Aboussekhra et al., 1992). Indeed, *srs2* cells have elevated levels of recombination, suggesting cells promote recombination-based DNA damage tolerance in the absence of Srs2 helicase (Rong et al., 1991). Finally, the suppression of UV and MMS sensitivity by *srs2* appears to act either specifically on the error-free branches (Broomfield and Xiao, 2002) or on the entire *RAD6* pathway (Broomfield and Xiao, 2002; Ulrich, 2001). Srs2 has been suggested to act upstream of the *RAD6*-dependent repair machinery. However, suppression of *rad6/rad18* null mutants by *srs2* results in a phenotype where the double mutants are more sensitive to UV than the *srs2* single mutant (Rong et al., 1991; Schiestl et al., 1990). Further investigation into the genetic interactions between *SRS2* and the other members of the *RAD6*-dependent pathways showed members to be differentially affected by deletion of the *SRS2* gene. For example, suppression of *rad5*, *ubc13* and *mms2* mutants is to the level of the *srs2* single mutant itself (Broomfield and Xiao, 2002; Ulrich, 2001), whereas suppression of *pol30-46* by *srs2* is complete in one report (Broomfield and Xiao, 2002) but only partial in another report (Ulrich, 2001). These observations suggest that when Srs2 is inactive, at least some of the lesions will be processed by HR. However, when mutations occur in genes involved in the *RAD6* pathway, neither PRR nor HR is able to process the replication blocks, resulting in the extreme cellular sensitivity to DNA-damaging agents. Under this condition, inactivation of Srs2 would allow the lesions to be accessed by the *RAD52* recombination pathway and suppress the PRR mutant phenotypes. In response to MMS

damage, progression of wild-type cells through S phase is reduced (Paulovich and Hartwell, 1995; Paulovich et al., 1997a) due to a slowing of replication fork progression and the inhibition of late replication origin firing (Tercero and Diffley, 2001). Both the *rad6* and *rad52* mutant strains display substantially slower progression through S phase in the presence of MMS-induced DNA damage compared with the wild-type strain (Chang et al., 2002). This suggests that both bypass repair and recombination repair are required for replication in the presence of MMS-induced DNA damage. In the absence of both recombination and PRR the cell would appear to have no means to overcome a stalled replication fork. However, the *rad6 srs2 rad52* triple mutant defective for both PRR and recombination is still viable (Schiestl et al., 1990), suggesting that there must be an alternative pathway for reinitiating spontaneously stalled replication forks. (Figure 1.5.)

1.5.3. Role of Srs2 in Replication Bypass

1.5.3.1 Model for Srs2 as a Molecular Switch between PRR and Recombination

The above observations pertaining to *srs2* phenotypes suggest that Srs2 acts as a molecular switch between the PRR and the recombination pathway. This can be achieved by either inhibition of recombination or by the promotion of PRR. Srs2 possesses 3' to 5' DNA helicase activity (Rong and Klein, 1993), which is crucial for recombination (Rong et al., 1991) and suppression of PRR defects (Broomfield and Xiao, 2002). Single strand gaps could result in potentially dangerous intermediates that cannot be processed normally by the cell and thus are toxic or result in genomic instability. In order to prevent inappropriate processing of such intermediates, cells

must have developed a survey system to determine whether recombination will result in such deleterious events. Genetic data has shown that Srs2 negatively regulates recombination (Aboussekhra et al., 1989; Aguilera and Klein, 1988), and it is suggested that this occurs by reversal of intermediate recombination structures (Chanet et al., 1996; Kaytor et al., 1995; Milne et al., 1995; Schild, 1995). Recent biochemical evidence shows that DNA strand exchange mediated by Rad51 is inhibited by Srs2 through disruption of the Rad51 filaments formed on ssDNA (Krejci et al., 2003; Veaute et al., 2003). Moreover, Srs2 has an ATPase activity that is dependent on ssDNA (Krejci et al., 2003; Veaute et al., 2003) and Srs2 interacts with Rad51 genetically (Chanet et al., 1996; Milne et al., 1995; Schild, 1995) and physically (Krejci et al., 2003). Although replication protein A (RPA) can function as a cofactor in the assembly of the Rad51 presynaptic filament (Sugiyama et al., 1997; Sung, 1994), it appears that RPA may compete with Rad51 for binding to ssDNA (Sugiyama et al., 1997; Sung, 1994; Sung, 1997) to promote the anti-recombination function of Srs2 by preventing reassembly of the presynaptic filament (Krejci et al., 2003).

Formation of a presynaptic filament by Rad51 is analogous to the HR mechanism used for replication fork reversal in *E. coli*. HR is triggered when ssDNA cannot be replicated, resulting in the recruitment of SSB (SSB in *E. coli*; gp32 in T4 and RPA in yeast). SSB is displaced from ssDNA by DNA replication and degradation enzymes but also helps prevent binding of recombinases and helicases. Under conditions where replication is stalled, replication enzymes are replaced by recombinases through the use of recombination-mediator proteins. The mechanism of recombination repair mediated by a ssDNA gap appears to be regulated by the RecF

pathway in *E. coli* (Horii and Clark, 1973; Tseng et al., 1994; Wang and Smith, 1984), in which RecF, RecO and RecR proteins facilitate the loading of RecA protein onto SSB-coated ssDNA gaps (Morimatsu and Kowalczykowski, 2003). However, whether and how Srs2 actively promotes PRR in yeast is still unclear.

1.5.3.2. Cell Cycle Regulation of Srs2

The above model predicts that Srs2 activity should be tightly regulated during the cell cycle and upon DNA damage. Investigation into additional phenotypes of *srs2* mutant cells and the regulation of Srs2 activity may provide some clues regarding the interaction between DNA replication, repair and recombination functions at stalled replication forks. *SRS2* is expressed at a low level but rises slightly in S-phase, likely in response to transcriptional induction at, or near, the G1/S boundary. In addition, Srs2 is induced by UV irradiation of exponentially growing cells, but this inducibility is limited to G2 cells (Heude et al., 1995). Interestingly, *srs2* haploids and diploids are sensitive to UV only in G1, indicating that Srs2 is essential for processing of lesions or repair intermediates at this stage. Perhaps post-translational modification of Srs2 is a major means of its regulation. Indeed, Srs2 is phosphorylated in response to intra-S-phase DNA damage and this phosporylation requires a functional checkpoint pathway, including Dun1 (Liberi et al., 2000). In response to DNA damage, the Dun1 protein kinase, along with Mec1 and Rad53, allow cell survival by delaying cell cycle progression and by promoting transcription of DNA metabolism genes (Foiani et al., 2000). In turn, *SRS2* appears to be required for the S-phase DNA damage checkpoint, since *srs2* mutants fail to activate Rad53 in response to MMS treatment (Liberi et al.,

2000), suggesting that Srs2 is indeed a component of the damage checkpoint pathway. Additional supporting evidence comes from observations that *srs2* partially rescues *rad17* cells from lethality (Liberi et al., 2000) and that SCE is partially dependent on the *RAD9* and *RAD17* checkpoint genes (Paulovich et al., 1998). Wild-type yeast cells respond to alkylation damage and UV irradiation by decreasing the rate at which cells progress through S phase (Paulovich et al., 1998; Paulovich and Hartwell, 1995), possibly indicating a requirement for additional time to replicate over DNA lesions using either TLS or lesion bypass. Srs2 is also required to recover from a checkpoint-mediated G2/M arrest after induction of ds breaks and for recovery following HO-induced ectopic gene conversion; the failure to recover from checkpoint arrest can be suppressed by mutations in *RAD51* (Vaze et al., 2002). Taken together, it is conceivable that Srs2 is a primary candidate for a molecular switch through interaction with cell cycle checkpoints, possibly by channeling intra-S DNA damage into a SCE or template switching mode of replication.

1.6. Control of Genomic Stability by Helicases

DNA helicases are ubiquitous enzymes that catalyze the unwinding of duplex DNA during replication, recombination and repair. Although not all helicases unwind duplex DNA in an identical way, many helicases possess similar properties, likely to be of importance to their mechanism of action. Helicases play essential roles in nearly all DNA metabolic transactions and have been implicated in a variety of human genetic disorders. DNA helicases catalyze the disruption of the hydrogen bonds that hold the two strands of dsDNA together. This energy-requiring unwinding reaction results in the

formation of the ssDNA required as an intermediate in DNA replication, repair and recombination (Matson et al., 1994).

1.6.1. Involvement of Helicases at Stalled Replication Forks

In order for repair proteins to be able to access DNA damage sites, the replication machinery needs to be cleared away from the block to facilitate subsequent repair and/or bypass. The use of helicases is an attractive means of unwinding the stalled fork. *In vitro* studies revealed that RecG in *E. coli* can specifically unwind forked DNA structures to form HJs (McGlynn and Lloyd, 2000; McGlynn et al., 2001; Singleton et al., 2001). This process would facilitate access to ssDNA gaps opposite the lesion to allow replication to resume. RuvAB can also unwind a HJ (Parsons et al., 1992). The exact mechanism for determining which structure each helicase works on has yet to be elucidated. However, it has been determined that a synergistic increase in the DNA repair defect occurs in *recG ruv* double-mutant strains as compared with either single mutant (Bolt and Lloyd, 2002; Gregg et al., 2002), suggesting that there are two alternative mechanisms to deal with DNA damage, and that these two activities may deal with distinct and overlapping types of replication-fork damage.

Although fork-unwinding activities analogous to that of RecG have yet to be detected in eukaryotes, a family of helicases that are related to *E. coli* RecQ has been implicated in the processing of damaged forks. Recent studies in *S. pombe* implicate the RecQ homolog, *rqh1*, in the bypass or stabilization of DNA damage at replication forks. Firstly, mutations in *rqh1* enhance UV sensitivity (Doe et al., 2002), and *rqh1*$^-$ cells are unable to segregate their chromosomes during mitosis after S-phase arrest,

resulting in a decreased viability in recovery from hydroxyurea (HU) treatment. Only recovery, not checkpoint control, is abolished by mutation of *rqh1* (Stewart et al., 1997). Rqh1 is required in cells with defects in DNA synthesis (Doe et al., 2000), again implying a role for Rqh1 in stabilization of replication intermediates. Secondly, *S. pombe* cells lacking both the Mus81 endonuclease and the Rqh1 helicase are inviable (Boddy et al., 2001; Boddy et al., 2000; Doe et al., 2002), suggesting that Mus81 is required for a step in the resolution of HJs that accumulate in *rqh1*$^{-}$ cells. This is further supported by the evidence that expression of a bacterial HJ resolvase partially rescues the DNA repair defects of *rqh1*$^{-}$, implying that HJs formed from stalled forks might be resolved by Rqh1-catalysed unwinding (Boddy et al., 2001; Boddy et al., 2000; Doe et al., 2002). Thirdly, Mus81 binds to the checkpoint kinase Cds1 and is phosphorylated in a Cds1-dependent manner during S phase and hyperphosphorylated in response to replication arrest (Boddy et al., 2000). Finally, cells lacking either Mus81 or the recombination protein Rhp54 exhibit a checkpoint-dependent delay in the cell cycle (Boddy et al., 2000; Muris et al., 1996). These observations suggest that *rqh1* and *mus81* function in a recombination-dependent pathway for DNA damage tolerance in response to delays or stalling of the replication machinery.

The *S. cerevisiae* RecQ homolog, Sgs1, unwinds stalled forks in the same polarity (3' to 5') as Rqh1 (Bennett et al., 1999; Laursen LV et al., 2003). Sgs1 is responsible for unwinding various branched DNA structures *in vitro* (Bennett et al., 1999), and appears to act as part of a complex with topoisomerase III (Gangloff et al., 1994). An *sgs1* null mutant exhibits normal growth but is characterized by increased genomic instability (Watt et al., 1996; Yamagata et al., 1998). Exponentially growing

haploid cells treated with γ-rays are proficient in recombination between sister chromatids in *sgs1* mutants. Conversely, diploid mutants are highly sensitive to IR suggesting the importance of Sgs1 in the recovery of radiation-induced recombinants through recombination involving homologous chromosomes (Gangloff et al., 2000). These results suggest that Sgs1 may play a role in promoting maturation of recombination intermediates.

1.6.2. Overlapping Functions of Sgs1 and Srs2

Unlike *sgs1*, *srs2* cells show evidence of increased UV-stimulated heteroallelic recombination (Aboussekhra et al., 1992). Deletion of *SRS2* results in synthetic lethality (Lee et al., 1999) or very poor growth with *sgs1* (Gangloff et al., 2000), and deletion of the recombination genes *RAD51*, *RAD55* or *RAD57* suppresses the growth defect of the *srs2 sgs1* double mutant (Gangloff et al., 2000). It has been suggested that Sgs1 and Srs2 may be functionally redundant, based on the fact that either single mutant is viable, but the double mutant has a severe-to-lethal growth defect (Lee et al., 1999), and overexpression of *SGS1* partially suppresses *srs2* phenotypes. However, complete redundancy can be ruled out due to some different phenotypes exhibited by each single mutant, and overexpression of *SRS2* does not complement the genotoxicity of *sgs1* mutants (Mankouri et al., 2002). Although Sgs1 may have functions that cannot by complemented by Srs2, the lack of complementation of *sgs1* by overexpression of *SRS2* may be partially due to the detrimental effects of higher levels of Srs2 in wild-type strains (Kaytor et al., 1995; Mankouri et al., 2002). It is conceivable that the

overlapping function of Sgs1 and Srs2 may be involved in the processing of recombination intermediates formed during replication.

1.6.3. Cell Cycle Regulation of Sgs1

Recent observations suggest Sgs1 is an important component of the S-phase checkpoint response. Firstly, like *SRS2*, expression of *SGS1* peaks during S phase of the cell cycle. However, unlike Srs2, Sgs1 levels undergo sharp fluctuations, being most abundant in S phase and barely detectable through metaphase and early G1 (Frei and Gasser, 2000). Secondly, Sgs1 appears to be required for a functional S-phase checkpoint and co-localizes with Rad53 to S-phase-specific nuclear foci during replication, whereas the G1/S and G2/M DNA damage checkpoints are fully functional in *sgs1* cells (Frei and Gasser, 2000). Analysis of the Rad53 phosphorylation state in the presence of HU places Sgs1 upstream of Rad53 (Frei and Gasser, 2000). Thirdly, *sgs1* was found to be epistatic to a mutation in DNA polymerase ε (Frei and Gasser, 2000), which prevents cell cycle arrest in response to an unscheduled block in DNA replication (Navas et al., 1995). The Sgs1/Polε-dependent checkpoint most likely works in a parallel pathway to Rad17 and Rad24, and is responsible for a portion of the signaling cascade that activates Mec1 and Rad53 in response to DNA damage in S phase. Loss of both Sgs1 and Rad24 prevents Rad53 phosphorylation, and allows passage through the cell cycle prior to completing DNA replication (Frei and Gasser, 2000). Finally, it was recently shown that replication checkpoints suppress the formation of HJ-like replication intermediates at stalled replication forks (Sogo et al., 2002). Indeed, Sgs1 is able to bind branched DNA structures, and its DNA helicase activity has been assayed

on HJs (Bennett et al., 1999). Since *mms4* and *mus81* are synthetic lethal with *sgs1* (Mullen et al., 2001), it is reasonable to hypothesize that, in the absence of Sgs1, yeast cells have to rely on a HJ resolvase-like activity mediated by Mus81-Mms4 (Kaliraman et al., 2001) to resolve or prevent the formation of recombination intermediates.

In summary, Srs2 and Sgs1 have distinct as well as overlapping functions and they are both involved in the regulation of recombination bypass of replication forks. Both *sgs1* and *srs2* phenotypes appear to be a consequence of unrestrained recombination (Gangloff et al., 2000). It is unclear which substrates or subset of substrates each helicase acts on; however, it is attractive to hypothesize that both helicases are responsible for recognizing DNA damage and preventing undesirable recombination, or possibly in the case of Sgs1, promoting appropriate recombination (Fig. 1.5.).

1.7. Topoisomerases

Unwinding of DNA by helicases introduces topological stress in the DNA. Cells are equipped with DNA topoisomerases to solve the topological problems associated with DNA replication, transcription, recombination and chromatin remodeling. To relieve topological problems, topoisomerases temporarily introduce single- or DSBs in the DNA. In addition, topoisomerases are responsible for altering the degree of DNA supercoiling during replication and transcription and to resolve joint DNA molecules that arise during mitosis and meiosis. (Champoux, 2001). Evidence now suggests that RecQ helicases can act with topoisomerases to disrupt joint DNA molecules. Two classes of DNA topoisomerases exist; Type I topoisomerases create

ssDNA nicks to relax supercoiled DNA, whereas Type II topoisomerases make dsDNA breaks and pass one dsDNA molecule through another. Four topoisomerases have been identified in the yeast *S. cerevisiae*, three of which are nuclear and one mitochondrial (Champoux, 2001). The three nuclear topoisomerases are encoded by *TOP1*, *TOP2* and *TOP3*. Deletion of *TOP1* (a type II enzyme) has no effect on growth rate or viability under normal conditions (Holm et al., 1985; Thrash et al., 1985). Top1 is required for DNA replication (Kim and Wang, 1989), mitotic chromosome condensation (Castano et al., 1996) and general transcription repression in stationary phase (Choder, 1991). A second type II enzyme, Top2, is essential for viability in yeast and is required for the resolution of intertwined chromosomes during mitosis and meiosis (Holm et al., 1985; Holm et al., 1989; Uemura et al., 1987). Top3 (a type I enzyme) acts on negatively supercoiled ssDNA (Kim and Wang, 1992). Phenotypically, cells lacking *TOP3* grow poorly (Kim and Wang, 1992; Wallis et al., 1989), accumulate in G2/M, are sensitive to DNA damaging agents (Chakraverty et al., 2001; Saffi et al., 2001), have severe meiotic defects (Bailis et al., 1992; Gangloff et al., 1999) and exhibit hyper-recombination between repetitive sequences such as telomeres and rDNA (Bailis et al., 1992; Kim and Wang, 1989; Wallis et al., 1989).

Evidence indicates that *SGS1* interacts with all three nuclear topoisomerases to achieve different tasks. Originally, the *SGS1* gene was identified as a loss-of-function mutation that suppressed the slow growth of *top3* mutants and was cloned by its interaction with Top3 in a two-hybrid system (Gangloff et al., 1994; Watt et al., 1996). Sgs1 and Top3 interact in a DNA independent manner (Watt et al., 1996), which imply the possibility that Sgs1 may be involved in recruiting Top3 to the DNA. Also, deletion

of *SGS1* partially suppresses the hyper-recombination and meiotic defects of *top3* cells (Gangloff et al., 1999) but not their sensitivity to DNA damaging agents (Fricke et al., 2001). *SGS1* was also identified in a 2-hybrid screen for Top2, and a genetic interaction has been observed between *SGS1* and *TOP1* with the double mutant displaying a slow grown rate (Watt et al., 1995).

1.8. DNA Replication and Repair

Eukaryotic RecQ-like helicases are implicated in several aspects of DNA metabolism. These include the resolution of aberrant DNA structures, suppressing illegitimate recombination and restarting DNA replication. It is also becoming apparent that RecQ helicases function in numerous mechanisms associated with DNA repair and replication. Three separable functions for the eukaryotic RecQ helicases in replication have been proposed: the restart of stalled replication, recombination repair of lesions at stalled forks and the resolution of aberrant DNA secondary structures. Co-localization of Sgs1 with the checkpoint kinase Rad53 in S-phase specific foci suggests that Sgs1 is required for proper association of Rad53 with chromatin (Frei and Gasser, 2000) and places Sgs1 at the stalled fork. Additionally, *in vitro* assays have established RecQ family members as promiscuous helicases capable of acting on a variety of substrates that include synthetic HJs (Constantinou et al., 2000; Kamath-Loeb et al., 2001; Karow et al., 2000). Given the variety of known proteins that interact with Sgs1 it is possible to speculate as to the cooperation of these proteins in the resolution of aberrant DNA structures. These proteins include the SSA protein Mgs1, Rad51, which binds ssDNA and aligns it with duplex DNA, and the topoisomerases required for decatenation of

duplex DNA. Theses DNA structures may form *in vivo* at regions of ssDNA during DNA replication and their resolution by RecQ helicases may be critical for the restart of replication.

1.9. Evidence for a Novel Damage Avoidance Pathway

As discussed before, the fact that *rad6 rad52* double mutants defective in both PRR and HR are viable suggests that yeast cells may employ alternative mechanism(s) to resolve lethal replication blocks. The best evidence for an alternative pathway is seen in mutants in the *MGS1* gene. Firstly, *MGS1* encodes a protein with homology to *E. coli* RuvB, required for HJ branch migration, and to the eukaryotic clamp loader protein Rfc (Hishida et al., 2001). Mgs1 possesses DNA-dependent ATPase and DNA-annealing activities (Hishida et al., 2001; Hishida et al., 2002). Secondly, like *sgs1* and *srs2*, *mgs1* mutants have an increased rate of HR, which is further elevated when combined with a mutation in *TOP3* (Hishida et al., 2001). *mgs1* was also found to confer a severe growth defect with *sgs1*, and the double mutant has a high percentage of cells arrested as large budded cells with a single nucleus at the neck of the bud (Branzei et al., 2002b). The *mgs1 sgs1* double mutant exhibits elevated recombination and SCE frequencies (Branzei et al., 2002b). These results suggest that, in the absence of both Mgs1 and Sgs1, replication forks collapse and some of the resulting ds breaks are repaired through SCE and HR. However, additional pathways are involved in repair of the lesions that accumulate in *mgs1 sgs1* cells, since deletion of *RAD52* in the double mutant does not result in a lethal or significantly elevated growth defect (Branzei et al., 2002b). Thirdly, *mgs1* is synthetic lethal with members of the PRR pathway, including

rad18 and *rad6*, and causes a synergistic growth defect with *rad5* (Hishida et al., 2002). Overexpression of Rad52 or deletion of *SRS2* results in suppression of the severe growth defect of *mgs1 rad18* cells (Hishida et al., 2001). In addition, the *mgs1 rad18 srs2* triple mutant does not display a growth defect, but results in a synthetic lethal phenotype when combined with *rad52* or *rad51* mutations (Hishida et al., 2001). Therefore, the *RAD52* HR pathway is essential when neither Mgs1 nor Rad18 is functional. The severe growth defect of the *mgs1 rad5* double mutant is seen in response to HU treatment, whereas its sensitivity to UV and MMS is similar to that of the *rad5* single mutant (Hishida et al., 2002). HU is responsible for the depletion of nucleotide pools, thus resulting in stalled replication. This suggests that the growth defect in the *mgs1 rad5* double mutant is a consequence of the inability to process stalled replication forks, not a consequence of a defect in DNA repair. Conversely, overproduction of Mgs1 sensitizes cells to MMS and HU, but does not cause a growth impairment (Hishida et al., 2001), suggesting that *MGS1* overexpression does not create a specific kind of DNA lesion. Fourthly, overexpression of Mgs1 causes lethality in *mec1* and *rad53* mutants (Branzei et al., 2002a). Since Mgs1 interacts functionally with DNA polymerase δ (Branzei et al., 2002a), this suggests that *MGS1* overexpression may cause the DNA polymerase δ complex to become unstable and stall. This would have detrimental effects in *mec1* and *rad53* cells, which are prone to form HJ intermediates at stalled replication forks (Lopes et al., 2001). The *mgs1-18 rad18* double mutant experiences a delay in S phase and arrests at late S/G2, which often indicates leaky DNA synthesis and suggests that completion of DNA replication is blocked in these cells (Hishida et al., 2002). Finally, deletion or overexpression of *MGS1* suppresses a

POL3 temperature sensitive mutation (Hishida et al., 2001). These results indicate that Mgs1 participates in an alternative pathway responsible for regulating the processivity and fidelity of replication catalyzed by DNA polymerase δ, and is essential in restoring arrested replication forks when *RAD6*-dependent PRR and HR pathways are impaired (Figure 1.5).

1.9.1 Regulation of Replication Fork Restart by Three Proteins

Current studies indicate that stalled replication forks, either due to external DNA damage or internal cellular influences, are tightly regulated in a complicated system involving DNA damage checkpoints, cell cycle stage, chromosomal content and replication machinery. Current research supports the idea that cells have the option of utilizing different replication fork bypass mechanisms, and that these mechanisms are tightly regulated. The exact mechanisms determining the mode of action preferred by cells under different cell cycle stages and conditions of stress have yet to be elucidated. It would be attractive to speculate that there exists a cooperative or coordinated regulatory mechanism for Sgs1, Srs2 and Mgs1 in replication fork restart/bypass. Cells encountering DNA damage while replicating DNA have different options. Ideally, cells would prefer a mechanism that will ensure a pristine DNA structure being preserved. By sensing the DNA content of the cell, either by monitoring cell cycle proteins (e.g., their expression and modification), or haploid- and diploid- specific proteins, the cell may be able to choose the most appropriate mechanism to bypass the obstruction. Cells could restart replication downstream of the lesion by repriming the replication

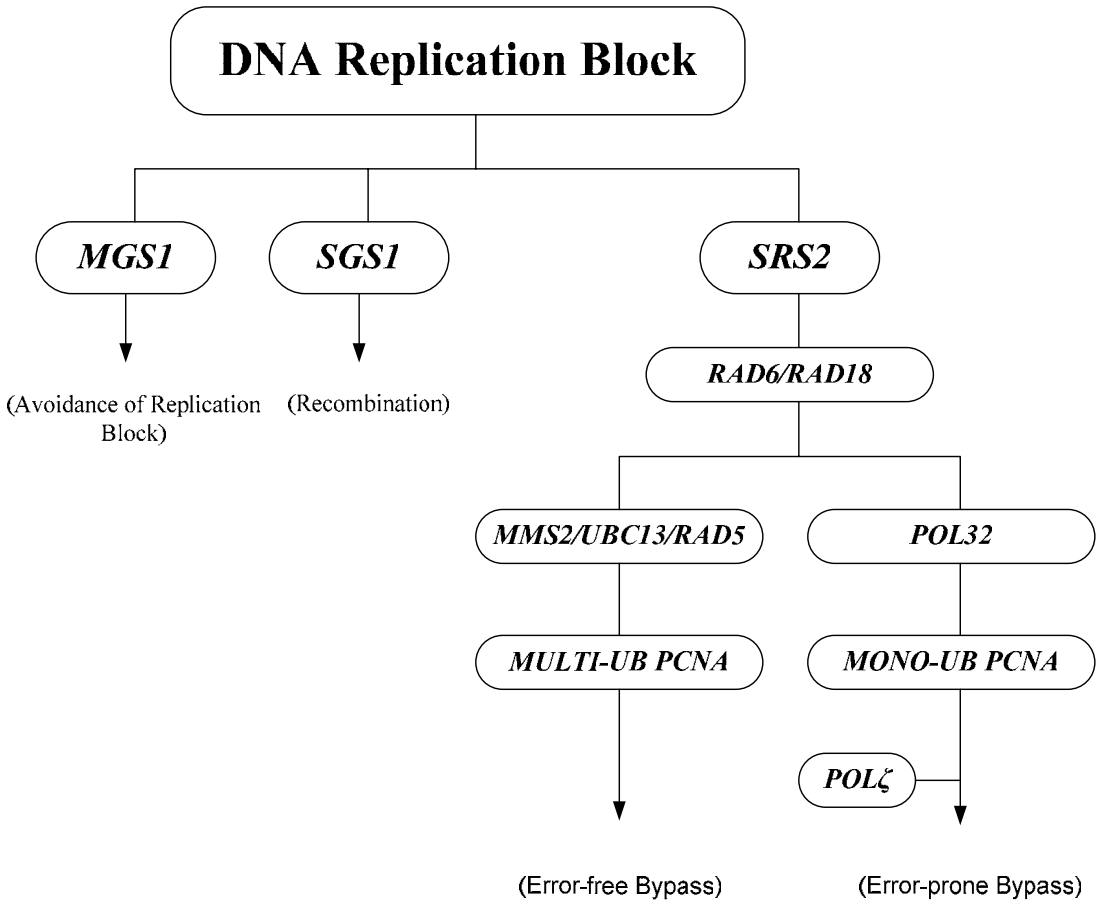

Figure 1.5. Proposed pathways for processing blocked replication forks. Srs2 can shuttle cells into the *RAD6*-dependent pathway when homologous templates are not available and also prevent inappropriate recombination. Sgs1 helps stabilize stalled forks and prevents inappropriate recombination. It may also play a role in resolving Holliday intermediates. In the absence of both recombination and the *RAD6*-dependent pathway, Mgs1 may facilitate fork progression through an alternative damage avoidance mechanism.

machinery; however, this option would inevitably lead to the formation of highly recombinogenic gaps jeopardizing genome integrity. The alternative option would be unwinding of the stalled replication fork, allowing the formation of recombination intermediates by annealing newly synthesized leading and lagging strands through template switching. These DNA structures could then migrate through the damage by allowing the leading strand to copy the newly synthesized lagging strand. This recombination-dependent replication would allow for error-free DNA synthesis. This process would be dependent on the resolution of the HJs and would allow reorganization of the replication fork through branch migration.

Deciding which protein/pathway will be required for stabilizing the stalled replication structure would depend on the type of obstruction and the type of DNA damage intermediate produced. The redundant functions of Sgs1, Srs2 and Mgs1 are likely involved in the stabilization of these stalled replication structures. Cells mutated in *SRS2* show unrestrained recombination in response to DNA damage, which can be rescued by preventing certain recombination pathways. Srs2 has also been shown to disrupt Rad51 nucleofilaments (Krejci et al., 2003), suggesting that Srs2 is responsible for preventing the binding of recombination proteins, or removing them from ssDNA, leaving a substrate for a DNA polymerase most likely mediated by the *RAD6*-dependent PRR pathway. The Rad18-Rad6 complex would be able to bind the damaged site and recruit Rad5-Mms2-Ubc13. This would allow for the polyubiquitination of PCNA and might promote limited synthesis through the replicative gaps via a copy-choice type of DNA synthesis. Although TLS is not the preferred mechanism used in yeast, it provides an important tolerance mechanism. This activity is carried out by non-essential DNA

polymerases. TLS would be essential in situations where the damage cannot be removed or bypassed, but it is costly with respect to genomic stability and mutagenesis. In order for a cell to bypass the damage using TLS, the cell must be equipped with a mechanism to switch between error-free bypass and error-prone bypass. The non-essential subunit of Polδ, encoded by *POL32*, may be a key player in this switch. Under ideal conditions, PCNA would be polyubiquitinated by members of the error-free *RAD6*-dependant pathway. However, when the error-free branch is disabled, mono-ubiquitination of PCNA would result in the binding of Pol32 at the site of DNA damage. This might allow for recruitment of the mutagenic bypass polymerase Polζ, which can extend a nascent DNA strand across damaged portions of the template strand. Recent evidence in *S. pombe* suggests that an alternate form of PCNA (Rad1-Rad9-Hus1) is involved in the checkpoint response. The *E. coli* translesion synthesis polymerase DinB physically interacts with Hus1 and Rad1, and the association of DinB with chromatin is dependent on functional Rad17 (Kai M and Wang, 2003).

Stalled replication forks can result in the formation of HJs through annealing of the two newly synthesized strands. HJs are the key intermediates of both homologous and site-specific recombination. Resolution of HJs may result in the collapse of the fork arm and the generation of recombinogenic substrates. Under conditions where a homologous template is not available, attempted recombination may produce additional damage, resulting in death. In order to avoid inappropriate recombination, the cell must have a surveillance mechanism in place to determine whether suitable substrates are available. Activation of Mec1 and Rad53 by Sgs1 could occur after detection of aberrant DNA structures. This would lead to the recruitment and stabilization of Rad51

on the resulting ssDNA. Sgs1 may also play a role in stabilizing and promoting the maturation of recombination intermediates by acting downstream of Rad51, possibly as a reverse branch migration enzyme. In the case of topological stress, Sgs1 may be required to stabilize the replication forks by unwinding topologically constrained domains, allowing completion of replication.

In a situation where both PRR and recombination are disabled, the cell can use an alternate replication fork arrest avoidance mechanism to ensure survival. Mgs1 may participate in an alternative pathway, which, in the absence of PRR and recombination, would be essential in restoring arrested replication forks, perhaps by regulating the processivity and fidelity of replication catalyzed by DNA polymerases. Mgs1 may be involved in this process by promoting the loading of Polδ and Polδ-mediated synthesis. Whether Mgs1 is involved in normal DNA replication in growing cells, or plays a specific role in DNA replication only when the replication fork is blocked by damage, has yet to be elucidated. However, the mutant phenotypes of *MGS1* indicate that this is probably not the only function for this gene (Branzei et al., 2002a; Branzei et al., 2002b; Hishida et al., 2001). Mgs1 may also have a redundant function with other cellular enzymes in regulating DNA topology during DNA replication.

Based on the phenotypes of *sgs1* and *srs2* mutants, it is attractive to hypothesize complementary roles for Sgs1 and Srs2 in dealing with stalled or collapsed replication forks. In circumstances where inappropriate recombination would result in deleterious results, Srs2 can prevent recombination by removing recombination proteins and stabilizing the ssDNA. This would channel the lesion into a *RAD6*-dependent PRR pathway. However, when recombination is required for resolution of the blocked

replication fork, Sgs1 would recruit and promote recombination and possibly play a role in resolution of the resulting HJ. Both Sgs1 and Srs2 are tightly regulated and involved in the cell cycle checkpoint pathway, allowing the cell to assess the best means to overcome stalled replication. For example, phosphorylated Srs2 would signal that a homologous template is not available for recombination, and therefore the cell should use alternate means to bypass the damage. In G1/S, cells would be able to use template switching and utilize a SCE mechanism to bypass the damage. In G2, cells would have access to the homologous template and could undergo HR. Understanding the regulation of Sgs1, Srs2 and Mgs1 will help to clarify the complex relationships between DNA replication, repair and recombination in ensuring genome stability.

1.10. Rationale of the Project

There are three major DNA radiation damage repair pathways in the organism *S. cerevisiae,* namely NER, recombination repair, and PRR. The *RAD3* epistatic group controls NER. NER defective mutants characteristically show sensitivity to UV and agents that generate bulky DNA lesions and DNA crosslinks (Friedberg et al., 1995). The *RAD52* epistatic group functions in genetic recombination and the recombinational repair of DNA DSBs (Friedberg et al., 1995). The least characterized of the repair pathways is the *RAD6* group of genes responsible for PRR and mutagenesis. Mutations in the *RAD6* and *RAD18* genes display an elevated sensitivity to UV, γ-rays, and alkylating agents (Prakash and Prakash, 1977) (Table 1.1).

Prakash and Prakash isolated mutants sensitive to MMS in 1977. In a search to

Table 1.1. Epistasis groups for yeast genes involved in cellular responses to DNA radiation damage

RAD3 (Nucleotide Excision Repair)	***RAD52*** (Homologous Recombination)	***RAD6*** (Damage Bypass)
RAD1	*RAD50*	*RAD5*
RAD2	*RAD51*	*RAD6*
RAD3	*RAD52*	*RAD18*
RAD4	*RAD54*	*RAD30*
RAD7	*RAD55*	*REV1*
RAD10	*RAD57*	*REV3*
RAD14	*RAD59*	*REV7*
RAD16	*MRE11*	*MMS2*
RAD23	*XRS2*	*UBC13*
		POL3
		POL30
		SRS2

identify mutants sensitive to methylating agents but not to radiation, they isolated five complementation groups: *mms1, mms2, mms4, mms5,* and *mms22.* These complementation groups are sensitive to MMS but not to UV or X-rays (Prakash and Prakash, 1977). The gene of interest in this study is *MMS2*, which is involved in the error-free pathway of postreplication repair. PRR includes *RAD18*, *RAD6, REV1*, *REV3*, *REV7*, *MMS2*, *RAD*5 (*REV2*), *SRS2*, *RAD30* and *POL30* divided into error-free and error-prone mutagenesis pathways. The *rad6* mutant does not carry out PRR, the *rad18* and *rad5* mutants show great inhibition while the *rev3* mutation does not affect PRR. Studies in prokaryotes and eukaryotes suggest that both recombinational and nonrecombination repair mechanisms may function in PRR and most of PRR is error-free (di Caprio and Cox, 1981; Kadyk and Hartwell, 1993; Prakash, 1981; Zou and Rothstein, 1997). However, the ability of the cell to determine when to utilize the PRR pathway or when to engage other repair pathways has yet to be elucidated.

Upon DNA damage, cells activate DNA repair pathways, cell cycle checkpoints and tolerance pathways that facilitate survival. Previous studies have reported that the lethality of the *srs2 rad54* and *srs2 rdh54* double mutants can be rescued by mutations in the DNA damage checkpoint functions *RAD9*, *RAD17*, *RAD24* and *MEC3*, which suggests that intermediates generated in the double mutants are sensed by these checkpoint functions. Intact checkpoints result in a nonreversible arrest, but loss of the checkpoints releases the arrest, most likely at the detriment of genomic instability and chromosome loss (Klein, 2001a).

The cellular mechanism used to determine the appropriate means of DNA repair has thus far not been expounded. It is attractive to speculate that the type of lesion and

the stage of cell cycle progression are both crucial in signaling which repair mechanism can best repair the damage. Previous studies have also established that the Ubc13-Mms2 complex functions as a signal for the error-free PRR pathway. It is now known that one signal for Ubc13-Mms2 is PCNA; however, it is likely that other effector genes also respond to the signal. A mutation in *MMS2* does not result in severe sensitivity to MMS, but combined with a mutation from error-prone PRR results in a synergistic phenotype. By taking advantage of the synergism between error-free and error-prone PRR mutations, it is possible to search for additional genes involved in PRR.

The synthetic lethal screen is a powerful genetic screen that relies on finding secondary molecular targets. In principle, a synthetic lethal screen can identify any gene that, if mutated, causes the death of cells with a nonlethal 'primary' mutation. With synthetic lethal screening, the entire genome of an organism can be scanned to identify mutations of related pathways or proteins with redundant functions. It is anticipated that the use of a synthetic lethal screen will help identify new genes involved in the regulation of PRR. Characterization of these genes will aid not only in the understanding of DNA repair mechanisms in general, but by studying DNA repair genes in lower eukaryotes we hope to contribute to the understanding of the repair process employed by the cell in higher eukaryotes including humans. It is very difficult to directly study repair pathways in humans, thus it is essential to have an excellent model to study in the lower eukaryotes. With a quick generation time of two hours, the ease of manipulating its genome and the ability to maintain yeast in a haploid state makes yeast an ideal model. Thus, studying novel PRR genes in *S. cerevisiae* is likely to contribute to the understanding of how human cells cope with DNA damage.

Chapter 2: Materials and Methods

2.1. Yeast Genetics

2.1.1. Yeast Strains and Cell Culture

The yeast strains used in this study are listed in Table 2.1. *S. cerevisiae* haploid parental strains used in this study are as follows: CH1305, obtained from Dr. J. Kranz (Harvard University), HK-578-10A and HK-580-10D, from Dr. H. Klein (New York University), PY39-0 and PY39-46 strains from Dr. P. Burgers (Washington University, St. Louis MI), and DBY747, from D. Botstein (Stanford University). The FY86 strain was provided by Dr. F. Winston (Harvard University), LSY390 and LSY391, were received from Dr. L. Symington (Columbia University), TWY176, from T. Weinert (University of Washington), U963-61A and U960-5C, obtained from R. Rothstein (Columbia University) and BY4741 and the haploid deletions from the *Saccharomyces* Genome Deletion Project. The *URA3* selectable marker, *hisG-URA3-hisG* was removed by selection on a plate containing 5-fluoro-orotic acid (FOA). Other strains are all isogenic derivatives of the above strains created by targeted gene disruption or by mating and tetrad dissection. See Table 2.1. for genotypes and modifications to the strain backgrounds.

Media used in this study for yeast cell culture include a rich YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose), YPGal (as YPD but with 2% galactose

instead of glucose) and YPRaf (as YPD but with 2% raffinose instead of glucose). SD medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose) was supplemented with amino acids and bases at recommended concentrations (Sherman et al., 1983). For long term storage, yeast cells were grown on plates (rich or minimal media) at 30^{o}C. After 2-3 days growth the yeast cells were removed from the plate with a sterile tooth-pick and inoculated into 1.0 ml of sterile 15% (v/v) glycerol. The cells were then stored at -70^{o}C.

2.1.2. Special Media

MMS and ethyl methane sulfonate (EMS) were purchased from Aldrich (Milwaukee, USA) as an aqueous solution. 4-Nitroquinoline-N-oxide (4-NQO) was purchased from Sigma (St. Louis, MO). The stock solution was made at a concentration of 10 mg/ml acetone. HU was purchased from Sigma (St. Louis, MO). The stock solution was made at a concentration of 2 M in double distilled water (ddH_2O). To prepare drug plates, the required drug was added immediately before plating to reduce the amount of drug degradation. FOA was purchased from US Biologicals (Swampscott, MA). FOA plates (0.67% yeast-nitrogen base, 0.1% FOA, 2% glucose, 2x uracil plus required amino acids and bases) were used to positively select *ura3* mutant cells (Boeke et al., 1984). For plating, the above media were solidified with 2% Bacto-agar. To make FOA plates the agar was autoclaved in half the final volume of ddH_2O, while the rest of the components were filter sterilized in half the final volume of ddH_2O. The above solutions were combined and poured into petri dishes.

Table 2.1 *Saccharomyces cerevisiae* strains

Strain	Genotype	Source
BY4741	*Mat**a** his3D1 leu2D0 met15D0 ura3D0*	ResGen
CH1305	*MATa ura3-52 lys2-801 ade2 ade3 leu2*	J. Kranz
DBY747	*Mat**a** his3-Δ1 leu2-3,112 trp1-289 ura3-52*	D. Botstein
FY86	*Matα his3-Δ200 ura3-52 leu2-Δ1 GAL+*	F. Winston
HK1031-1A	HK578-10A with *chk1Δ::HIS3*	H. Klein
HK1031-6B	HK578-10D with *chk1Δ::HIS3*	H. Klein
HK578-10A	*Mat**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*$^+$	H. Klein
HK578-10D	*Matα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*$^+$	H. Klein
HK590-1D	HK578-10A with *srs2Δ::HIS3*	H. Klein
HK590-6D	HK578-10D with *srs2Δ::HIS3*	H. Klein
HK845-1A	HK578-10A with *rad9Δ::HIS3*	H. Klein
HK845-3A	HK578-10D with *rad9Δ::HIS3*	H. Klein
LSY386	*Mat**a** rad52Δ::TRP1*	L. Symington
LSY387	*Matα rad52Δ::TRP1*	L. Symington
LSY390	*Mat**a** leu2-3,112 trp1-1 ura3-1 his3-11,15 ade 2-1 can1-100*	L. Symington
LSY391	*Matα leu2-3,112 trp1-1 ura3-1 his3-11,15 ade 2-1 can1-100*	L. Symington
LSY395	*Matα rad50Δ::HUH*	L. Symington
LSY396	*Mat**a** rad50Δ::HUH*	L. Symington
LSY401	*Matα rad51Δ::LEU2*	L. Symington
LSY402	*Mat**a** rad51Δ::LEU2*	L. Symington
LSY403	*Mat**a** rad54Δ::LEU2*	L. Symington
LSY404	*Matα rad54Δ::LEU2*	L. Symington
LSY405	*Mat**a** rad55Δ::LEU2*	L. Symington
LSY406	*Matα rad55Δ::LEU2*	L. Symington
LSY407	*Mat**a** rad57Δ::LEU2*	L. Symington
LSY408	*Matα rad57Δ::LEU2*	L. Symington
LSY977	*Mat**a** rad51-K191R-URA3-rad51-K191R*	L. Symington
LSY979	*Mat**a** rad51-K191R*	L. Symington
LSY983	*Mat**a** rad51-K191A*	L. Symington
PY39-0	*Matα ura3-52 trp1-Δ901 leu2-3,112 can1 pol30-Δ1* [pBL230 (POL30 TRP1)]	P. Burgers
PY39-46	PY39-0 with [pBL230-46(TRP1 pol30-46)]	P. Burgers
TWY176	*Matα mec1-1 leu2 his3 his7 ura3*	T. Weinert
TWY281	*Matα rad17-1 ura3 trp1 his7*	T. Weinert
TWY297	*Mat**a** rad24-1 ura3 his3 trp1*	T. Weinert

U960-5C	*Mat**a** rad53Δ::HIS3 sml1-1*	R. Rothstein
U963-61A	*Mat**a** mec1Δ::TRP1 sml1Δ::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
WXY1202	W303 with *mms2Δ::TRP1 rad9Δ:: HIS3*	This Study
WXY1203	W303 with *mms2Δ::TRP1 rev3Δ::HUH rad9Δ::HIS3*	This Study
WXY1204	W303 with *rad9::HIS3 rad18Δ::LEU2*	This Study
WXY1205	W303 with *rev3Δ::HUH rad9Δ::HIS3*	This Study
WXY1224	DBY747 with *mms2Δ::HIS3 rad9Δ::HUH*	This Study
WXY1225	DBY747 with *mms2Δ::HIS3 rev3Δ::LEU2 rad9Δ::HUH*	This Study
WXY1226	DBY747 with *rad18Δ::LEU2 rad9Δ::HUH*	This Study
WXY1227	DBY747 with *rev3Δ::LEU2 rad9Δ::HUH*	This Study
WXY1228	W303 with *Mat**a** mms2Δ::TRP*	This Study
WXY1229	W303 with *rev3Δ::hisG rad17Δ::HIS3*	This Study
WXY1230	W303 with *rev3Δ::hisG rad24Δ::URA3*	This Study
WXY1231	W303 with *rev3Δ::HUH rad53Δ::HIS3 sml1-1*	This Study
WXY1232	W303 with *Mat**a** rev3Δ::HUH* (FOA)	This Study
WXY1233	W303 with *Matα rev3Δ::HUH*	This Study
WXY1234	W303 with *chk1Δ::HIS3 rev3Δ::HUH*	This Study
WXY1235	U960-5C with *rev3Δ::HUH*	This Study
WXY1236	W303 with *matαΔ::LEU2* / YCp50-MATα	This Study
WXY1237	W303 with *matαΔ::LEU2 mms2Δ::TRP1*	This Study
WXY1238	W303 with *mms2Δ::TRP1 sir3Δ::LEU2*	This Study
WXY642	DBY747 with *mms2Δ::HIS3*	W. Xiao
WXY665	DBY747 with *mms2Δ::HIS3 rev3Δ::LEU2*	W. Xiao
WXY901	W303 with *Mat**a** mms2Δ::HIS3*	W. Xiao
WXY902	W303 with *Matα mms2Δ::HIS3*	W. Xiao
WXY906	W303 with *MATα ubc13Δ::LEU2*	Wei Xiao
WXY918	HK-578-10A but *ade3Δ::hisG-URA3-hisG*	This Study
WXY919	HK-578-10A but *ade3Δ::hisG mre11Δ::HIS3*	This Study
WXY920	W303 with *mms2Δ::HIS3 rev3Δ::HUH*	W. Xiao
WXY930	W303 with *Matα rad18Δ::LEU2*	This Study
WXY9382	DBY747 with *rev3Δ::LEU* (pAM56)	W. Xiao
WXY9444	DBY747 with *rad18Δ::LEU2*	W. Xiao
WXY9519	DBY747 with *rad9Δ::HUH*	W. Xiao
W303	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*$^{+}$	H. Klein

Table 2.2. Plasmids

Plasmid	Source
p*sir2Δ::LEU2*	C. Bennett
p*sir3Δ::LEU2*	C. Bennett
p*sir4Δ::LEU2*	C. Bennett
YCp50	M. Rose
pJH318 (*matαΔ::LEU2*)	J. Haber
YCp50-MATα	F. Fabre
YCp50-MAT**a**	F. Fabre
pGAD-POL32	This Study
YCpL-REV1	This Study
YCpL-REV3	This Study
YCpL-REV7	This Study
pSLS-MMS2	This Study
p*ade3Δ::hisG-URA3-hisG*	This Study
p*ade2Δ::hisG-URA3-hisG*	This Study
pSLS1-MRE11	This Study
pSLS2-MRE11	This Study
YEpGAL1-hMMS2	This Study
YEpCUP1-hMMS2	This Study
YEpADH1-hMMS2	This Study
YCpU-ADE3-P_{CUP1}	This Study
YCpU-ADE3-P_{ADH1}	This Study
YCpU-ADE3-P_{GAL1}	This Study
YCpU-ADE3	This Study
YRpU-ADE3	This Study
YRpU33	D. Gietz
YCplac33	D. Gietz
YCp-ADE3-MRE11	This Study
pSLS1	This Study
pSLS2	This Study

2.1.3. Yeast Transformation and Targeted Disruption

Yeast cells were transformed using a dimethyl sulfoxide (DMSO)-enhanced method as described (Hill et al., 1991). A 2 ml culture of *S. cerevisiae* was grown overnight at 30^{o}C in rich media (or appropriate minimal media). The next day the cells were subcultured into 5 ml of fresh media, and allowed to grow until the yeast cells reached a mid-logarithmic phase of growth. The yeast cells were collected by centrifugation, washed in LiOAc solution (0.1 M lithium acetate, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and resuspended in 100 µl of the LiOAc solution. 4 µl of carrier DNA (single stranded salmon sperm) and 1-5 µl of transforming DNA were added. After incubation at room temperature for 5 minutes, 280 µl of PEG_{4000} solution (5% polyethylene glycol 4000 in LiOAc solution) was added and the contents were mixed by inverting the tube 4-6 times. The transformation mixture was incubated for 45 minutes at 30^{o}C. Following incubation, 39 µl of DMSO was added and followed by a 5 minute heat shock at 42^{o}C. Yeast cells were then washed with sterile ddH_2O and resuspended in 100 µl of ddH_2O. The resuspended cells were plated on the appropriate minimal media. For targeted gene deletion, plasmid DNA was digested with the appropriate restriction enzymes, precipitated by ethanol and resuspended in ddH_2O prior to transformation.

2.1.4. Yeast Plasmid Extraction

Yeast plasmid extraction were performed as previously described (Hoffman and Winston, 1987). Yeast cells were obtained from plates or from a liquid culture. Cells grown on plates were scraped with a sterile toothpick and resuspended in 230 µl of

extraction buffer (2% TritonX-100, 1% SDS, 0.1 M NaCl, 1 mM EDTA, 0.01 M Tris HCl pH 8.0) in a screw top microcentrifuge tube. Cells from a liquid culture were collected by centrifugation and resuspended into 230 µl of extraction buffer. 0.1 ml of phenol, 0.1 ml of chloroform and 0.3 g of acid-washed beads were added to the cell mixture. The tube was vortexed at high speed for 2-3 minutes. The mixture was centrifuged for 5 minutes and the aqueous layer was transferred into a clean microcentrifuge tube. The plasmid DNA was precipitated by 2 volumes of 95% ethanol. In order to obtain a pure DNA preparation, the precipitated DNA was resuspended in 200 µl of TE (10 mM Tris HCl, 1 mM EDTA pH 8.0) and treated with 5 µl of RNase (10 mg/ml stock) at 37^{o}C for 10 minutes. After the RNase treatment, the DNA was precipitated in 2 volumes of 95% ethanol.

2.1.5. Yeast RNA Isolation

One ml of overnight culture was used to inoculate 4 ml of fresh medium and cells were cultured for 2 hours. For MMS induction, MMS was added to a final concentration of 0.05% and incubation continued for 30 minutes before RNA isolation. RNA was isolated by a glass bead method (Carlson and Botstein, 1982). Briefly, yeast cells were washed in DEPC (Diethylpyrocarbonate)-treated H_2O once and resuspended in 350 µl of lysis buffer (0.5 M NaCl, 10 mM EDTA, 1% SDS and 0.2 M Tris-HCl at pH 7.6) in a microcentrifuge tube. 0.3 g of acid-washed glass beads (D=0.4-0.5 mm) and 350 µl of phenol/chloroform were added. The contents were vortexed at high speed for 2 to 2.5 min. The tubes were spun in a microcentrifuge for 30 seconds and the aqueous phase was extracted with chloroform. After centrifugation, the aqueous phase

was transferred into a new tube. The RNA was precipitated by adding 1 ml of 95% ethanol, and the contents were mixed and centrifuged immediately for 5 min. The RNA pellet was washed in 70% ethanol once and briefly dried by vacuum.

2.1.5.1. Northern Hybridization

RNA (10 µl) was mixed with 8 µl of formamide, 4.5 µl of formaldehyde, 1 µl of EtdBr solution, 1 µl of loading dye (containing sucrose or glycerol and bromophenol blue) and 2.5 µl of 10x MOPS (3-(*N*-morpholino) propanesulfonic acid) buffer. The RNA was separated in a 1% denaturing agarose gel containing 1x MOPS buffer (20 mM MOPS, 5 mM Na acetate, 1 mM EDTA pH 7.0) and formaldehyde (2 ml in 40 ml gel). After electrophoresis, the separated RNA was blotted onto a GeneScreen Plus nylon (DuPont) membrane with 20x SSC overnight. The membrane was treated in a UV crosslinker and was hybridized with DNA probes made by the Random Primer Labeling Kit (Invitrogen). The blots were hybridized overnight with the [α-^{32}P] dCTP-labeled fragment containing the appropriate coding region, washed, and exposed to an X-ray film. The probe was stripped off the membrane and the membrane was subsequently hybridized to a 1.6-kb *ACT1* probe as the internal control. The *ACT1* probe was isolated as a 1.6-kb *Bam*HI-*Hind*III fragment from pAA93 (F. Sherman, Rochester University).

2.1.6. Sporulation and Yeast Tetrad Dissection

Two haploid strains with opposite mating types were cross-streaked in an X-formation on minimal selective plates which would support growth of the diploid, but

not support the growth of either of the haploid strains. The cells were mixed and streaked out to isolate for individual colony growth. The plates were incubated at 30°C for 2-3 days to obtain diploid cells, which were then inoculated into 2 ml of YPD medium. After overnight growth at 30°C, the cells were collected and washed twice in sterile ddH_2O, resuspended in 3 ml of sporulation media (0.5% potassium acetate, 0.5 x auxotrophic nutrients), and incubated at room temperature for 3-7 days with agitation and aeration.

Sporulation was checked by visual inspection with a light microscope. Dissection of tetrads was carried out as follows; 10 µl of diploid mixture was incubated for 5 minutes with 10 µl of NEE-154 glusulase (Dupont Company, Wilmington, DE, USA); 20 µl of ice-cold ddH_2O was added and the mixture was placed on ice. The tetrads were dissected on YPD plates by a Singer MSM micromanipulator (Singer Instrument Co. Sumerset, England). The plates were incubated for 3 days at 30°C. The individual tetrads were replicated on SD minimal media to determine their genotypes.

2.1.7. Co-Segregation Test

This test was used to determine whether the isolated clone was responsible for restoration of the MMS resistant phenotype in the synthetic lethal strains. The MMS sensitivity of the cells was determined with and without the plasmid.

Yeast cells were inoculated into 2 ml of YPD liquid media and grown overnight at 30°C. 10 µl of the culture was subcultured into 5 ml of fresh YPD liquid media and again incubated overnight. These cells were diluted and plated on YPGal plates and incubated for 2-3 days at 30°C. After incubation, the colonies were selected and

replica-plated onto minimal media including (+Ura) or excluding (-Ura) uracil and on various concentrations of MMS. Ura^+, MMS resistant colonies are an indication of plasmid-dependent drug resistance.

2.1.8. Measurement of Plasmid Stability

Yeast transformants carrying plasmids YCplac33 and its derivatives were cultured overnight in either YPD or SD-Ura selective medium. The cultures were diluted, plated on YPD or YPGal media as indicated, and incubated at 30°C for 5-7 days. The strain carrying YCpU-P_{CUP1} was induced by plating an overnight culture on YPD containing 0.1 mM $CuSO_4$. Once the color was fully developed (usually 5-7 days of incubation), the colonies were scored for evidence of sectoring. Individual colonies containing any visible white sectors are considered as sectoring colonies, whereas those without detectable white sectors are recorded as non-sectoring colonies.

2.1.9. Measurement of Promoter Strength

The *hMMS2* gene (Xiao et al., 1998) carried in a plasmid was used to examine the strength of *GAL1*, *CUP1* and *ADH1* promoters in yeast cells. Plasmids YEpGAL1-hM2 and YEpADH1-hM2 have been described previously (Xiao et al., 1998). To create YEpCUP1-hM2, the 0.55-kb *Eco*RI fragment containing the entire *hMMS2* coding region was cloned in the correct orientation into pJN40. All three plasmids are YEp-based multicopy plasmids with similar length of *hMMS2* insert. Furthermore, all three promoters used in these constructs are the same as used for pSLS plasmids. Hence, the level of *hMMS2* mRNA in this study is believed to represent the promoter

strength of transcription, and reflect the ability of various promoters to drive *CEN4* transcription of pSLS plasmids under given conditions.

The promoter strength was measured by Northern hybridization. Wild-type CH1305 cells were transformed with YEpCUP1-hM2, YEpADH1-hM2, YEpGAL1-hM2 or a control plasmid pYES2.0 (YEp, *URA3*, P_{GAL1}), and the transformants were selected on SD-Ura plates. Independent colonies from each transformation were incubated in 4 ml liquid SD-Ura overnight until the cultures reached a titer of 2-5 x 10^7 cells/ml. For galactose induction, YEpGAL1-hM2 and pYES2.0 transformants were grown overnight in SD-Ura. Cells were harvested by centrifugation, resuspended and incubated for two hours in an equal volume of YPRaf, and then transferred into an equal volume of YPGal for 2-hour incubation. After incubation, total RNA was isolated from the above transformants, and a Northern hybridization was used to determine *hMMS2* mRNA. The *hMMS2* probe consisted of a 0.55 kb *Eco*RI fragment containing the entire *hMMS2* coding region.

2.1.10. Synthetic Lethal Screen

Yeast cells harboring the pSLS-based plasmids were grown overnight in 10 ml of SD medium lacking uracil. The cells were harvested by centrifugation, resuspended in the same volume of YPD medium, and incubated for another four hours. The cells were collected, washed twice in 50 mM potassium phosphate buffer and resuspended in 10 ml of the same buffer. EMS was added to a final concentration of 3% and the culture was incubated at 30^{o}C for 30 minutes. Ten percent (w/v) filter-sterilized sodium thiosulfate was added to stop the reaction. The cells were washed twice, diluted and

plated onto YPD or YPGal medium, and incubated for four days at 30°C. Individual nonsectoring colonies were picked and further characterized by two steps. First, they were streaked onto the same medium to monitor color segregation. Cells from the nonsectoring colonies were then used to inoculate 2-ml liquid YPD. After an overnight incubation, cells were diluted and plated onto YPD to record colony-color segregation.

2.1.11. Cell Killing and Mutagenesis Assays

2.1.11.1. Cell Killing

MMS-induced liquid killing was performed as previously described (Xiao et al., 1996). Briefly, overnight yeast cultures were used to inoculate fresh YPD at approximately 5×10^6 cells/ml and allowed to grow until the culture contained about 2×10^7 cells/ml. MMS was added to the culture at a final concentration as specified and aliquots were taken at given intervals. Cells from each sample were collected, washed, diluted and plated on YPD. The colonies were counted after 3-days of incubation and scored as percent survival with untreated cells as a control. For UV treatment, cells were plated at different dilutions and then exposed to 254-nm UV light in a UV crosslinker (Fisher Sci. model FB-UVXL-1000 at ≈2,400 $\mu W/cm^2$) at specified doses. Cells were plated in duplicate on YPD to score cell survival, and the plates were incubated at 30°C for 3 days. Irradiation and the subsequent incubation of the cells was performed in the dark to prevent photoreactivation.

2.1.11.2. Spontaneous Mutagenesis Assay

Spontaneous Trp^+ reversion rates of DBY747 derivatives were measured by a modified Luria and Delbruck fluctuation test as described (Von Borstel, 1978). Trp^+ reversions were measured using the *trp1-289* amber allele. An overnight yeast culture was used to inoculate five tubes, each containing 10 ml of fresh YPD, to a final titer of 20 cells/ml. Incubation was continued until the cell titer reached $2x10^7$ cells/ml. Cells were collected, washed, resuspended and plated. Each set of experiments contained five independent cultures of each strain, and each culture was plated onto YPD in duplicate to score total survivors and onto SD-Trp plates to score Trp^+ revertants. Spontaneous mutation rates (number of revertants per cell per generation) were calculated as previously described (Williamson et al., 1985). The following formula was used to calculate the frequency of spontaneous mutagenesis:

$$\textbf{Frequency (F)} = \frac{\textbf{total cell number of TRP}^+\textbf{ cells}}{\textbf{total number of viable cells}}$$

To calculate the rate of spontaneous mutagenesis, the following formula was used:

$$\textbf{Rate} = \frac{\textbf{0.4343 x Frequency}}{\textbf{log(total cell number) – log(initial cell number)}}$$

The formula was derived to determine mutation rate for a replication system, where 0.4343 is approximately $\log_{10}$ e.

2.1.11.3. DNA Damage Induced Mutagenesis Assays

For MMS-induced mutagenesis the same protocol was used as for spontaneous mutagenesis with minor changes. Cells were diluted and plated on YPD to score for total cell count. The remaining cells were treated with 0.05 to 0.1% MMS for 30 minutes, washed twice with ddH_2O, and diluted and plated on YPD plates to score for

total cell survival, and onto selective plates to score for Trp^+ revertants. The cells were incubated at 30°C for three days.

2.1.12. Cell Growth by a Plate Assay

FY86m2L cells (Xiao et al., 1998) transformed with YEpCUP1-hM2, YEpADH1-hM2, YEpGAL1-hM2 or pYES2.0 grown on SD-Ura selective plates were evenly streaked onto a YPD or YPGal plate containing 0.025% MMS. The plates were incubated at 30°C for four days before photographing.

2.1.13. Analysis of MMS Sensitivity

The gradient plate assay was performed as a semi-quantitative measurement of relative MMS sensitivity. Thirty ml of molten YPD agar was mixed with the appropriate concentration of MMS to form the bottom layer. The gradient was created by pouring the media into tilted square petri dishes. After brief solidification for one hour, the petri dishes were returned flat and 30 ml of the same molten agar without MMS was poured to form the top layer. A 0.1 ml sample was taken from an overnight culture, mixed with 0.4 ml sterile water and 0.5 ml of molten YPD agar, and then immediately imprinted onto freshly made gradient plates via a microscope slide. Gradient plates were incubated at 30°C for time as indicated.

MMS sensitivity was also determined by a serial dilution assay. Yeast cells were inoculated in 3 ml of YPD medium (or selective medium if required) overnight and subcultured into 3 ml of fresh medium the next day. Cells were incubated at 30°C until the reached a mid-logarithmic phase. The cell density was adjusted to 2×10^6

cells/ml as determined by a hemocytometer, and further 10-fold serially diluted with ddH_2O. The relative MMS sensitivity was determined using freshly made YPD plates containing the indicated amount of MMS. Five µl aliquots of each dilution were applied onto YPD and YPD + MMS plates. The plates were incubated at 30°C for 2 days and photographed.

2.1.14. Cell Cycle Arrest

A *MAT***a** strain was grown to logarithmic (asynchronous) growth, and then cells were either arrested at G1 phase by the addition of 5 µg/ml of alpha factor or allowed to continue asynchronous growth. After two hours, cells were treated with 0.05 % MMS for 20 min. The cells were washed, diluted and plated onto YPD medium. The remaining cells were treated with Nocadazole (20 µg/ml final concentration) to arrest the cells at the G2 phase of the cell cycle. The cells were incubated for 3 hours, diluted and plated on YPD. The cells were incubated at 30°C for three days and scored for survival.

2.2. Molecular Biology Techniques

2.2.1. Bacterial Culture and Storage

The *E. coli* strains DH5α and DH10B (Gibco BRL, Grand Island, NY USA) were used for bacterial transformations. All plasmids used in this study contained the ampicillin resistance marker gene, *bla*. Transformed strains were cultured in Luria broth (LB: 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) containing 50 µg/ml of Ampicillin. Plates were solidified using 1.2% agar. For long term storage,

transformed cells were grown overnight in 900 µl of LB + Ampicillin (50 µg/ml), 100 µl of DMSO was added, and the cells were immediately placed in a -70^{o}C freezer.

2.2.2. Preparation of Competent Cells

For chemical transformation, *E. coli* DH5α or DH10B were treated as previously described (Chung et al., 1989). Cells were grown in LB media to an OD_{600nm} of 0.3-0.4. The cells were diluted 1:1 in TSS solution (1x TSS: LB with 10% PEG_{8000}, 5% DMSO, and 50 mM Mg^{+2} ($MgSO_4$ or MgCl, pH 6.5). The cells were aliquoted, 50 µl/tube, and placed in -70^{o}C for storage.

For electroporation transformations, *E. coli* cells were prepared as indicated in the BioRad *E. coli* Pulser manual. The cells were incubated in 1 liter of LB medium until an OD_{600nm} of 0.6 was reached. The culture was collected by centrifugation at 3500 rpm in a Beckman GSA rotor and the pellet was resuspended in 500 ml of 10% sterile glycerol. The centrifugation was repeated 4 times, each time reducing the resuspending volume, with the final volume being 4 ml of cold 10% glycerol. Aliquots of 25 µl were placed into 1.5 ml microcentrifuge tubes and quickly placed in the -70^{o}C freezer for storage.

2.2.3. Bacterial Transformation

2.2.3.1. Chemical Transformation

Competent *E. coli* cells for chemical transformations were prepared as previously described. The volume of transforming DNA was added to the cells at no greater than 10% of the final volume. The cells were incubated on ice for 30 minutes

then heat shocked for 1 minute at 42°C. After heat shocking, 450 µl of SOC media (2% Bacto-tryptone, 0.5% Yeast extract, 10 mM NaCl, 20 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) was added to the cells, and the cells were incubated at 37°C for 1 hour. The cells are plated onto LB + Ampicillin (50 µg/ml) plates.

2.2.3.2. Electroporation

Competent *E. coli* cells for electroporation were prepared as previously described. Transforming DNA was added to the competent cells to a final concentration no greater than 10% of the final volume. After a brief incubation on ice, the cell mixture was transferred to a chilled 1 mm width electroporation cuvette (BioRad). The cells were exposed to a voltage of 1.8 kV using the *E. coli* Pulser (BioRad). After electroporation, 280 µl of SOC was added to the cuvette, and the cells were transferred to a 1.5 ml microcentrifuge tube. The cells were incubated for 45 minutes at 37°C and plated onto LB + Amp plates for incubation at 37°C overnight.

2.2.4. Plasmid DNA Isolation

2.2.4.1. Boiling Method

Plasmid amplification and isolation was performed as described in Maniatis et al. (Maniatis et al., 1982). Cells were inoculated into LB + Amp liquid media and grown overnight at 37°C. Cells were collected by centrifugation and resuspended into 350 µl of STET solution (8% sucrose, 0.5% TritonX-100, 50 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0). Twenty-five µl of lysozyme (10 mg/ml; Sigma, St. Louis MI) was added, the mixture was boiled for 45 seconds, and the mixture was centrifuged for

10 minutes. The pellet was removed and the DNA was precipitated with 8 µl of 5M NaCl and 2 volumes of 95% ethanol.

2.2.4.2. Alkaline-lysis Method

Plasmid amplification and isolation was performed as descried in Maniatis et al. (Maniatis et al., 1982). Cells were inoculated into LB + Amp liquid media and grown overnight at 37^{o}C. The cells were collected by centrifugation and resuspended in 100 µl of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) by vigorous vortexing. Two hundred µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added, and the contents were mixed by inverting the tube five times. One hundred and fifty µl of ice-cold Solution III (5 M potassium acetate, 11% glacial acetic acid) was added and the contents mixed by inverting the tube 5 times. The tube was stored on ice for 3-5 minutes and then centrifuged at 12,000 g for 5 minutes. The supernatant was transferred to a new tube, and the DNA was precipitated by adding 2 volumes of 95% ethanol.

2.2.4.3. Large Scale DNA Isolation (Maxi-prep)

A 5 ml overnight culture of the transformed bacterial cells was subcultured into 500 ml of fresh LB + Amp medium. The culture was grown until an OD_{650nm} reached 0.6. At mid logarithmic growth, 2.5 ml of chloramphenicol (0.033 g/ml in alcohol) was added and the culture was further incubated overnight at 37^{o}C. The cells were harvested by a 10 minute centrifugation at 6700 rpm in a Beckman GSA rotor, and resuspended in 12 ml of a sucrose solution (10% sucrose w/v, 50 mM Tris-HCl pH 8.0).

The cells were transferred to a 40 ml centrifuge tube where 2 ml of lysozyme (10 mg/ml) and 2.4 ml of 0.5 M EDTA pH 8.0 were added to the mixture. The cells were incubated on ice for 10 minutes. To lyse the cells, 1 ml of 2% sarkosyl was added. The solution was centrifuged for 70 minutes at 12,000 rpm in a 30 ml Corex tube using the Beckman SS34 rotor. The supernatant was transferred into a 40 ml plastic centrifuge tube, 8 ml of both phenol and chloroform were added, and the mixture was centrifuged for 5 minutes at 5000 rpm (Beckman SS34 rotor). The supernatant was transferred to a clean tube, and the plasmid was further purified with another 16 ml of chloroform and centrifuged for 5 minutes. The supernatant was evenly divided between two glass centrifuge tubes and precipitated with 2 volumes of 95% ethanol at -20^{o}C for 3 hours. The tubes were centrifuged at 10,000 rpm (Beckman SS34 rotor) for 20 minutes, and the pellets were air dried by inverting the tube. The pellets was dissolved in 9.5 ml of TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0), combined into one tube, and then mixed with 9 g of cesium chloride. The mixture was transferred to heat sealer tubes using a 10 ml syringe. Three hundred and fifty µl of ethidium bromide (10 mg/ml) was added, and the volume was adjusted with a solution of TE with cesium chloride. The tubes were heat sealed and centrifuged in the Beckman ultracentrifuge for 16 hours at 55000 rpm.

The DNA band of interest was detected using a UV lamp, and the band was extracted using a syringe. The ethidium bromide solution was extracted twice with equal volumes of butanol. The aqueous layer was dialyzed in a large volume of TE, the DNA was precipitated with ethanol, and the precipitated DNA was resuspended in 500 µl of TE.

2.2.5. PCR Amplification

The *mms2::TRP1* disruption cassette was produced using a polymerase chain reaction (PCR) method. The plasmid pJJ280 (MT148) was amplified using the primers YGL87 (5'-TTCTTATTCTGTATATGCAACGTAGAAGAAGCAGCGTTTACACA AACAGCTATGACCATG-3') and YGL88 (5'-GTGGCTTGGAATGCTGCAAAT ACTGTTTAGGAAAAAGTAGATAACGTTTTCCCAGTCACGAC-3'). The PCR product contained the 5' and 3' terminus of the *MMS2* (underlined) coding sequence disrupted by the *TRP1* coding sequence. The PCR mixture consisted of 10 µl of *Taq* Reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 8 µl of 2.5 mM dNTP's, 2 µl of YGL85 and YGL86 oligonucleotides at 5 µM, 4 µl of $MgCl_2$ (50 mM), 1 µl of Taq DNA polymerase (Invitrogen), 1 µl of pJJ280, and ddH_2O; 100 µl total volume. The PCR parameters were as follows: Step 1, 95°C for 5 minutes; Step 2, 95°C for 1 minute; Step 3, 45°C for 1 minute; Step 4, 72°C for 1 minute; Step 5, return to step 2 five times; Step 6, 95°C for 1 minute; Step7, 48°C for 1 minute; Step 8, 72°C for 1 minute; Step 9, return to step 6 twenty five times; Step 10, 72°C for 10 minutes; Step 11, hold indefinitely at 4°C.

2.2.6. Agarose Gel Electrophoresis and DNA Fragment Isolation

For analysis of plasmid and genomic DNA, a 0.75% agarose gel was used. Electrophoresis was performed in 1x TAE (24% Tris-base, 5.7% glacial acetic acid, 10% EDTA pH 8.0) and the gel was stained in 0.5 µg/ml ethidium bromide for viewing.

Isolation of DNA fragments from an agarose gel was modified from the protocol previously described (Wang and Rossman, 1994). After restriction enzyme digestion,

the sample was electrophoresed through 0.6% agarose and stained with ethidium bromide. A 0.5 ml microcentrifuge tube was pierced at the bottom, and the tube was packed with a small piece of glass wool. Two hundred µl of TE (10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) saturated Sephadex G-10 beads were placed into the tube and the contents were packed by centrifugation. The agarose containing the DNA fragment was placed into the prepared tube, which was placed into another 1.5 ml tube, and the contents were centrifuged at high speed for 10 minutes. The flow through was then treated with phenol/chloroform extraction and ethanol precipitated.

Alternatively, a small piece of sterile cheese cloth could be used to purify the fragment. The 0.5 ml microcentrifuge tube, pierced at the bottom, was packed with a small piece of cheese cloth. The agarose containing the DNA fragment of interest was placed into the tube and was frozen at -70^{o}C for at least 20 minutes. The contents of the tube were collected into a second 1.5 ml tube by centrifugation for 10 minutes. The flow through was treated with a phenol/chloroform extraction and ethanol precipitated.

2.2.7. Radioactive Labeling of DNA Fragments

DNA fragments isolated from an agarose gel or from PCR reactions were used for labeling and hybridizations. A Random Primer Labeling System (Gibco/BRL) was used as per manufacturer's instructions. Radioactive probe was resuspended in 120 µl of water and used as required and stored at -20^{o}C.

2.2.8. Southern Transfer and Hybridization

After electrophoresis, the agarose gel was treated for approximately 10 minutes in 0.25 M HCl, 30 minutes in 0.4 M NaOH/0.6 M NaCl, and 30 minutes in 1.5 M NaCl/0.5 M Tris-HCl pH 7.5. The DNA was transferred to a nylon-based membrane (GeneScreen by NEN) using 10x SSC (20x SSC: 3 M NaCl, 0.3 M tri-sodium citrate pH 7.0) overnight. After transfer, the membrane was UV crosslinked.

Membranes were incubated in prehybridization solution (2x SSC, 10% dextran sulfate, 5x Denhardt's solution, 50% formamide, 1% SDS) for at least 1 hour at 42^{o}C. Fifty µl of ssDNA (10 mg/ml) and 30 µl of the probe of interest were denatured by boiling for 5 minutes, and cooled on ice before adding to the prehybridization mixture. Hybridization was carried out overnight at 42^{o}C.

The membrane was washed twice for 5 minutes each at room temperature in 2x SSC/0.1% SDS, followed by two 30 minute washes at 65^{o}C in 0.2x SSC/0.1% SDS. The membrane was exposed to x-ray film at -70^{o}C and developed after an appropriate amount of time.

2.2.9. Construction of Plasmids

2.2.9.1. Plasmids for Synthetic Lethal Screen and Promoter Studies

Restriction and modifying enzymes were purchased from New England Biolabs and Gibco-BRL, and used as instructed by the manufacturers. The synthetic lethal screen cloning vectors (pSLS) used in this study were made based on plasmid YCplac33 (*CEN4*, *URA3*, *ARS1*, (Gietz and Sugino, 1988)). A 1.25-kb *Spe*I-*Nhe*I fragment containing *CEN4* was deleted from YCplac33 to form YRpU33. A 0.7-kb *Eco*RI-*Eco*RV fragment containing the *ADH1* promoter was isolated and cloned into

the *Eco*RI-*Sma*I sites of YCplac33 to form YCpU-P_{ADHI}. A 0.53-kb *Spe*I-*Bam*HI fragment containing the *GAL1* promoter was isolated from pYES2.0 (Invitrogen) and cloned into the *Xba*I-*Bam*HI sites of YCplac33 to form YCpU-P_{GALl}. A 0.4-kb *Bam*HI fragment containing the *CUP1* promoter was isolated from pJN40 (a gift from Dr. C. Lawrence, University of Rochester, N.Y.) and cloned into the *Bam*HI site of YCplac33 to form YCpU-P_{CUP1}. A 0.7-kb *Eco*RI-*Tth*111I (for YCpU-P_{ADH1} and YCpU-P_{CUP1}) or *Bam*HI-*Tth*111I (for YCpU-P_{GAL1}) fragment was deleted to bring the above promoters in close proximity to the *CEN4* sequence. The resulting plasmids YCpU-$P_{ADH1\Delta}$, YCpU-$P_{CUP1\Delta}$ and YCpU-$P_{GAL1\Delta}$, along with YCplac33 and YRpU33, were used to clone the 5.4-kb *Sal*I fragment from pJM555 (from Dr. S. Brill, Rutgers University, Piscataway, N.J.) containing the *ADE3* gene. A diagram depicting regions of interest in the resulting plasmids is shown in Figure 3.1.

To create additional unique cloning sites in the above plasmids, one of the two *Sal*I sites was removed from YcpU-ADE3-P_{GAL1} and YCpU-ADE3 to form pSLS1 and pSLS2, respectively. The *Sal*I site upstream of *ADE3* in YCpU-ADE3-P_{GALl} was destroyed by *Sal*I partial digestion, followed by isolation of the linearized plasmid, filling the cohesive end, and self ligation. Plasmid pSLS2 was made by deletion of a small *Bam*HI fragment flanking the *Sal*I site upstream of *ADE3* in YCpU-ADE3 (Figure 3.1).

To compare two synthetic lethal screen protocols, plasmids pSLS1-MRE11 and pSLS2-MRE11 were made by cloning a 4.3-kb *Bam*HI fragment from pNGS1-10 (Chamankhah and Xiao, 1998) containing the *MRE11* gene into the *Bam*HI site of pSLS1 and pSLS2. Plasmid pSLS-MMS2 was made by cloning a 1.1-kb *Bgl*II

fragment from YCpM2 (Broomfield et al., 1998) containing the MMS2 gene into the *Bam*HI site of pSLS1.

2.2.9.2. Plasmids for Targeted Gene Deletions

The *ade2Δ::hisG-URA3-hisG* cassette was made as follows: First, the unique *Bam*HI site in pASZ10 (Stotz and Linder, 1990) was destroyed. The resulting pASZl0B was used to delete a 1.5-kb *Eco*RV-*Bsr*GI fragment containing 90% of the *ADE2* coding region and to insert a *Bam*HI linker during religation. This *Bam*HI site was used to clone the 3.8-kb *Bam*HI-*Bgl*II fragment from pNKY51 (Alani et al., 1987) containing the *hisG-URA3-hisG* sequence. The *ade2Δ::hisG-URA3-hisG* cassette can be released by *Bgl*II digestion of the resulting plasmid pade2Δ::HUH.

To make the *ade3Δ::hisG-URA3-hisG* cassette, the 5.4-kb *Sal*I fragment containing *ADE3* was cloned into pTZI8R (Pharmacia). The resulting pTZ-ADE3 was then used to delete a 2.1-kb *Xho*I-*Hpa*I fragment containing 76% of the *ADE3* coding region and a *Bgl*II linker was inserted during religation. This *Bgl*II site was used to clone the 3.8-kb *Bam*HI-*Bgl*II *hisG-URA3-hisG* fragment. The *ade3Δ::hisG-URA3-hisG* cassette can be released by *Bam*HI-*Eco*RI digestion of the resulting plasmid pade3Δ::HUH.

2.2.9.3. Plasmids for Complementation

YCpL-REV1, YCpL-REV3 and YCpL-REV7 were created to test for complementation of the *REV* genes in the synthetic lethal mutation strains. The 4.4 kb *Xho*I-*Sal*I fragment containing *REV1* was obtained from plasmid pFL41 and cloned into

a *Sal*I digested YCplac111 vector. For *REV3*, a 5.5 kb *Bam*HI fragment from pJA6B was cloned into the *Bam*HI site of YCplac111. The 2.5kb *REV7* fragment was released from pLT39-1 using *Bgl*II-*Sph*I and directionally cloned into YCplac111 using the same enzymes.

Chapter Three - Improving Synthetic Lethal Screens by Regulating the Yeast Centromere Sequence

3.1. Abstract

The synthetic lethal screen is a useful method for the identification of novel genes functioning in an alternative pathway to the gene of interest. The current synthetic lethal screen protocol in yeast is based on a colony-sectoring assay that allows direct visualization of mutant colonies among a large population by its inability to afford plasmid loss. This method demands an appropriate level of stability of the plasmid carrying the gene of interest. YRp-based plasmids are extremely unstable and complete plasmid loss occurs within a few generations. Consequently, YCp plasmids are the vector of choice for synthetic lethal screens. However, we found that the high-level stability of YCp plasmids resulted in a large number of false positives that must be further characterized. In this study, we attempt to improve the existing synthetic lethal screen protocol by regulating the plasmid stability and copy number. It was found that by placing a yeast centromere sequence under the control of either inducible or constitutive promoters, plasmid stability can be significantly decreased. Hence, altering the culture conditions under which yeast cells carrying the plasmid P_{GAL1}-CEN4 allowed us to develop a method that eliminated virtually 100% of false positives and drastically reduced the time required to carry out a synthetic lethal screen.

3.2. Introduction

A synthetic lethal screen is a method of isolating novel mutants whose survival is dependent on the presence of the gene of interest. Studies by Koshland et al. (1985), Hieter et al. (1985), Kranz and Holm (1990), and Bender and Pringle (1991) have provided a means to screen for synthetic lethal mutants using a convenient colony-color assay.

The colony-color assay relies on the ability to visually identify colonies in which plasmid loss has occurred. Cells carrying an *ade2* mutation are deficient in the purine biosynthetic pathway and accumulate a red pigment. The *ade3* mutation is epistatic to *ade2* by blocking the pathway at a point prior to pigment accumulation, resulting in an *ade2 ade3* double mutant that forms white colonies (Hieter et al., 1985; Koshland et al., 1985). Introducing a plasmid carrying *ADE3* into an *ade2 ade3* strain generates mostly red colonies containing white sectors where the plasmid has been lost. A synthetic lethal screen works on the premise that a desired mutant is reliant on a plasmid containing the gene of interest to survive and form colonies. Combining the colony-color assay with a synthetic lethal screen offers a means to visually detect a mutant dependent on a plasmid by screening for solid red colonies as opposed to sectoring colonies (Bender and Pringle, 1991; Kranz and Holm, 1990) (Figure 3.1).

The efficiency of this method is strongly influenced by the stability of the plasmid carrying the gene of interest. A plasmid containing an autonomously replicating sequence (ARS) is unstably maintained in host cells and segregates with a frequency of 6-18% per generation. This usually results in complete loss of the plasmid

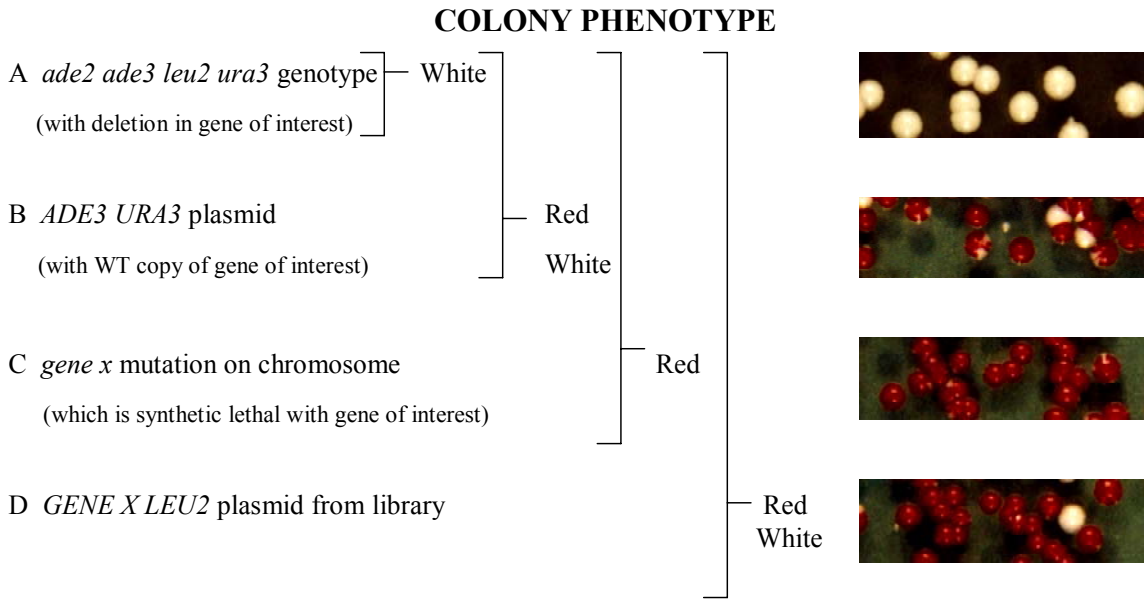

Figure 3.1. Genotypes and expected phenotypes for the synthetic lethal screen and colony color assay. **(A)** Wild-type strain, SLS, deleted for the gene of interest showing a solid white phenotype. **(B)** Transformation of SLS with a plasmid containing the gene of interest and *ADE3*. Phenotypically cells are red when carrying the plasmid with white sectors where the plasmid is lost. **(C)** Mutagenesis of SLS. When mutation x is synthetic lethal with the gene of interest the plasmid is required for survival and shows the solid red phenotype. **(D)** A library screen is used to recover the synthetic lethal mutation. When the library plasmid complements the mutation the original plasmid (step B) is lost resulting in the red and white sectoring phenotype.

in a cell population within 20 generations in a nonselective medium (Kingsman et al., 1979) making this plasmid difficult to work with. In contrast, a YCp plasmid containing both the ARS and a centromere sequence (CEN) can be maintained at one or two copies in 90% of cells in nonselective medium for 20 generations (Clarke and Carbon, 1980). A centromere is an important element of eukaryotic chromosomes and is essential for proper chromosome segregation during cell division. Studies have shown that yeast centromeres improve stability and segregation of the YRp plasmid, so that it behaves as a mini-chromosome (Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982; Hsiao and Carbon, 1981; Tschumper and Carbon, 1983). This stability of centromere plasmids makes them ideal for genetic manipulations.

Unfortunately, the high stability of centromere plasmids can generate undesirable results when used in synthetic lethal screens. As stated, synthetic lethal screens are carried out by screening for colonies that are contingent on the plasmid for viability, thus all colonies maintaining a plasmid would be considered putative synthetic lethal mutants (Kranz and Holm, 1990). Since the YCp plasmid can be sustained in a large number of cells, a vast number of false positives are often produced. This problem becomes more severe if loss of the gene of interest reduces cell growth, a situation one often encounters during a synthetic lethal screen.

This study aimed to improve the synthetic lethal screen protocol, to eliminate the high number of false positives encountered during the screen. We took advantage of the fact that the CEN function can be disrupted when transcription is directed into the centromere sequence. It has been shown that when driven by a strong promoter, yeast centromeric DNA no longer exerts mitotic stability (Panzeri et al., 1984). Transcription

through CEN appears to interfere with its function, resulting in the plasmid behaving more like a YRp plasmid than a CEN plasmid (Chlebowicz-Sledziewska and Sledziewski, 1985). YCp plasmids containing either inducible or constitutive promoters located upstream of the CEN were used to regulate the centromere function of the plasmid to reduce their stability. This plasmid is useful when experiments require different levels of plasmid stability at different points throughout the experimental protocol. We demonstrate that under ideal conditions virtually 100% of false positive can be eliminated, thus increasing the efficiency and drastically reducing the time required to carry out a synthetic lethal screen. The application of this protocol is compared with the conventional method in the isolation of yeast synthetic lethal mutants in the absence of *MRE11*, a gene involved in several DNA metabolic pathways (Haber, 1998a).

3.3. Results

3.3.1. Rationale for Improving the Synthetic Lethal Screen Efficacy

During the application of the conventional synthetic lethal screen method to our research, we routinely obtained as high as several percent of non-sectoring colonies, and realized that a large number of false positive clones had escaped from detection. Strategies, such as pre-incubation nonselectively, and plating fewer cells per plate to allow larger colony formation for further segregation, did not effectively reduce false positives. Furthermore, during screening synthetic lethal mutants with mutations such as *mre11* (Chamankhah and Xiao, 1998), more false positive colonies were obtained. This is presumably due to the fact that the *mre11* mutation severely affects cell growth

and morphology (data not shown). Hence, cells that lost the YCp-ADE3-MRE11 plasmid (white sector) grew slower than those that retained the plasmid (pink sector). A YRp-based plasmid was examined; however, the plasmid was so unstable that the transformants were unable to grow in the liquid selective medium. It has been reported that a strong promoter placed in front of a centromere sequence is able to disrupt the CEN function (Panzeri et al., 1984). Thus, we investigated the possibility of enhancing YCp plasmid instability by regulating its CEN function.

3.3.2. YCp Plasmid Stability Regulated by *GAL1*, *CUP1* and *ADH1* Promoters

Three commonly used and well characterized promoters were utilized to examine their effects on YCp plasmid stability under various growing conditions, and compared with their parental constructs YCplac33 and YRpU33 (Figure 3.2). An *ADE3* gene was cloned into the above plasmids to monitor plasmid loss by colony-color sectoring. Wild-type CH1305 contains *ade2 ade3* markers and produces white colonies on a YPD plate. Harboring an *ADE3* gene allows CH1305 cells to form pink colonies (Figure 3.3). Kingsman et al. (Kingsman et al., 1979) showed that YRp-based plasmids are unstably sustained in rich medium and segregate with a frequency of 6-18% per generation. Indeed, when a YRpU-ADE3 transformant was allowed to grow selectively overnight and plated onto YPD plates, 100% of the colonies were sectored (Table 3.1) and the vast majority were actually completely white colonies (Figure 3.3 A), indicating that the YRpU-ADE3 plasmid is segregated at a very high rate. In contrast, YCp plasmids containing a centromere sequence could be maintained at one or two copies in

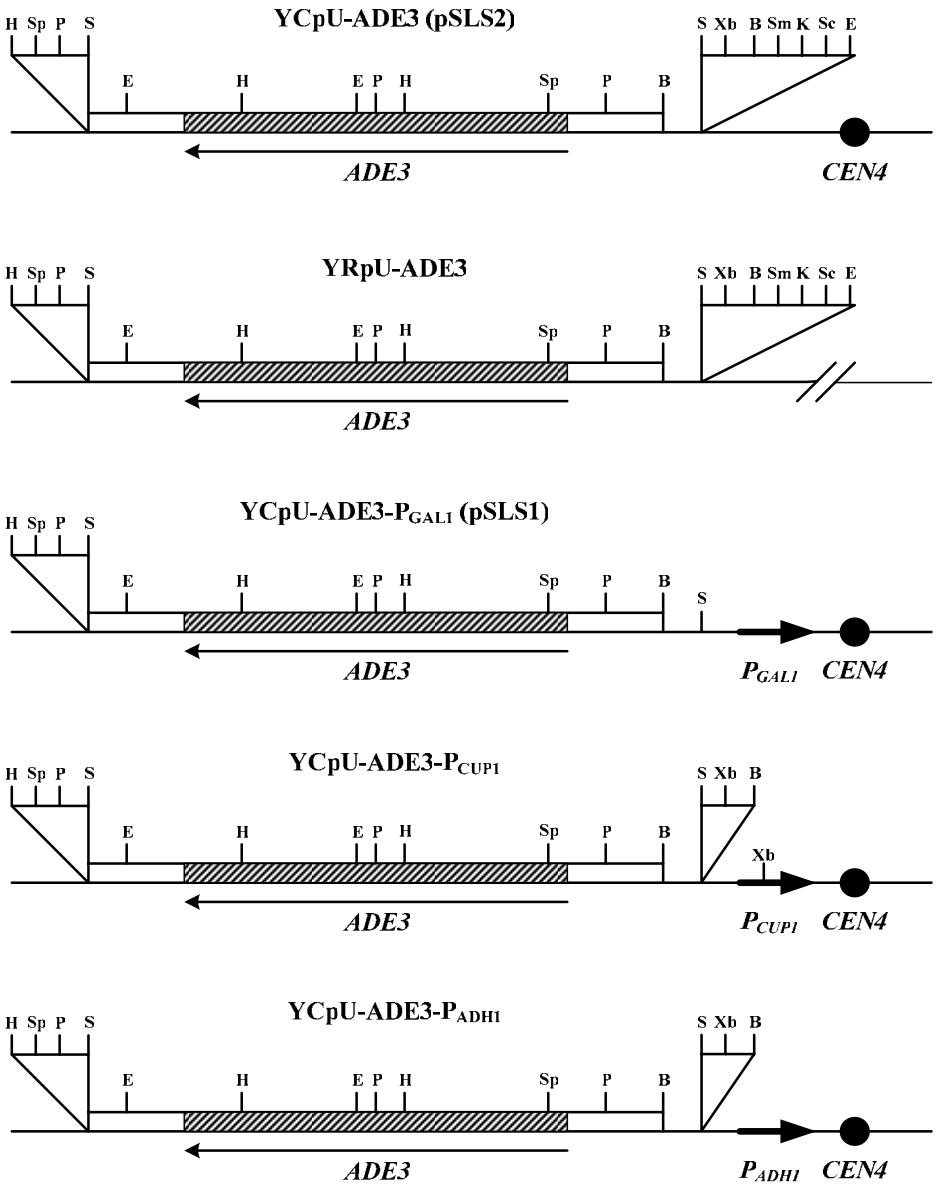

Figure 3.2. Schematic diagrams of plasmid constructs used in this study. Only areas of interest, including *ADE3*, *CEN4*, the promoter and selected restriction sites in this region are shown. Boxed area indicates yeast DNA. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I.

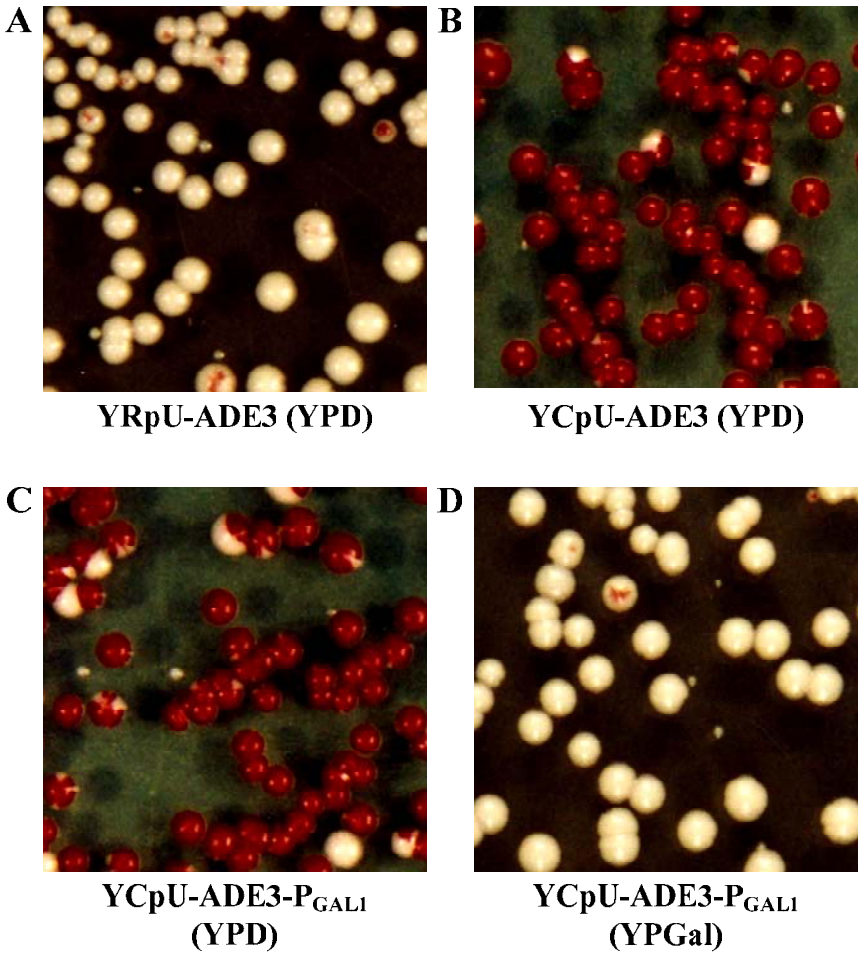

Figure 3.3. Representative colony-color sectoring plates. CH1305 transformants were grown in a SD selective medium overnight, diluted and plated on nonselective media as indicated and incubated at 30^{o}C for 5 days (YPD) or 7 days (YPGal) before taking photographs. **(A)** YRpU-ADE3 transformants plated on YPD; **(B)** YCpU-ADE3 transformants plated on YPD; **(C)** YCpU-ADE3-P_{GAL1} transformants plated on YPD; **(D)** YCpU-ADE3-P_{GAL1} transformants plated on YPGal. YCpU-ADE3-P_{ADH1} and YCpU-ADE3-P_{CUP1} transformants plated on YPD behave like plate **(A)**. Note that some bright spots shown in red colonies on plates **(B)** and **(C)** are from the reflection of light during photographing.

Table 3.1. Plasmid Stability as determined by a colony-sectoring assay.

Plasmid	Pre-culture medium	Plating medium	Nonsectoring red colonies[a]	Total colonies	% Nonsector
YRpU-ADE3	SD-URA	YPD	0	4893	0
YCpU-ADE3	SD-URA	YPD	321	4518	7.1
	YPD	YPD	123	2396	5.1
	SD-URA (O/N) YPD (5 hrs)[b]	YPD	450	6448	6.9
YCpU-ADE3-P_{GAL1}	SD-URA	YPD	267	3430	7.8
	SD-URA	YPGal	0	2698	0
YCpU-ADE3-P_{CUP1}	SD-URA	YPD	0	3928	0
	SD-URA	YPD 0.1 mM $CuSO_4$	0	2181	0
YCpU-ADE3-P_{ADH1}	SD-URA	YPD	0	2532	0

[a] All plates were incubated 5-7 d at 30°C before counting colonies

[b] The transformants were incubated overnight in SD-URA and transferred to YPD for an additional 5-h incubation

up to 90% of cells in a nonselective medium for 20 generations (Clarke and Carbon, 1980). Under our experimental conditions, about 7% of colonies did not display a visible sign of YCpU-ADE3 plasmid segregation (Figure 3.3 B and Table 3.1). Growing the transformants overnight in a nonselective (YPD) medium only slightly increased the plasmid segregation rate (Table 3.1).

The *GAL1* gene encodes an enzyme responsible for galactose utilization in *S. cerevisiae*. In a wild-type Gal^+ strain, expression of the *GAL1* gene is barely detectable when grown in the absence of galactose. Upon addition of galactose, a 1,000-fold induction is achieved (Adams, 1972; Douglas and Hawthorne, 1966; St John and Davis, 1979). To see whether or not the *GAL1* promoter could destabilize the YCp plasmid, we constructed YCpU-ADE3-P_{GAL1} (Figure 3.2), grew the transformant in selective SD medium and plated cells onto either YPD or YPGal. After a 5-day incubation, 7.8% of colonies growing on YPD plates were nonsectoring, whereas those on YPGal showed 0% nonsectoring colonies (Figure 3.3 C,D and Table 3.1). This result demonstrates that the *GAL1* promoter efficiently destabilized the CEN sequence under inducible conditions, and that indeed the YCp plasmid stability can be regulated.

Yeast *ADH1* encodes an alcohol dehydrogenase involved in ethanol production (Williamson et al., 1980). The *ADH1* gene is considered to be one of the most constitutively expressed genes in yeast (Fowler et al., 1972), although its expression may be altered by carbon source (Denis et al., 1983). *CUP1* is an inducible gene encoding metallothionein (Butt et al., 1984b; Karin et al., 1984). When induced, *CUP1* confers a resistance to copper directly proportional to the copy number of *CUP1* in the cell (Fogel and Welch, 1982). When resistant cells are exposed to 0.3 mM $CuSO_4$,

CUP1 mRNA is rapidly produced, whereas sensitive strains containing a single copy of the gene produce little mRNA and are unable to grow (Butt et al., 1984a). Plasmids YCpU-ADE3-P_{ADHI} and YCpU-ADE3-P_{CUP1} were created in this study and their effects on YCp plasmid stability were examined. We found that under all experimental conditions, 100% of the colonies had sectored to the degree comparable to the YRpU-ADE3 transformants (Table 3.1). In the case of YCpU-ADE3-P_{CUP1} transformants, the presence or absence of 0.1 mM $CuSO_4$ induction did not make significant difference with respect to colony-color sectoring (Table 3.1 and data not shown).

The above experiments were also performed in strain 10A*ade3Δ* and the results are similar as shown in Table 3.1 and Figure 3.2.

3.3.3. Relative Promoter Strength of *GAL1*, *ADH1* and *CUP1* in Yeast Cells

To address whether or not the effects of the above promoters on YCp plasmid stability are closely related to their strength of transcription initiation, we examined the ability of these promoters to express a heterologous gene. The *hMMS2* cDNA was used as a reporter gene because it does not share significant nucleotide homology with any yeast DNA, and yet, it is able to complement a yeast *mms2* defect (Xiao et al., 1998). *hMMS2* was cloned downstream of each promoter in a similar fashion as constructing YCp-promoter plasmids (see Materials and Methods). Our Northern hybridization results (Figure 3.4 A) show that, as expected, the *ADH1* promoter supports a high level of constitutive expression, and the *GAL1* promoter displays strict carbon source dependent expression of the *hMMS2* mRNA. In contrast, a noticeable level of *hMMS2* expression from the *CUP1* promoter was observed even under noninducing conditions.

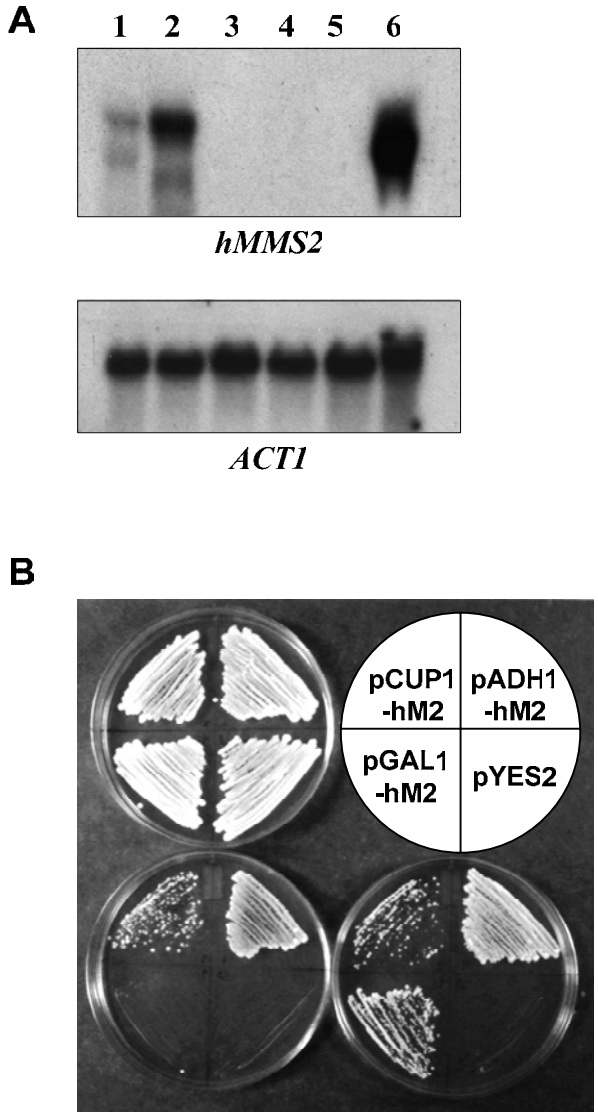

Figure 3.4. Promoter strength of P_{GAL1}, P_{ADH1}, and P_{CUP1} examined by expression of *hMMS2*. **(A)** Northern hybridization. Total RNA was isolated from CH1305 cells transformed with YEpCUP1-hM2 (lane 1), YEpADH1-hM2 (lane 2), pYES2.0 (lanes 3 and 5), and YEpGAL1-hM2 (lanes 4 and 6). The transformants were grown either in YPD (lanes 1-4) or YPGal (lanes 5 and 6) prior to RNA isolation. The membrane was sequentially hybridized with the *hMMS2* and the *ACT1* probes. **(B)** Functional expression of the *hMMS2* gene driven by various promoters. FY86m2L cells harboring YEpADH1-hM2, YEpCUP1-hM2, YEpGAL1-hM2, or vector alone (pYES2) were streaked onto YPD (two on left) or YPGal (bottom right) plates with (two on bottom) or without (top left) 0.025% MMS, and the plates were incubated at 30^{o}C for 4 days.

The promoter strength was further examined by the ability of the above *hMMS2* constructs to functionally complement the yeast *mms2* null mutant. The yeast *mms2* mutant is defective in DNA PRR and displays enhanced sensitivity to DNA damaging agents such as UV and MMS (Broomfield et al., 1998). In the presence of 0.025% MMS, the *mms2* mutant was unable to grow; however, *mms2* cells harboring YEpADH1-hMMS2 or YEpCUP1-hMMS2 were able to grow, regardless of carbon source. In contrast, the YEpGAL1-hMMS2 transformants grew on the YPGal plate, but not on the YPD plate (Figure 3.4B). These results collectively confirmed that under our experimental conditions, YCp plasmid instability is relatively correlated with the promoter strength and appears to have a threshold and that only the *GAL1* promoter can be regulated to effectively alter plasmid stability.

3.3.4. Comparison of Two Synthetic Lethal Screen Protocols

MRE11 is involved in repairing double-strand chromosome breaks, HR and NHEJ, and has been shown to play a role in telomere maintenance (Haber, 1998a). The *mre11* mutation is known to be synthetic lethal with *rad27* (Symington, 1998) and we suspect that it may also be synthetic lethal with other unknown mutation(s). A conventional synthetic lethal screen with *mre11* resulted in 90% nonsectoring colonies (Table 3.2), presumably due to the fact that *mre11* mutant cells grow slowly and aggregate in the culture medium. To determine whether or not the *GAL1* promoter is able to improve the efficiency of this synthetic lethal screen we used pSLS1-MRE11 instead of pSLS2-MRE11 and plated the transformants on YPGal and YPD after EMS

Table 3.2. Characterization of *mre11* synthetic lethal screens by different methods.

Plasmid	Pre-culture medium	Plating medium	Total colonies[a]	Nonsectoring colonies (%)	Colonies characterized	Total false positives
pSLS1-MRE11	YPD	YPGal	5000	7 (0.14)	7	1
pSLS1-MRE11	YPD	YPD	5000	4500 (90)[b]	64	64
pSLS2-MRE11	YPD	YPGal	5000	4500 (90)[b]	30	30

[a]Approximate number of colonies appeared on five 150-mm plates after a 5-day incubation at 30°C.
[b]Number and percentage are approximate.

mutagenesis. When cells carrying pSLS1-MRE11 were plated onto a rich galactose medium, the vast majority of colonies were completely white with only seven colonies showing the solid red phenotype. All but one did not segregate after vigorous testing. In contrast, both pSLS1-MRE11 transformants plated on YPD and pSLS2-MRE11 transformants plated on YPGal predominantly showed a solid red phenotype. Further vigorous tests of randomly picked small samples showed that they were all false positives (Table 3.2). These results clearly demonstrate that the *GAL1* promoter can efficiently reduced the amount of false positives and eliminate successive rounds of screening.

3.3.5. The *ade2* and *ade3* disruption cassettes

To carry out a synthetic lethal screen using the colony-sectoring assay, the transforming strain must carry the *ade2 ade3* marker. The *ADE2* gene is required in purine biosynthesis to convert P-ribosylaminoimidazole (AIR) to P-ribosylaminoimidazolecarboxylate (CAIR). Strains that harbor the *ade2* mutation accumulate the intermediate AIR producing the red pigment. *ADE3* is involved in the metabolism of tetrahydrofolate (THF); three enzymes, methyleneTHF dehydrogenase, methenylTHF cyclohydrolase, and formylTHF synthetase, are encoded by the *ADE3* locus (Jones and Fink, 1982). The *ade3* mutation blocks the purine pathway at a point prior to *ade2*, thus making the *ade3* phenotype epistatic to *ade2* (Hieter et al., 1985; Koshland et al., 1985). This mutation also blocks a branch of the histidine pathway making the *ade3* strain a histidine auxotroph (Luzzati, 1975). Initial synthetic lethal screens utilized naturally isolated *ade2 ade3* mutants that may be revertable in each

allele and affect colony-color sectoring. In addition, a certain mutational background may be required for a given screen where an *ade2 ade3* double mutant may not be readily available. We thus created plasmids containing *ade2Δ::hisG-URA3-hisG* and *ade3Δ::hisG-URA3-hisG* cassettes to facilitate the strain creation. First, the *ADE2* and *ADE3* genes are deleted by using these cassettes to avoid undesired revertants. Second, the *hisG-URA3-hisG* allows repeated use of the *URA3* gene as the selectable marker (Alani et al., 1987) by positively selecting *URA3-hisG* pop-outs on an FOA plate (Boeke et al., 1984). Finally, deletion of the *ADE2* and *ADE3* genes can be sequentially identified by first isolating pink *ade2Δ* colonies, and then isolating white *ade2Δ ade3Δ* colonies.

3.4 Discussion

In eukaryotes such as *S. cerevisiae*, the completed genome sequence has greatly facilitated and accelerated research with regard to gene functions. It is now practical to systematically analyze the lethality and any other given phenotypes through each gene deletion (Shoemaker et al., 1996). However, currently the synthetic phenotypes of more than two mutations rely heavily on conventional genetic analyses. The synthetic lethal screen is an extremely useful method in the identification of related pathway mutations, and can be potentially extended to the identification of genetic interactions that are conditionally lethal under certain growth conditions (i.e. absence of nutrients, resistance to antibiotics or DNA damaging agents). Given the fact that the majority of yeast genes have been found to have orthologs in higher eukaryotes, the exploration of this

organism has made, and will continue to make, significant impacts on agricultural and medical research.

In this study, we wished to further improve the existing synthetic lethal screen protocol by reducing and eliminating large number of false positive clones. To do this, we examined the effects of two inducible (*GAL1*, *CUP1*) and one constitutive (*ADH1*) promoters on the YCp plasmid stability. The *CUP1* and *GAL1* promoters can be readily induced by adding $CuSO_4$ or using galactose instead of glucose as the sole carbon source, respectively, in the culture medium. However, in comparison with transformants carrying parental YCp and YRp plasmids, it appears that only the *GAL1* promoter can be utilized to regulate YCp plasmid stability, whereas *CUP1* and *ADH1* promoter-based plasmids behave like the YRp plasmid, regardless of culture conditions. The unique feature of the *GAL1* promoter seems to be intrinsic to its tight regulation in glucose vs. galactose media. The *GAL1-GAL10* dual promoters are known to be repressed at the transcriptional level by glucose through several mechanisms (Johnston and Carlson, 1992). In contrast, the *CUP1* promoter, although inducible, displays a significantly higher basal transcriptional activity, so that it is sufficient to disrupt the CEN function in the absence of induction. Although such a high basal activity of the *CUP1* promoter may be dependent on the host strain background, the YCpU-ADE3-P_{CUP1} exhibited instability in two different strains tested, suggesting that this phenomenon is common in most laboratory yeast strains. The GAL1-CEN4-based vector was compared with a standard CEN4-based vector in the screen of mutations synthetic lethal with *mre11*. Our improved protocol is apparently superior to the conventional protocol under this condition.

Regulating the plasmid stability may confer several advantages during the synthetic lethal screen. First and foremost, it effectively eliminates all false positives resulting from inefficient plasmid loss rate. Our results show that under inducible conditions, all colonies are not only sectoring, but become predominantly white. Hence, it becomes extremely easy to identify the nonsectoring, entirely pink colonies among those clearly sectored ones. Second, it does not inherit some drawbacks associated with YRp plasmids, such as undesired plasmid loss during mutagenesis and recovery, as well as difficulties in growing cells in the selective medium. Third, although cells grown on YPGal plates may carry various copies of the plasmids (due to the disruption of CEN function) that may affect the efficacy of the synthetic lethal screen, subsequent replicating of putative positives onto YPD plates will return their single-copy state, which appears to be as stable as a typical YCp plasmid, and is expected to be one copy per cell. Finally, the regulated YCp construct may confer additional advantages when mutations of the gene of interest severely retard cell growth, which is the case for many mutants employed in synthetic lethal screening.

Several practical observations made in this study are also worth noting. The YCpU-ADE3-P_{GAL1} transformants were incubated, mutagenized and post-incubated all in the glucose medium, and plated onto YPGal. While the cell growth on galactose medium is slightly slower than on glucose medium, the colonies develop enough color to be clearly scored during a 5-7 day incubation. The relatively small size of colonies sufficient for scoring affords an increase in cell plating density. We routinely plate up to 3,000 cells per large (150 mm diameter) petri dish. We also found that less expensive, low-grade galactose containing up to 2% glucose does not affect the colony

sectoring efficiency. Finally, plasmids YCpU-ADE3-P_{GAL1} and YCpU-ADE3 have been further modified to form pSLS1 and pSLS2, respectively, which allow the genes of interest to be cloned into unique sites such as *Bam*HI and *Sal*I. We have used these vectors to successfully clone several genes and found that these plasmids behave as expected.

Chapter Four – Involvement of DNA Damage Checkpoints for Efficient PRR

4.1. Abstract

The *S. cerevisiae* Mms2 protein is involved in PRR. In order to gain better insight into the roles of PRR, we performed a screen to identify mutations that are synergistic with an *mms2* deletion. *MMS2* encodes a ubiquitin-conjugating enzyme variant involved in the error-free branch of PRR. *REV3* encodes a DNA polymerase required for mutagenic TLS and belongs to the error-prone branch of PRR. We have shown that in the presence of 0.005% MMS, *rev3* is conditionally synthetic lethal with *mms2*. Characterization of a conditional synthetic lethal mutation with *mms2* identified the *RAD9* checkpoint gene. Epistatic analysis showed that *rad9* is synergistic to both *mms2* and *rev3* with respect to killing by MMS. In addition, *rad18* is epistatic to *rad9*, suggesting that *rad9* belongs to the PRR pathway. In addition, *mms2* spontaneous mutagenesis is partially dependent on the *RAD9* gene, and the induced mutagenesis of *mms2* is completely abolished in cells carrying a *rad9* mutation. These results suggest that checkpoint mechanisms are essential for efficient repair in both the error-free and error-prone branch of PRR.

4.2. Introduction

Since the late 1950s and early 1960s, molecular biology has allowed scientists to characterize, isolate, and manipulate the molecular components of cells and organisms. Genetic interactions can occur between two or more mutations that result in a new phenotype. Studying these interactions can reveal gene function, the nature of the mutations, functional redundancy and protein interactions. Cells and organisms defective in processing DNA damage typically show increased sensitivity to DNA damaging agents. This phenotype has proved to be particularly useful for the isolation and genetic characterization of DNA repair mutants. Yeast mutants hypersensitive to UV or IR are designated as *rad* mutants (Haynes and Kunz, 1981). Many of the corresponding mutations have been grouped into multiple complementation and allelic groups (Haynes and Kunz, 1981). In addition, mutations from several of the established complementation groups have been tested for epistatic interactions by comparing the sensitivity of double mutants to each of the relevant single mutants. Epistasis is inferred when an allele of one gene eliminates expression of alleles of another gene and expresses its own phenotype instead. Generally, when a gene acts "upstream" in a biochemical pathway, we would expect there to be an epistatic effect of a defective allele on genes later in the sequence. Therefore, finding a case of epistasis provides insight about the sequence in which genes act. In contrast, additive effects reflect genetic or environmental perturbations that elicit independent cellular responses. A synergistic effect or synergy is used to describe the effect obtained when two mutations in an organism is greater than the effect of the sum of the individual effects.

To help define the PRR pathway, yeast cells were screened for mutations that are conditionally lethal in the absence of *MMS2*. By taking advantage of the synergism between error-free PRR and error-prone mutagenesis pathway, we used a synthetic lethal screening protocol in the presence of extremely low does of MMS (0.005%) that will not affect the ability of single mutant growth, but effectively kill the double mutants. One of the synthetic lethal mutations identified in this screen revealed a role for checkpoint proteins in the PRR pathway.

Eukaryotic cells are constantly challenged by environmental stresses and normal cellular processes that can cause DNA damage and compromise the integrity of the genome. Organisms have evolved surveillance mechanisms that sense and respond to genome damage. This surveillance mechanism, known as DNA-damage checkpoints, was initially identified when inactivation of genes resulted in defects in cell cycle arrest in response to genotoxic treatments.

DNA checkpoints play a significant role in cancer pathology. The *p53* tumor suppressor gene is mutated in sporadic human cancers (Bargonetti and Manfredi, 2002). p53, like many checkpoint proteins, is not essential for cell viability, but instead has a role in G1 arrest and apoptosis (Taylor and Stark, 2001). *ATM* (ataxia telangiectasis mutated), required for most DNA maintenance responses, when mutated is responsible for the human disease ataxia telangiectasis (AT) (Savitsky et al., 1995). The number of checkpoint genes that are now recognized to be involved in human pathology clearly demonstrates the importance of understanding the molecular mechanisms of DNA checkpoints and how they are involved in maintaining genomic integrity. The DNA damage checkpoint requires a set of proteins that have been highly conserved during

evolution (Foiani et al., 2000). Studies in *S. cerevisiae* revealed that the cellular response to DNA lesions results in the induction of a signal transduction cascade which has been divided into three major groups of proteins that act together allowing cells to respond by inducing cell cycle arrest and repair processes. These groups include sensor proteins, transducer proteins and effector proteins.

The DNA damage checkpoint was initially discovered by Weinert and Hartwell (1988) when analyzing the *rad9* mutant of *S. cerevisiae*. In addition to *RAD9* several genes have been found to be involved in the DNA damage response pathway; the order of function of the genes in the cascade has been mainly inferred by monitoring the phosphorylation of proteins belonging to the pathway (Carr, 2002; Longhese et al., 1998; Lowndes and Murguia, 2000). The G1, G2 and intra-S DNA damage checkpoints engage several groups of proteins that function in combination with a central signal transduction cascade. Rad17, Mec3 and Ddc1 form a heterotrimeric complex with a structural similarity to PCNA (Kondo et al., 1999; Thelen et al., 1999). Rad24 is related to replication factor C (RFC), a protein complex responsible for loading PCNA onto DNA during replication (Waga and Stillman, 1998). The interaction of Rad24 with the four smaller RFC subunits acts to load the Rad17-Mec3-Ddc1 complex close to DNA lesions enabling the detection of DNA damage (Majka and Burgers, 2003). The sensor proteins are thought to directly associate with damaged DNA; however, the type of lesion(s) recognized by these proteins has yet to be expounded. Once the damage has been detected, the checkpoint pathway transmits the signal through a kinase cascade.

The *RAD9* gene functions predominantly in the G1/S and G2/M transitions of the DNA damage checkpoints. Rad9 is phosphorylated during normal cell-cycle

progression (Vialard et al., 1998), and hyperphosphorylated after DNA damage in a Mec1- and Tel1-dependent manner (Emili, 1998; Vialard et al., 1998). It is proposed that Rad9 recruits and catalyses the activation of Rad53 by acting as a scaffold that brings Rad53 molecules into close proximity, facilitating *in trans* autophosphorylation of Rad53 (Toh and Lowndes, 2003). Phosphorylation of Rad53 depends on the function of several DNA damage checkpoint gene products (Sanchez et al., 1996; Sun et al., 1996). Activated Rad53 is involved in cell cycle arrest, transcriptional induction of repair genes, inhibition of late replication origin firing and stabilization of stalled replication forks (de la Torre Ruiz and Lowndes, 2000; Lopes et al., 2001; Santocanale and Diffley, 1998; Santocanale et al., 1999; Tercero and Diffley, 2001).

Like Rad53, Mec1 plays a central role in the DNA damage checkpoint at all cell cycle stages. Mec1 is a member of the evolutionarily conserved subfamily of phosphatidylinositol 3-kinase (PI3-kinase) (Elledge, 1996) and plays a critical role in the DNA damage checkpoint control throughout the cell cycle (Longhese et al., 1998). Mec1 functions in a partially redundant manner with Tel1, another member of the PIK family (Lowndes and Murguia, 2000). Several species have orthologs of *MEC1* and *TEL1*, including humans (*ATR* and *ATM*, respectively) and fission yeast *S. pombe* (*rad3*) (Abraham, 2001). Several cellular proteins become rapidly phosphorylated in a Mec1/Tel1-dependent manner in response to DNA damage, including Rad53 and Chk1 (Lowndes and Murguia, 2000). Mec1 constitutively interacts with Ddc2 (Rouse and Jackson, 2002). Recent evidence shows that Mec1/Ddc2 and the Rad17 complex can be independently recruited onto damaged DNA (Kondo et al., 2001; Melo et al., 2001; Zou et al., 2002).

The checkpoint proteins arrest the cell cycle in the presence of DNA damage, allowing the cell time for repair. Checkpoint proteins may also be able to directly recruit DNA repair machinery or play a direct role in repairing damage. General mechanisms linking checkpoint protein function and genomic stability have been illustrated in budding yeast. For example, checkpoint mutants spontaneously lose chromosomes (Klein, 2001b), and defects in the checkpoints lead to an increased frequency of ectopic recombination in meiotic and mitotic cells (Fasullo et al., 1998; Grushcow et al., 1999). Furthermore, *RAD9, RAD17, RAD24* and *MEC3* are required for UV-induced mutagenesis, and *RAD9* and *RAD17* are required for maximal UV induction of SCE in *rad1Δ* cells (Paulovich et al., 1998).

We report here the conditional synthetic lethality of *rad9* and PRR mutants. Our results show the partial requirement of *RAD9* in the spontaneous mutagenesis observed in the *mms2Δ* mutants. These findings suggest a role for the checkpoint pathway in PRR, potentially for delaying the cell cycle and allowing time for the cells to repair the damage using the PRR pathway.

4.3. Results

4.3.1. *rad9* Is Conditionally Synthetic Lethal with *mms2* and *rev3*

The colony color assay of Bender and Pringle (1991) was used to identify mutants that require the error-free PRR gene *MMS2* for viability on plates containing 0.005% MMS. An *mms2Δ ade2-1 ade3Δ* strain was constructed and transformed with pSLS-MMS2, which contains the *MMS2*, *ADE3* and *URA3* genes. Cells carrying an *ade2* mutation are red while cells with *ade2 ade3Δ* mutations are white; the transformed

strain acquires a red color due to *ADE3* function (Hieter et al., 1985; Koshland et al., 1985). When grown without selection, pSLS-MMS2 may be lost and the strain's color changes from red (*ade2*) to white (*ade2 ade3*) with colonies exhibiting a sectoring morphology (Figure 3.2). Following mutagenesis we screened for nonsectoring colonies that retain the plasmid in the presence of 0.005% MMS. Of 50,000 mutagenized colonies screened, 15 potential conditional synthetic lethal mutants were identified, of which 12 were placed into known error-prone PRR pathway genes, including *REV3* and *REV1*. One of the strains recovered in the screen had reduced transformation efficiency and was therefore not further characterized. Two of the conditional synthetic lethal mutants were not placed into known error-prone PRR genes and were chosen for further characterization. The SLM-11 mutant will be discussed below and the SLM-9 mutant will be discussed in the next chapter.

The mutant SLM-11 was obtained in the synthetic lethal screen and was transformed with a genomic library to recover DNA which complements the MMS sensitivity observed in this strain. Functional cloning of a library plasmid obtained from the SLM-11 transformants revealed the *RAD9* gene. To investigate the role of *RAD9* within the *RAD6* pathway, we measured MMS-induced killing of the *rad9Δ mms2Δ* double mutant by both a plate assay and liquid assay. While the *rad9Δ* mutant showed no sensitivity and the *mms2Δ* single mutants showed some sensitivity to the DNA-damaging agents, the *rad9Δ mms2Δ* double mutant was extremely sensitive to MMS (Figure 4.1 A,C). The effect of the two mutations was clearly synergistic using either the plate assay or a liquid killing assay. To determine if *rad9Δ* is only involved

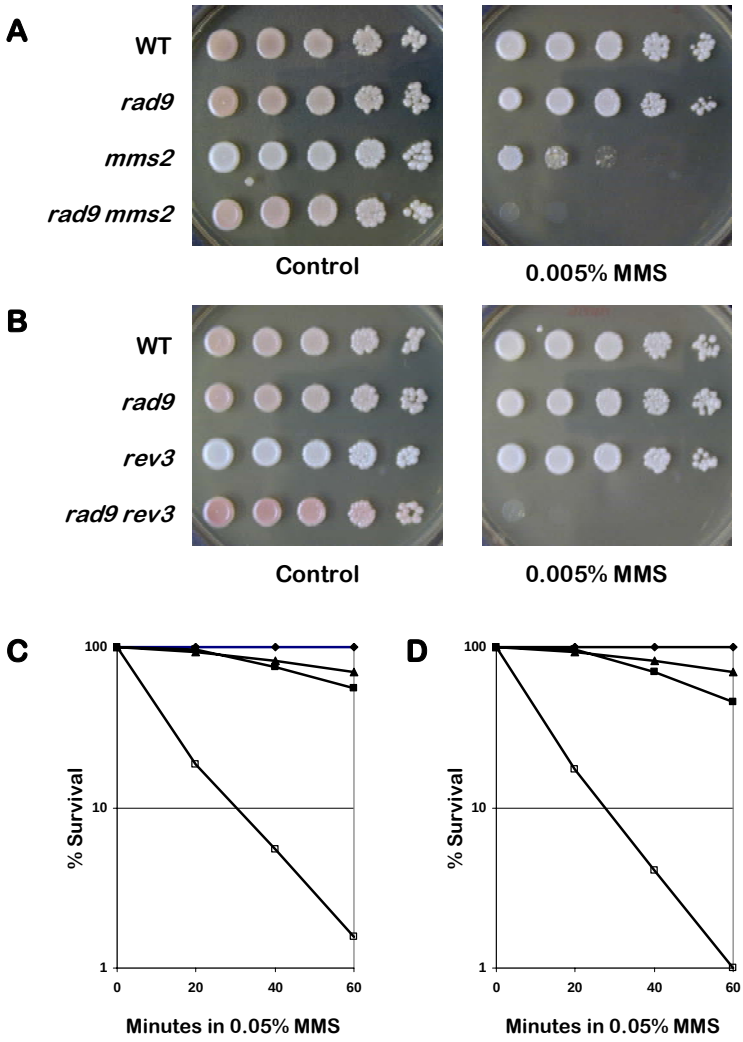

Figure 4.1. Sensitivity of *rad9* and PRR mutants to MMS. For the plate assay, cells were cultured in YPD at 30°C until they reached log-phase. The 10-fold serial dilutions of the cell suspension were spotted onto YPD plates or YPD plates containing the indicated concentration of MMS. Incubation was carried out at 30°C for 2 days before the plates were photographed. For the MMS-induced liquid killing, the wild-type strain and its isogenic derivatives were treated with MMS for the given time and plated onto YPD plates to score for cell survival and compared with untreated cells. These results show a typical experiment. Each experiment has been repeated at least three times. **(A)** WXY1228 (*mms2Δ*), HK845-1A (*rad9Δ*), WXY1202 (*mms2Δ rad9Δ*); **(B)** WXY1233 (*rev3Δ*), HK845-1A (*rad9Δ*), WXY1205 (*rev3Δ rad9Δ*); **(C)** (♦) DBY747 (wild-type), (▲) WXY642 (*mms2Δ*), (■) WXY9519 (*rad9Δ*), (□) WXY1224 (*mms2Δ rad9Δ*); **(D)** (♦) wild-type, (▲) WXY9382 (*rev3Δ*), (■) WXY9519 (*rad9Δ*), (□) WXY1227 (*rev3Δ rad9Δ*).

in one branch of the PRR pathway, we combined *rad9Δ* with *rev3Δ* and determined the MMS-induced killing of the *rad9Δ rev3Δ* double mutant. Both single mutants alone showed no sensitivity on 0.005% MMS. However, the *rad9Δ rev3Δ* double mutant showed a synergistic effect to the DNA damaging agents (Figure 4.1 B,D). This suggests that *RAD9* genetically interacts with both the error-free and error-prone branches of PRR and may constitute a third branch of the PRR pathway.

Different DNA damaging agents produce specific types of lesions which are more detrimental to the cell at specific stages of the cell cycle. Cells in the G1 phase of the cell cycle are most sensitive to UV damage which causes predominantly thymine-thymine dimers. In contrast, γ-irradiation produces DSBs, lesions causing the most effect to cells in the G2 phase of the cell cycle (Friedberg et al., 1995). To determine if the synergistic effect seen in the *mms2Δ rad9Δ* and *rev3Δ rad9Δ* double mutants is specific to MMS damage and thus during intra S-phase of the cell cycle, the sensitivity of these mutants to UV and γ-irradiation was determined (Figure 4.2 A,B). When treated with UV irradiation, the *rad9Δ*, *mms2Δ* and *rev3Δ* mutants all display sensitivity to the DNA damaging agent. When combining the *rad9Δ* mutation with either the *mms2Δ* or *rev3Δ* mutation the double mutants display an additive phenotype to UV-irradiation (Figure 4.2 A). This suggests that the *RAD9* gene does not have a strong genetic interaction with either *MMS2* or *REV3* in response to UV irradiation. The *rad9Δ* mutant shows a 10 fold elevated level of sensitivity to γ-irradiation than the *mms2Δ* or *rev3Δ* single mutants. When the *rad9Δ* mutation is combined with *rev3Δ*, the double mutant shows an additive phenotype to γ-irradiation when compared to the

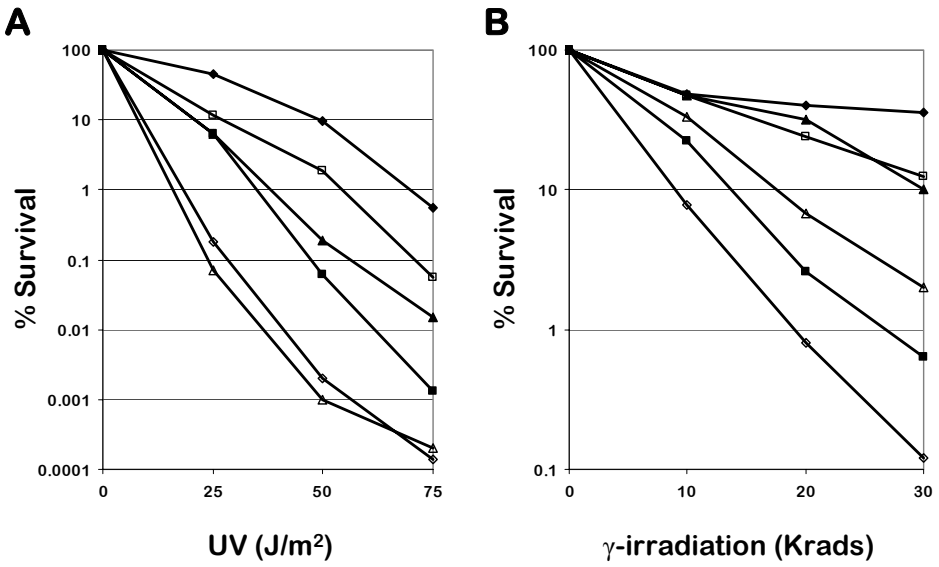

Figure 4.2. Sensitivity of *rad9Δ* and PRR mutants to UV- and γ-irradiation. For the UV- and γ-induced killing, the wild-type strain and its isogenic derivatives were plated onto YPD plates and treated with either UV **(A)** or γ-irradiation **(B)** at the dose indicated. The plates were scored for cell survival and compared to untreated cells. These results show a typical experiment. Each experiment has been repeated at least three times. (♦) DBY747 (wild-type), (■) WXY9519 (*rad9Δ*), (▲) WXY642 (*mms2Δ*), (□) WXY9382 (*rev3Δ*), (Δ) WXY1224 (*mms2Δ rad9Δ*), (◊) WXY1227 (*rev3Δ rad9Δ*).

single mutants. In contrast, combining the *rad9Δ* mutation with the *mms2Δ* mutation resulted in a slight rescuing affect of the *rad9Δ* phenotype (Figure 4.2 B); however, this effect is not significant and no genetic interactions can be concluded from this phenotype. It is possible that a small portion of the lesions caused by γ-irradiation are repaired by means of the error-free branch of PRR, and it is essential that the cell contains an active checkpoint when repairing γ-irradiation damage through the PRR mechanism. Nevertheless, these results suggest a weak genetic interaction for both UV and γ-irradiation when the *rad9Δ* mutation is combined with *mms2Δ* or *rev3Δ*.

4.3.2. *rad18* Is Epistatic to *rad9*

The relative level of MMS sensitivity of the *mms2Δ rad9Δ* and *rev3Δ rad9Δ* double mutants led us to speculate that *RAD9* may function in the PRR pathway. To further determine the genetic involvement of *RAD9* in the PRR pathway we combined the *rad9Δ* mutation with both *rad18Δ* and *mms2Δ rev3Δ* mutations. We found the *rad9Δ rad18Δ* double mutant and the *mms2Δ rev3Δ rad9Δ* triple mutant was no more sensitive to 0.0005% MMS than the *rad18Δ* single mutant (Figure 4.3 A). The *rad9Δ* mutant has no growth defect on this small amount of MMS and looks like wild-type cells (data not shown). Due to the high number or revertants in the *rad18Δ* single mutant it is difficult to obtain accurate survival levels in a liquid killing assay. However, by using a plate assay it is possible to distinguish the revertants from the *rad18Δ* mutant cells. Using a plate assay we found consistent results when treating the cells with 0.0005% MMS (Figure 4.3 B). Interestingly, *rad18Δ* is epistatic to the

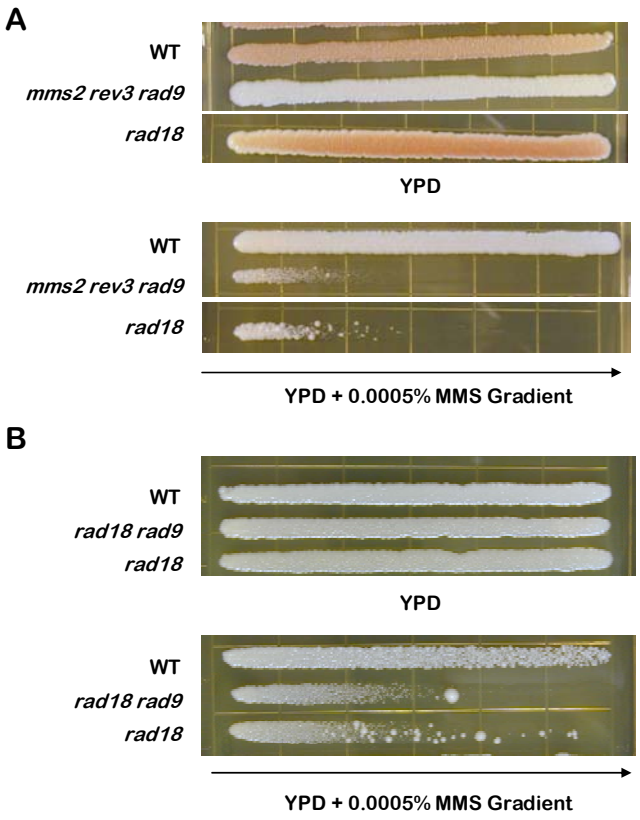

Figure 4.3. Epistasis analysis of *rad18Δ* with *rad9Δ* or *mms2Δ rev3Δ rad9Δ*. Sensitivity of mutants to MMS by a gradient plate. YPD and YPD + 0.0005% MMS gradient plate were incubated at 30°C for 2 days. The arrow points towards the higher MMS concentration. **(A)** DBY747 (WT), WXY1225 (*mms2Δ rev3Δ rad9Δ*), WXY9444 (*rad18Δ*); (**B)** DBY747 (WT), WXY9444 (*rad18Δ*), WXY1226 (*rad18Δ rad9Δ*). The *rad9Δ* strain is not sensitive on 0.005% MMS and looks like WT (data not shown).

mms2Δ rev3Δ rad9Δ triple mutant, whereas the *mms2Δ rev3Δ* double mutant shows a higher level of resistance to MMS than the *rad18Δ* mutant. This result is consistent with a role for *RAD9* in PRR, where *RAD9* may define a third sub-pathway with *MMS2* and *REV3,* and deletion of the three genes results in an MMS sensitivity equivalent to the *rad18Δ* single mutant.

4.3.3. *RAD9* Is Required for Mutagenesis in PRR

We further predicted that if the *rad9Δ* mutation affects all branches of the PRR pathway, it may play a role in the mutagenesis seen when lesions are channeled into the error-prone pathway. The spontaneous mutation rates of the mutants were determined using the *trp1-289* reversion assay. As expected, the *mms2Δ* single mutant had an increased spontaneous mutation rate by ~22 fold at the *trp1-289* allele compared to wild-type cells, whereas the spontaneous mutagenesis was abolished in the *mms2Δ rev3Δ* double mutant (Table 4.1). The spontaneous mutation rate of *rad9Δ* was ~1.6 fold higher than wild-type. As expected, this minor spontaneous mutagenesis was abolished when *rad9Δ* is combined with *rev3*Δ. Interestingly, the *mms2Δ* mutant, which has a spontaneous mutagenesis ~22 fold compared to the wild-type cells, was reduced to ~12 fold when combined with the *rad9Δ* mutation. This suggests that the *RAD9* gene is partially responsible for the spontaneous mutagenesis seen in the absence of *MMS2* (Table 4.1).

We also determined the involvement of the *rad9Δ* mutation on induced mutagenesis. As seen in the spontaneous mutagenesis assay, the MMS-induced reversion of the *trp1-289* allele was also elevated in the *mms2Δ* mutant (Table 4.2). As

Table 4.1 Spontaneous mutation rate of *S. cerevisiae* strains

Strain	Mutation Rate 10^{-9} (SD)	Relative Rate[a]
WT	7.0 (3.5)	1.0
mms2Δ	155.0 (21)	22.1
rev3Δ	3.7 (2.5)	0.5
rad9Δ	11.1 (2.3)	1.6
mms2Δ rev3Δ	6.2 (1.4)	0.9
mms2Δ rad9Δ	84.0 (16)	12.0
rev3Δ rad9Δ	4.5 (2.9)	0.6
mms2Δ rev3Δ rad9Δ	7.4 (4.1)	1.1

All strains are isogenic and carry the revertable *trp1-289* amber mutation. Rates are expressed as number of revertants per cell per generation.
[a] Relative to the wild-type strain

Table 4.2 Induced mutation frequency of *S. cerevisiae* strain

Strain	% Survival	Mutation Frequency (10^{-7} Viable Cells)
WT	92	76.0
mms2Δ	68	110.0
rev3Δ	75	0.29
rad9Δ	85	< 0.12
mms2Δ rev3Δ	2	< 5.0
mms2Δ rad9Δ	9	< 1.1
rev3Δ rad9Δ	11	< 0.9

All strains are isogenic and carry the revertable *trp1-289* amber mutation.

expected the induced mutagenesis was abolished in the *rev3Δ* mutant. Interestingly, when the cells were deleted for *RAD9*, the mutation frequency could not be scored when plating 10^{-8} surviving cells. The mutation frequency seen in the *mms2Δ* strain was completely abolished when combined with *rad9Δ*, and again we found that we could not score a single mutation event among 10^{-8} surviving cells. These results suggest that the *RAD9* gene is required for induced mutagenesis seen in the *mms2Δ* mutant. These results are consistent with a role for *RAD9* in participating in the PRR pathway.

4.3.4. The DNA Damage Checkpoint Is Required for Efficient PRR

It can be speculated that the involvement of the *RAD9* gene in the PRR pathway is strictly to delay the cell cycle progression in response to DNA damage. To determine if *RAD9* has a unique role in PRR, we combined a *rev3Δ* mutation with additional checkpoint mutations, such as *rad17Δ* and *rad24Δ*. None of the *rev3Δ, rad17Δ* or *rad24Δ* single mutants displayed a sensitivity to 0.005% MMS. However, we observed a strong lethal phenotype in the *rad17Δ rev3Δ* and *rad24Δ rev3Δ* strains when exposed to 0.005% MMS on a plate assay (Figure 4.4 A,B). These results suggest that *RAD17* and *RAD24* checkpoint genes participate in the PRR pathway, most likely in a manner similar to *RAD9*.

4.3.5. *RAD53* but not *CHK1* Is Involved in the DNA Damage Response

The *RAD53* and *CHK1* genes are responsible for defining two parallel pathways

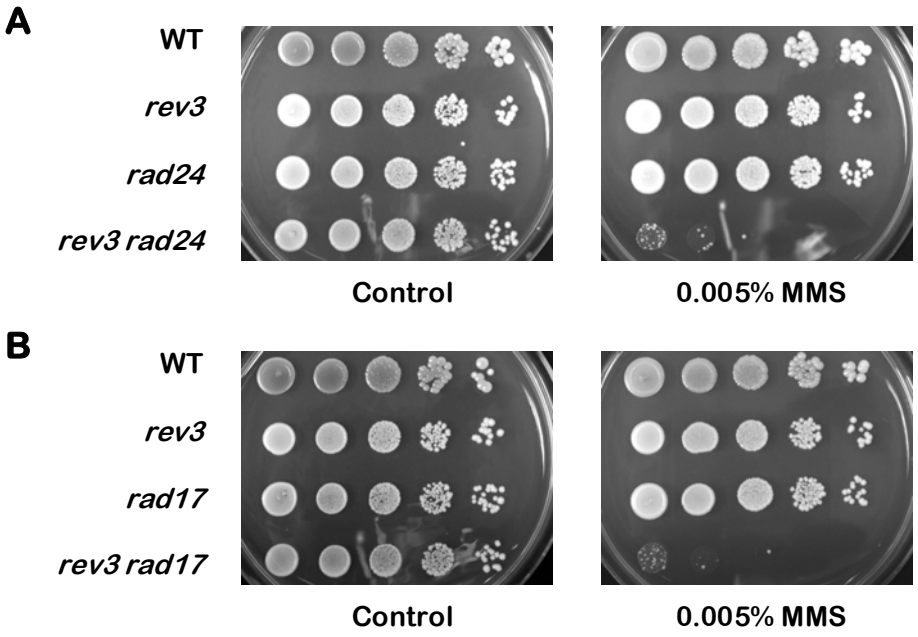

Figure 4.4. Sensitivity of *rev3Δ rad24Δ* and *rev3Δ rad17Δ* mutants to MMS. Cells were cultured in YPD at 30°C until they reached log-phase. The 10-fold serial dilutions of the cell suspension were spotted onto YPD plates or YPD plates containing the indicated concentration of MMS. Incubation was carried out at 30°C for 2 days before the plates were photographed. **(A)** BY4741 *rev3Δ*, BY4741 *rad24Δ*, BY4741 *rev3Δ rad24Δ*; **(B)** BY4741 *rev3Δ*, BY4741 *rad17Δ*, BY4741 *rev3Δ rad17Δ*.

that regulate multiple cell-cycle transitions. To determine if the involvement of checkpoints in the PRR response encompassed the entire DNA damage checkpoint pathway, *rad53Δ rev3Δ* and *chk1Δ rev3Δ* double mutants were tested for their sensitivity to MMS on a plate assay. The *rev3Δ* mutant showed no sensitivity on 0.01% MMS, and the *rad53Δ* mutant was slightly sensitive on the MMS plate. However, the *rev3Δ rad53Δ* double mutant was lethal on this concentration of MMS, which unmistakably shows a synergistic interaction between the two genes (Figure 4.5 A). In contrast, the *rev3Δ chk1Δ* double mutant displayed a phenotype no more sensitive than the single mutants (Figure 4.5 B). These results suggest that the *RAD53* branch, and not the *CHK1* branch, of the checkpoint pathway is responsible for facilitating the repair of the DNA lesion by the PRR pathway.

4.3.6. A G2 Arrest Partially Compensates for the Lack of the Checkpoint Response

Regulation of the eukaryotic cell cycle by checkpoints ensures that preceding processes be completed before initiation of the ensuing processes. Early studies showed that the genetic basis for this response in *S. cerevisiae* is dependent on the *RAD9* gene product and is essential for arrest of cell division induced by DNA damage (Weinert and Hartwell, 1988). Irradiated wild-type G1 and M phase cells arrest irreversibly in G2, whereas irradiated G2 phase haploid cells delay in G2 for a time proportional to the extent of damage before resuming cell division. In contrast, irradiated *rad9* cells do not delay cell division in G2, but continue to divide for several generations and die irregardless of the cell cycle phase at the time of damage. In spite of this, efficient DNA repair can occur in irradiated *rad9* cells if they are blocked for several hours in G2

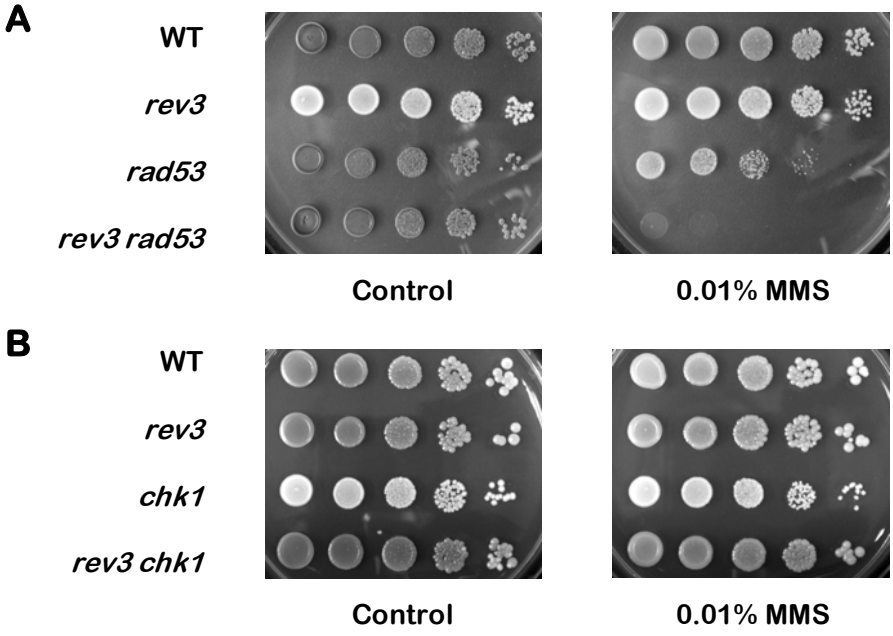

Figure 4.5. Sensitivity of *rev3Δ rad53Δ* and *rev3Δ chk1Δ* mutants to MMS. Cells were cultured in YPD at 30°C until they reached log-phase. The 10-fold serial dilutions of the cell suspension were spotted onto YPD plates or YPD plates containing the indicated concentration of MMS. Incubation was carried out at 30°C for 2 days before the plates were photographed. **(A)** WXY1233 (*rev3Δ*), U960-5C (*rad53Δ*), WXY1231 (*rev3Δ rad53Δ*); **(B)** WYX1233 (*rev3Δ*), HK1031-1A (*chk1Δ*), WXy1234 (*rev3Δ chk1Δ*).

by treatment with a microtubule poison (Weinert and Hartwell, 1988). It is reasonable to hypothesize that the PRR mutants deleted for a checkpoint gene are failing to slow S phase or delay at G2 in response to the DNA damage, and thus are entering M phase with damaged chromosomes. A failure to slow intra-S phase and arrest at G2 would result in the extreme sensitivity seen in the *rev3Δ* checkpoint double mutants.

To investigate the role of the checkpoint response in the PRR mutants, *rev3Δ, rad24Δ* and *rev3Δ rad24Δ* mutants were either treated with MMS under asynchronous or G1-arrested cell cycle conditions. The cells were allowed to resume cell division or were delayed at G2 for 3 hours using a microtubule poison. Wild-type cells showed no increase in survival when treated with MMS as either asynchronous or G1-arrested cultures (Table 4.3). The *rev3Δ* and *rad24Δ* mutants did not display an increase in survival when arrested at G2 and permitted time for recovery. Since both the wild-type and the *rev3Δ* strains contain a functional checkpoint, an increase in survival by chemically arresting cells at G2 is not expected. The *rad24Δ* mutants did not show an increase in survival when asynchronous or G1-arrested at the time of treatment and further held at G2 (Table 4.3). In contrast, the *rev3Δ rad24Δ* double mutant showed a modest increase in survival when treated as a G1-arrested culture and further held at G2 to allow time for repair. The cells showed an increase in survival from 22% to 36% when treated with MMS at G1 and further delayed at G2 for three hours (Table 4.3). However, this increase in survival was not evident in the asynchronous cultures treated with MMS. These results suggest that the checkpoint arrest plays a role in cell survival

Table 4.3. Recovery from 0.05% MMS damage after holding at G2 phase in *S. cerevisiae* strains

Strain	Asynchronous Culture			G1 Arrested Culture		
	% Survival	Released	G2 hold (3h)	% Survival	Released	G2 hold (3h)
WT	100	90	91	100	73	70
rev3Δ	100	92	92	100	61	63
rad24Δ	100	93	97	100	83	84
rev3Δ rad24Δ	100	38	32	100	22	36

in PRR mutants when exposed to MMS damage. This result is consistent with the checkpoints involvement in PRR, most likely as being responsible for delaying the cell cycle at intra-S and G2 to allow the PRR pathway time to repair the damage.

4.4. Discussion

Defects in DNA repair genes can result in the accumulation of spontaneous DNA damage that partially or completely arrest cells during or after DNA replication. Several lines of direct and indirect evidence support the assertion that the checkpoint genes play a role in allowing for efficient PRR. For example, *S. cerevisiae* cells containing a single unrepairable ds break exhibit a long, but transient arrest at G2/M. Cells lacking Ku70, responsible for NHEJ of DSBs, fail to escape from this checkpoint arrest and display accelerated 5' to 3' degradation of the broken chromosome. This arrest can be suppressed by a mutation in the SSB protein, RPA, which suggests a role for RPA in adaptation to the checkpoint arrest (Lee et al., 1998). Nevertheless, only circumstantial evidence exists for both the autonomy and interdependence of checkpoint controls on DNA repair and replication. Checkpoint genes appear to have no essential role in the DNA repair pathways, and no evidence exits for a detectable role for the DNA repair genes in cell cycle arrest (Weinert and Hartwell, 1988). However, a few repair and replication mutants, namely DNA Polε and specific subunits of RPA and RFC, display checkpoint defects (Elledge, 1996; Paulovich et al., 1997b). Whether these replication proteins are needed to signal arrest directly, or if they indirectly form a structure that checkpoint proteins recognize has yet to be elucidated.

In this study we used a synthetic lethal screen to identify novel genes involved in the error-prone branch of PRR. To do this, we utilized a conditional approach by supplementing the screening medium with a small dose of the DNA damaging agent MMS. Cells containing a deletion in either the error-free or error-prone branch of PRR grow efficiently, but cells containing a deletion in both sides of the PRR pathway are synergistic and are unable to grow on plates containing the drug. This approach allowed us to isolate mutations synergistic with the *mms2Δ* mutation. Most of these mutations fall into the *REV* genes, already classified into the error-prone branch of PRR. However, further investigation of the SLM-11 strain identified a synthetic lethal mutation in the *RAD9* gene. Several lines of evidence suggest the involvement of *RAD9* in the PRR pathway.

The results of the screen were confirmed by determining the MMS sensitivity of the *rad9Δ* and *mms2Δ* mutants. While both the individual mutants grow on MMS, the *mms2Δ rad9Δ* double mutant fails to grow on small amounts of MMS and shows the characteristic synergistic phenotype seen with the *mms2Δ rev3Δ* double mutant. The same phenotype was demonstrated with the *rev3Δ rad9Δ* double mutant. In contrast, treatment of the *rev3Δ rad9Δ* double mutants with either UV or γ-irradiation results in an additive phenotype as compared to either of the single mutants. The *mms2Δ rad9Δ* double mutant shows an additive phenotype compared to either of the single mutants when treated with UV irradiation, but displays a small rescuing effect of the *rad9Δ* single mutant phenotype upon exposure to γ-irradiation. These results suggest that the *RAD9* checkpoint gene genetically interacts with both error-free and error-prone PRR

upon specific types of DNA damage, namely MMS, and is important for prolonged S phase of the cell cycle.

We demonstrated that the *rad18Δ* single mutation is epistatic to both the *rad9Δ* and the *mms2Δ rev3Δ rad9Δ* triple mutations. Initial investigations into the epistatic analysis of the PRR pathway demonstrated that the *mms2Δ rev3Δ* double mutant does not display the same MMS sensitivity as the *rad18Δ* single mutant. The level of sensitivity in the double mutant, which was thought to remove all PRR activity, is suggestive for a third, unidentified member in the PRR pathway. The *rad9Δ rad18Δ* double mutant displays the same MMS sensitivity as the *rad18Δ* single mutant. In addition, *rad18Δ* is epistatic to the *mms2Δ rev3Δ rad9Δ* triple mutant and these mutants display the same sensitivity on an MMS gradient. The epistatic analysis of *RAD9* with members of PRR suggests a role for this gene in both the error-free and error-prone branches of the PRR pathway, and possibly constitutes a third, uncharacterized branch of PRR.

The spontaneous and induced mutagenesis in a *rad9Δ* mutant is indicative of a role in PRR. The *rad9Δ* mutant has a slightly elevated spontaneous mutagenesis rate as compared to the wild-type strain. Also, the spontaneous mutagenesis seen in *rad9Δ* is abolished when combined with the *rev3* deletion. In addition, the *mms2Δ* spontaneous mutagenesis is reduced when combined with *rad9Δ*. This suggests that the *RAD9* gene is partially responsible for the spontaneous mutagenesis observe in the absence of *MMS2*. Most interestingly, the MMS-induced mutagenesis seen in *mms2Δ* was completely abolished in the *mms2Δ rad9Δ* strain. These results are all consistent with the involvement of *RAD9* in PRR.

To fully understand the involvement of the checkpoint genes in PRR, we combined several checkpoint genes with the *rev3Δ* strain. The DNA damage sensor components of the checkpoint response, including *RAD17* and *RAD24,* display the same synergistic phenotype on MMS when combined with the *rev3Δ* mutation. The sensors elicit the checkpoint response by activating the signal transducer kinase *MEC1*. From here, two parallel pathways exist in *S. cerevisiae* that regulate multiple cell-cycle transitions. To determine if both pathways are involved with the PRR pathway, *rev3Δ* was combined with *rad53Δ* and *chk1Δ*. *RAD53* is required for several checkpoint responses, including the transcription of G1 cyclins, replication fork progression, replication origin firing during S phase and possibly mitotic exit. On the other hand, *CHK1* is required for the metaphase to anaphase transition by degradation of Pds1. The *rev3Δ rad53Δ* double mutant is synergistic to MMS damage as compared to either of the single mutants. However, the *rev3Δ chk1Δ* double mutant does not show sensitivity to MMS and is identical to the *rev3Δ* and *chk1Δ* single mutants. These results suggest that the *RAD53* component of the DNA checkpoints and not the *CHK1* component is required for efficient PRR.

It is easy to speculate that the involvement of the checkpoints in the PRR pathway is simply to delay the cell cycle to allow time for the DNA repair genes to remove the damage. By experimentally inducing a checkpoint delay at G2 using a microtubule poison, it should be possible to recover the dramatic sensitivity seen in the *rev3Δ rad24Δ* double mutant. As expected, the *rev3Δ* strain, which has a functional checkpoint, displayed no increase in viability by holding the cells at G2 after treating with UV irradiation. The *rad24Δ* mutant did not demonstrate an increase in survival

after MMS treatment when held in G2 when compared to the cells that were allowed to continue dividing. These cells are not exceptionally sensitive to MMS, and may have been able to repair most of the damage prior to the G2 checkpoint. The cells would therefore not need to arrest at G2, and can safely continue cell division. However, the most noticeable increase in viability by implementing a G2 checkpoint exists in the *rev3Δ rad24Δ* strain. Cells held at G1 during treatment with MMS showed a survival of 22% compared to the untreated controls. However, when a G2 arrest was experimentally induced in these cells, the survival increased to 36%. Although this increase in survival is modest, these results suggest that the checkpoint response is important in PRR mutants in order to help maintain viability.

Based on the results presented in this study, we conclude that the checkpoints employed in response to MMS damage are important for the efficient repair of DNA damage by the PRR mechanism. The *RAD9* gene was originally placed within the *RAD6* epistasis group based on its phenotypic response to various DNA damaging agents (Lawrence and Christensen, 1976; McKee and Lawrence, 1980). Further epistatic analysis of *rad9* with PRR mutants, and the partial requirement of *RAD9* for spontaneous and induced mutagenesis in *mms2Δ* mutants, suggests that checkpoint mechanisms are essential for efficient repair in both the error-free and error-prone branch of PRR. The involvement of *RAD9* in the PRR process is specific to MMS damage and thus the late S and G2 phase of the cell cycle. The checkpoint involvement in PRR can be further refined specifically to the *RAD53* branch of the checkpoint response pathway. We can further conclude that in the presence of MMS damage a cell cycle delay is required for efficient PRR, but in the absence of *RAD18* this delay has no

effect in cell survival. *RAD6* and *RAD18* may serve as a sensor specifically to MMS DNA damage and elicit a SOS type of response, including a DNA damage checkpoint response. In response to UV or γ-irradiation, the cell would elicit a different repair mechanism and would not rely on PRR for repair of the DNA damage. Therefore, it is likely the cell senses the type of lesion as well as the cell cycle phase and thus determines the best mechanism of DNA damage repair.

Chapter Five – Mating type genes play a role in cellular tolerance to DNA damage that is specific to the PRR and Mutagenesis Pathway

5.1. Abstract

Diploid cells heterozygous for mating type (**a**/α) show an increased resistance to UV damage and are more recombination-proficient as compared to haploid cells and diploids homozygous for mating type genes (**a**/**a** and α/α). Expression of both mating type genes in haploids causes cells to be non-mating and behave as if they were diploid. This effect has been demonstrated with mutants defective in PRR, including *rev3* and *rad18*, but is abolished when combined with mutations involved in recombination repair. In contrast to PRR genes, I found the *srs2* haploids expressing both mating types become more sensitive to DNA damaging agents. Either deletion of *SRS2* or altering cells to a psuedodiploid state increase the cells propensity for recombination. However, combination of the two states (an *srs2* psuedodiploid) results in a notable decrease in resistance, which suggests that deletion of *srs2* and expression of both mating type genes results in deleterious recombination intermediates that can no longer be repaired by the cell. Expression of mating type genes has no effect on recombination or excision repair genes.

5.2. Introduction

5.2.1. Mating Type Gene Switching in *Saccharomyces cerevisiae*

The budding yeast *S. cerevisiae* exists as two different mating types determined by the mating type (*MAT*) locus. Along with other fungi, budding yeast has acquired the capability to change from one haploid mating type to another. Cells of opposite mating type are able to self-diploidize, providing the fungi with a number of evolutionarily advantageous strategies unavailable to haploids, including the ability to undergo meiosis and spore formation under nutritionally limiting conditions. In many lower eukaryotes, such as fungi, sexual exchange of genetic material relies on the existence of simple cell identity mechanisms that stimulate outcrossing, thus increasing genetic diversity.

The two mating type alleles, *MAT*α and *MAT***a**, encode four open reading frames with functions for three gene products being identified. These include Mat**a**1, Matα1 and Matα2 (Haber, 1998b). The Matα1 protein interacts with Mcm1 to activate a set of α-specific genes (Bruhn and Sprague, 1994; Hagen et al., 1993), including those encoding the mating pheromone α-factor and Ste2, a trans-membrane receptor of **a**-factor (Haber, 1998b). *MAT*α*2* encodes a repressor protein, which in combination with Mcm1, Tup1 and Ssn6 is responsible for the repression of **a**-specific genes (Herschbach et al., 1994; Keleher et al., 1989). Mat**a**1 has no known role in haploid cells, but when complexed with Matα2 in diploid cells repression of both α-specific and **a**-specific genes occurs rendering the cells nonmating (Goutte and Johnson, 1988; Strathern et al., 1988). The haploid-specific genes repressed include *RME1*, a repressor of early meiosis

genes, and *HO*, which encodes the site-specific endonuclease necessary to induce mating type gene switching (Haber, 1998b).

5.2.2. Regulation of Mating type Gene Switching

An elaborate set of mechanisms has evolved in *S. cerevisiae* which enables cells to undergo mating type switching. *MAT* switching depends on four phenomena: a cell lineage pattern such that only half of the cells in a population switch at any one time; the occurrence of two silenced copies of mating type sequences that act as donors during *MAT* switching; site-specific DSB-induced recombination at the *MAT* locus; and regulation of the selective use of the two donors (Haber, 1998b).

The **a** and α information is present as intact, but unexpressed copies of mating type genes at transcriptionally silent domains of chromatin located at the silent mating type loci, *HMR* and *HML*. Silencing at the mating type loci is directed by specific *cis*-acting regulatory silencer sequences, *HML-E*, *HML-I*, *HMR-E* and *HMR-I* (Laurenson and Rine, 1992). Each of these elements interacts directly or indirectly with a number of *trans*-acting factors to establish or maintain silencing at the mating type loci. The *trans*-acting factors include the Silent Information Regulator (Sir) proteins, Rap1, histones H3 and H4 (Aparicio et al., 1991; Kurtz and Shore, 1991; Thompson et al., 1994) and the Origin Recognition Complex (ORC) proteins (Laurenson and Rine, 1992). Together these gene products and *cis*-acting sequences create short regions of heterochromatin in which the DNA sequences of *HML* and *HMR* are established as a highly ordered, continuous, nucleosome structure (Nasmyth, 1982; Weiss and Simpson,

1998), thus preventing transcription from both RNA PolII- and PolIII-transcribed genes (Brand et al., 1985).

The *SIR* genes were identified as mutations unlinked to *HML* or *HMR,* yet which phenotypically result in constitutive expression of both loci and are separated into four complementation groups (Rine and Herskowitz, 1987). Deletion of *SIR1* partially reduces silencing at the mating type loci, whereas deletion of *SIR2*, *SIR3* or *SIR4* completely abolishes silencing at both the silent mating type loci and telomeres (Rine and Herskowitz, 1987). *In vitro* and *in vivo* studies have shown a tight association between Sir2 and Sir4 and a weak association between Sir4 and Sir3 (Moazed et al., 1997). Recruitment of both Sir3 and Sir4 to the chromosome involves their interaction with Rap1 in a cooperative fashion (Moretti and Shore, 2001), as well as their interaction with the N-termini of histones H3 and H4 (Hecht et al., 1995). *SIR2* belongs to a family of NAD-dependent protein deacetylases (Landry et al., 2000), and is involved in silencing of rDNA, telomeres and at mating type loci.

Important mating type dependent differences exist that cannot simply be attributed to the haploidy versus diploidy state of the cell. For example, *MAT***a**/*MAT*α diploids are markedly different from diploids homozygous for either *MAT***a** or *MAT*α (**a**/**a** or α/α). Diploids heterozygous for mating type are able to initiate meiosis and spore formation, whereas diploids homozygous for mating type cannot. In addition, an axial pattern of budding designed to facilitate efficient mating occurs in haploids and diploids expressing one mating type allele, whereas *MAT***a**/*MAT*α diploids have a polar budding pattern (Chant, 1996).

5.2.3. DNA Repair and Mating type

Some aspects of DNA repair are also under mating type control. Heterozygous diploids are significantly more resistant to IR than diploids homozygous for mating type. As well, spontaneous rates of recombination are higher in *MAT***a**/*MAT*α cells than either **a**/**a** or α/α diploids (Friis and Roman, 1968; Heude and Fabre, 1993). The genetic source for these repair and recombination differences has yet to be elucidated. Recent studies have indicated that the DNA replication machinery is coupled to silencing of mating type loci and may be responsible for chromatin assembly and reestablishment of the parental states of gene expression in daughter cells. In this case, the *RAD6* pathway may be involved in maintaining mating status. Rad6 is the only E2 that cooperates with an E3, Ubr1, in an N-end-rule-dependent ubiquitin conjugation and protein degradation pathway (Dohmen et al., 1991). Rad6 is also required for the ubiquitination of histones H2B (Robzyk et al., 2000). In addition, *RAD6* has been implicated in *SIR*-dependent silencing (Huang et al., 1997). In contrast with the *rad6* mutation, deletion of *UBP3*, a ubiquitin hydrolase, results in an enhancement of silencing, and Ubp3 directly interacts with Sir4 (Moazed and Johnson, 1996). Thus, ubiquitin conjugation by Rad6 at these sites appears to be counteracted by Ubp3 ubiquitin deconjugation.

5.2.4. Regulation of the Switch Between PRR and Recombination by Mating Status

DNA damage and cell cycle stage may not be the only parameters governing the regulatory switch between alternative pathways. Other physiological conditions such as cell ploidy and mating status also appear to be important determinants. For example, deletions of the *SIR* genes causes *MAT***a** or *MAT*α haploid cells to have the non-mating

phenotype characteristic of *MAT***a**/*MAT*α diploid cells (Haber, 1998b). Diploid cells heterozygous for mating type (**a**/α) show an increased resistance to UV damage and are more recombination-proficient as compared to haploid cells and diploids homozygous for mating type genes (Friis and Roman, 1968; Heude and Fabre, 1993). This effect has been demonstrated between haploids and heterozygous diploids with mutants defective in *rev3* (Lawrence and Christensen, 1976) and *rad18* (Boram and Roman, 1976), but is abolished when combined with mutations involved in recombination repair (Saeki et al., 1980). The *SRS2* deletion shows the same sensitivity as either a haploid or a homozygous diploid, and this sensitivity is suppressed by mutations in *RAD51* (Aboussekhra et al., 1992), suggesting lethal recombination events are the probable cause of the UV sensitivity in these cells. Studies by Heude and Fabre (1993) demonstrated that this resistant effect in diploids is reproducible in G2 haploids by simultaneous expression of both *MAT* genes, thus allowing expression of both the **a**1 and α2 products of the *MAT* locus. This allows one to study the effects of mating type ploidy in a haploid yeast cell.

5.3. Results

5.3.1. Independent Segregation of the Synthetic Lethal Mutation in SLM-9

A second mutant recovered in the synthetic lethal screen, SLM-9, displays a synergistic sensitivity on plates containing 0.005% MMS as compared to the *mms2Δ* single mutant. To determine if the conditional synthetic lethal mutation can independently segregate from *mms2Δ*, and whether a single mutation is responsible for the added MMS sensitive phenotype seen in SLM-9, diploids were created and tetrad

analysis was undertaken to determine the segregation of the mutations in SLM-9. Either a wild-type or an *mms2Δ* strain was crossed with SLM-9; the resulting diploids were sporulated, dissected and scored for markers and MMS sensitivity. In both cases the tetrads recovered showed the independent segregation of *mms2Δ* from the synthetic lethal mutation. Dissection of the tetrads from a cross between the *mms2Δ* mutation and SLM-9 revealed a slow growth phenotype compared to the *mms2Δ* single mutant (Figure 5.1 B).

5.3.2. Molecular Cloning of SLM-9 Synthetic Lethal Mutation

5.3.2.1. Isolation of Clones from Genomic Library

To identify the SLM-9 synthetic lethal mutation, a single- and multi-copy yeast genomic library was screened for the functional complementation of the SLM-9 MMS-sensitive phenotype. The single-copy yeast genomic library was obtained from ATCC (Cat. #77162) and utilizes the centromeric YCp50 based plasmid ensuring low copy number and mitotic stability. The library vector, p366, contains a 2.23 kb *Sal*I-*Xho*I *LEU2* fragment instead of the *URA3* gene. The multi-copy yeast genomic library was obtained from ATCC (Cat. #37323) and utilizes the YEp13 plasmid as the vector (Nasmyth and Reed, 1980).

Over 20,000 transformants were screened to obtain the clones capable of suppressing the MMS sensitive phenotype of SLM-9. The plasmids responsible for the complementation were isolated from the SLM-9 mutant and were recovered in *E. coli* cells. Two multi-copy and one single-copy genomic clones were recovered containing inserts ranging from 5 kb to 10 kb.

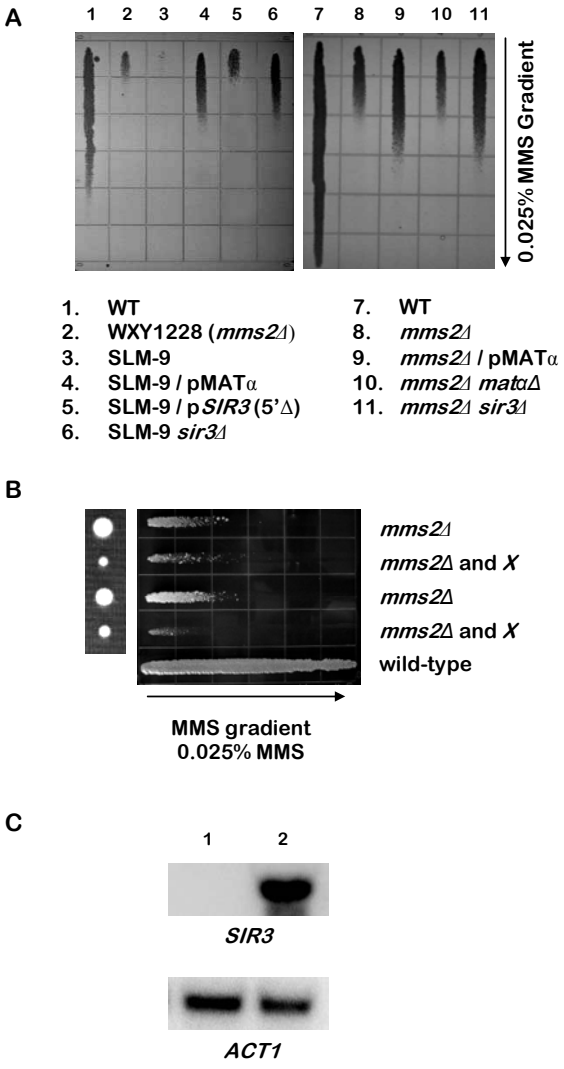

Figure 5.1. (A) Sensitivity of SLM-9 on 0.025% MMS. **(B)** Growth phenotype observed in the SLM-9 strain. Tetrads containing both the *mms2Δ* and the synthetic lethal mutation (X) have a decreased growth rate as compared to the *mms2Δ* single mutant. Tetrads containing the SLM were confirmed by MMS sensitivity. SLM- synthetic lethal mutation. Overnight cultures were replicated onto YPD and YPD + MMS and incubated at 30°C for 42 h. Arrow points towards a higher concentration of MMS. **(C)** Northern hybridization. Total RNA was isolated from W303 cells alone (lane 1) or transformed with YEp-SLM-9-1 (lane 2). The membrane was sequentially hybridized with the *SIR3* and *ACT1* probes.

To confirm that the yeast genomic library plasmids were responsible for the MMS resistance, a co-segregation test was performed. The SLM-9 colonies that were unable to grow on SD-Leu plates were sensitive to MMS. The SLM-9 colonies that were Leu^+ were resistant to MMS. These results confirm that the genomic library plasmids were indeed responsible for complementing the MMS sensitive phenotype of SLM-9 (Figure 5.1A).

5.3.2.2. Mapping the Synthetic Lethal Clone

Two YEp13 genomic library plasmids were recovered with one containing a 6.6 kb genomic fragment (YEp-SLM-9-1) and one having a 4.6 kb fragment (YEp-SLM-9-2). To determine the chromosomal location of the genomic DNA fragment, the insert was sequenced using the primers MAG-4 (5'-CTT CCC CAT CGG TGA TG-3') and pBR-1 (5'-GCT CGC TTC GCT ACT TG-3') flanking the genomic DNA insert. The sequencing results were compared to known sequences in the non-redundant GenBank CDS (coding DNA sequences) database using the BLAST (Basic Linear Alignment Research Tool) program to determine the chromosomal location (http://www.ncbi.nlm.nih.gov/BLAST/).

To determine which ORF within these clones was responsible for complementing the synthetic lethal mutation in SLM-9; various deletions were constructed and tested for their ability to complement the MMS sensitivity of SLM-9. The insert sequence obtained with YEp-SLM-9-1 is located on Chromosome 12 and contained the following ORFs: *YLR440c* (*SEC39*), *YLR441c* (*RPS1A*) and *YLR442c* (*SIR3*). To determine which ORF was responsible for complementation, two initial

deletions were constructed, both inactivating *YLR441c* but leaving *YLR440c* or *SIR3* intact. YEp-SLM-9-1 was digested with either *Nhe*I-*Nco*I or *Nco*I-*Pvu*II to create YEp-SLM-9-1ΔNN and YEp-SLM-9-1ΔNP, respectively. The YEp-SLM-9-1ΔNN fragment, containing the *SIR3* gene, was still able to complement the MMS sensitivity of SLM-9. Removal of the *SIR3* gene in the YEp-SLM-9-1ΔNP construct abolished complementation of the MMS sensitivity of SLM-9. Since both constructs disrupted the *RPS1A* ORF, it was concluded *SIR3* was responsible for the complementation of the synthetic lethal mutation in SLM-9 (Figure 5.2).

YEp-SLM-9-2 contains a 4.3 kb genomic insert located on Chromosome 3. This genomic fragment contains three hypothetical ORFs (*YCL069w*, *YCL068c* and *YCL065w*) and two characterized ORFs (*HMLALPHA1* and *HMLALPHA2*). Two initial constructs were created. YEp-SLM-9-2ΔE was created by digesting the genomic library plasmid with *Eco*RI. This construct deletes the hypothetical ORF *YCLO69w* and is still able to complement the MMS sensitivity in the SLM-9 mutant (Figure 5.3). The YEp-SLM-9-2ΔX was created by digesting the library plasmid with *Xba*I. This construct is deleted for all genes except *HMLα1*, and no longer complements for the MMS sensitivity of the SLM-9 strain. These initial deletions were able to limit the complementing genes to *HMLα2* or the hypothetical ORF, *YCL068c* (Figure 5.3). YEp-SLM-9-2ΔE was used for additional deletion analysis to further define which gene is responsible for the complementation of MMS sensitivity. The YEp-SLM-9-2ΔE plasmid was digested with *Eco*RI-*Spe*I and religated to create the construct YEp-SLM-9-2ΔESp (Figure 5.3). This deletion removes the C-terminal half of *YCL068c*, leaving *HMLα1* and *HMLα2* intact. The YEp-SLM-9-2ΔESp construct complements the MMS

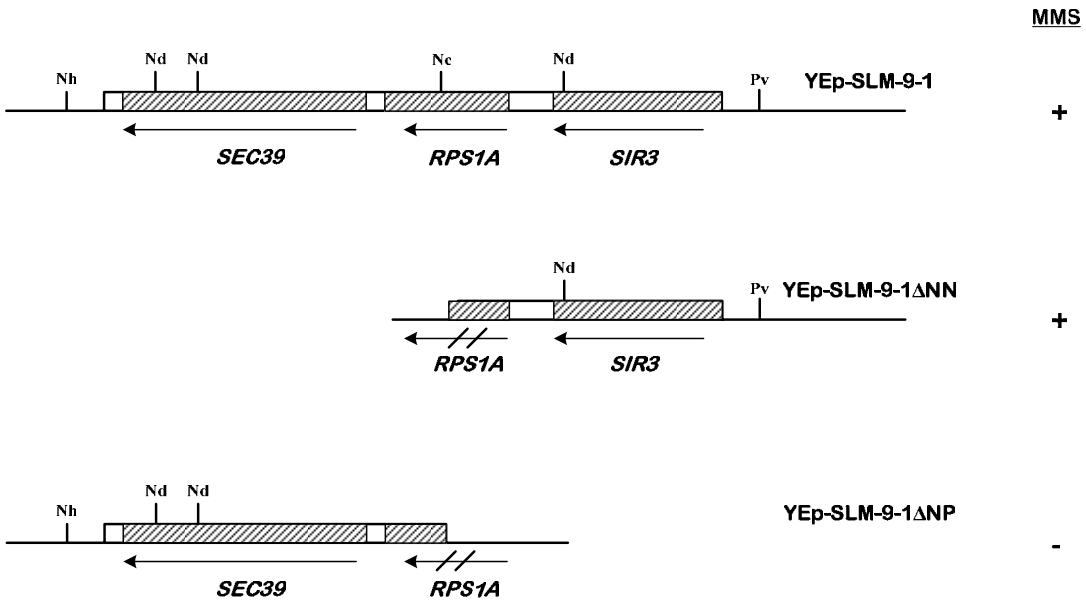

Figure 5.2. Deletion analysis of YEp-SLM-9-1. Schematic diagrams of plasmid constructs used in this study. Only areas of interest, including *SEC39*, *RPS1A* and *SIR3* and selected restriction sites in this region are shown. Boxed area indicates yeast DNA and hatched boxes indicate ORFs. Restriction sites: Nc, *Nco*I; NdI, *Nde*I; Nh, *Nhe*I; Pv, *Pvu*II. Results of complementation of SLM-9 mutation for growth on MMS plates are depicted by +/-.

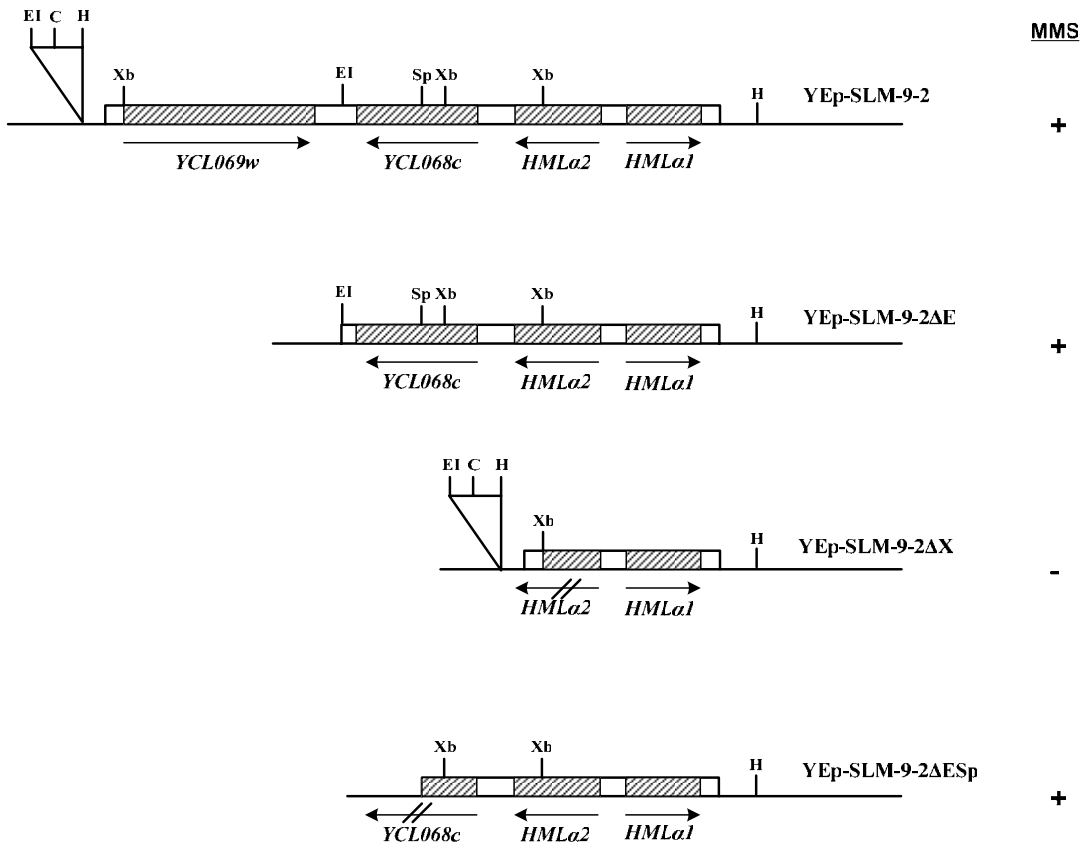

Figure 5.3. Deletion analysis of YEp-SLM-9-2. Schematic diagrams of plasmid constructs used in this study. Only areas of interest, including *YCLO69w*, *YCL068c*, *HMLα2* and *HMLα1* and selected restriction sites in this region are shown. Boxed area indicates yeast DNA and hatched boxes indicate ORFs. Restriction sites: C, *Cla*I; EI, *Eco*RI; H, *Hin*dIII; Sp, *Sph*I; Xb, *Xba*I. Results of complementation of SLM-9 mutation for growth on MMS plates are depicted by +/-.

sensitivity in the SLM-9 strain. Since we already determined that the *HMLα1* gene alone is not capable of complementing the MMS sensitivity in SLM-9, we concluded that *HMLα2* gene is either completely responsible or works with *HMLα1* in suppressing the MMS sensitivity in SLM-9.

The single-copy plasmid recovered in the library screen contained approximately a 10-kb insert and was labeled YCpL-SLM-9-1. Several ORFs were contained within the genomic insert including *MATα1* and *MATα2*. Although these genes belong to the mating type gene coding region on Chromosome 3 and not the silenced *HML* loci recovered in the multi-copy plasmid, the DNA sequences are identical and not under the control of the *cis*-acting regulator elements that act to silence the chromosomal *HML* loci. Therefore, both genomic inserts would act the same when expressed ectopically from a plasmid. Because deletion analysis confirmed the requirement for *MATα2* in complementing the MMS sensitivity in SLM-9, further deletion analysis was not carried out on this plasmid.

5.3.3. Complementation of SLM-9 by *MAT***a** and *MATα*

The results of the library screen suggest a role for mating type in increased resistance to MMS. The SLM-9 strain was transformed with the YCp50-MAT**a** and YCp50-MATα plasmids to determine if expression of the mating type gene from the plasmid will result in complementation of the MMS sensitivity seen in SLM-9. The YCp50-MATα plasmid results in an increased resistance to MMS, but this effect is not seen with YCp50-MAT**a**. The initial SLM-9 strain expresses the *MAT***a** gene at the mating type locus on Chromosome 3. Because only expression of *MATα*, and not

*MAT***a,** complements the synthetic lethal mutation in SLM-9, this suggests a role for mating type heterozygosity in the increased MMS resistance (Figure 5.1).

5.3.4. Deletion of the Mating type Locus in *mms2* Does Not Result in the SLM-9 Phenotype

To determine if deletion of the mating type locus will result in the same phenotype as seen in SLM-9 an *mms2Δ matαΔ* double mutation strain was constructed. Tetrad analysis distinguished the *mms2Δ matαΔ* phenotype from the SLM-9 phenotype in that no slow growth phenotype was observed in the *mms2Δ matαΔ* double mutants, which was observed in the SLM-9 strain. Also, gradient plate analysis of the MMS sensitivity revealed that *mms2Δ* and *mms2Δ matαΔ* have the same sensitivity to 0.025% MMS, whereas the SLM-9 synthetic lethal mutation is synergistic with *mms2Δ* (Figure 5.1 A). These results suggest that the synthetic lethal mutation in SLM-9 is not in the *MAT*α gene and that expression of *MAT*α in the SLM-9 strain suppresses the synthetic lethal phenotype.

5.3.5. Deletion of *SIR3* in the *mms2Δ* Mutants Does Not Result in an Additive Phenotype

To determine if the double mutation of *mms2Δ sir3Δ* results in the synthetic lethal phenotype as observed in the SLM-9 strain, the *SIR3* gene was deleted in the *mms2Δ* strain. The *mms2Δ sir3Δ* double mutants exhibited the same phenotype seen in *mms2Δ* strains expressing both mating type genes (Figure 5.1 A). This is the expected result given that disruption of the *SIR3* gene results in the loss of silencing at the *HML*

and *HMR* loci, thus allowing expression of both *MAT***a** and *MAT*α. Again, these results suggest that the synthetic lethal mutation in the SLM-9 strain is not a simple loss of function mutation in the *SIR* genes. Interestingly, deletion of *SIR3* or expression of both mating type genes in PRR mutants partially rescues the sensitivity to DNA damaging agents. This is similar to the effect seen with deletion of *SRS2* in PRR mutants.

5.3.6. A 5'-truncated *SIR3* Gene Complements the SLM-9 Mutation to the *mms2* Level.

The YEp-SLM-9-1 library plasmid complements the SLM-9 strain to an MMS sensitivity no greater than the *mms2Δ* single mutant. Sequencing of the genomic library fragment suggests that the 5' terminus of the *SIR3* ORF is truncated and the promoter region of the gene is absent. However, northern analysis indicates that *SIR3* mRNA is produced from this plasmid (Figure 5.1 C). When complementation of the YEp-SLM-9-1 plasmid was compared to the deletion of *SIR3* in SLM-9 a difference was observed on MMS gradient plates (Figure 5.1 A). As mentioned above the YEp-SLM-9-1 plasmid complements the MMS sensitivity to the *mms2Δ* level; conversely, deletion of *SIR3* in the SLM-9 strain results in an MMS sensitivity similar to that seen with YCp50-MATα. This is expected since *SIR3* is responsible for maintaining silencing at the *HML* and *HMR* mating type loci. What is unexpected is the complementation of the MMS sensitivity phenotype in SLM-9 with the YEp-SLM-9-1 plasmid due to the truncated genomic DNA in the library construct. The YEp library plasmid does not contain a promoter region, and since the *SIR3* gene is truncated at the N-terminus the promoter will also be absent. However, it is possible that an internal site within the

SIR3 gene, or the vector sequence provides a cryptic promoter element and mRNA is being produced (Figure 5.1 C). Due to the elimination of all but the *SIR3* ORF in the deletion analysis, it is assumed that a truncated Sir3 protein is being produced and acting in a dominant negative manner with respect to silencing at the *HML* loci or may be competing with endogenous Sir3 for binding at the *HML* loci. It must be noted that if the truncated protein was acting to fully disrupt the silencing at the *HML* loci, one would expect the phenotype to be identical to the expression of *MAT*α in the same strain. It is possible that since the *SIR* complex is assembled in a step-wise fashion, the assembly of the Sir2 and Sir4 proteins is unaffected by the truncated Sir3 protein. However, the efficient interaction of the assembled Sir4 protein with Sir3 is destabilized, which may result in altered nucleosome binding and silencing activity.

5.3.7. Mating Type Genes Confer Increased Resistance to PRR Genes

Although we have not identified the synthetic lethal mutation in SLM-9, we further investigated the ability of mating type genes to confer increased resistance to PRR mutants**.** It has been previously shown that **a**/α *rad18* homozygous diploids have an increased resistance to the lethal effects of both UV and γ-irradiation as compared to isogenic haploids or *MAT* homozygous diploids (Heude and Fabre, 1993). It was also demonstrated that the subpopulation corresponding to the G2 phase cells of *rad18* haploids could be reversed by the expression of both *MAT***a** and *MAT*α (Heude and Fabre, 1993). The increased resistance to UV and γ-rays seen in haploids expressing both mating type genes is dependent on a functional recombination pathway (Saeki et al., 1980). We wanted to further investigate the involvement of the **a**/α repair in PRR

haploid mutants. Several different mutants encompassing the error-free and error-prone branches of PRR were transformed with the YCp50-MAT**a** and YCp50-MATα plasmids to determine the response of theses mutants when exposed to MMS and UV irradiation. All genes surveyed within the PRR pathway including *mms2, ubc13, rad18, pol32, rev3, rad5* and *pol30-49* showed an increase in resistance to DNA damaging agents when expressing both mating type genes irregardless of the initial mating type of the mutant (Figure 5.4 and data not shown). The increased resistance to MMS damage appears to be more dramatic than the increased resistance seen after UV damage (Figure 5.4 C,D). These results suggest that expression of both mating type genes, which would be indicative of a diploid state, allows for the channeling of lesions into a recombination repair pathway, and a larger subset of cells are available for the recombination repair pathway in cells treated with MMS than cells treated with UV.

5.3.8. *SRS2* Haploids have an Increased Sensitivity in the Presence of *MAT* Heterozygosity

Previous studies have demonstrated that the *srs2* null mutation leads to a channeling of lesions away from PRR and into the recombinational repair process (Aboussekhra et al., 1989; Schiestl et al., 1990). *SRS2* deleted G1 treated diploids are as sensitive as G1 haploids. Also, the *srs2Δ* diploids homozygous for *MAT*α are more resistant than their isogenic **a**/α counterparts, indicating that the **a**/α channeling into recombination is partly responsible for the sensitivity of *srs2* null diploids (Heude and Fabre, 1993). Mating type heterozygosity in *srs2Δ* haploids showed an increase in

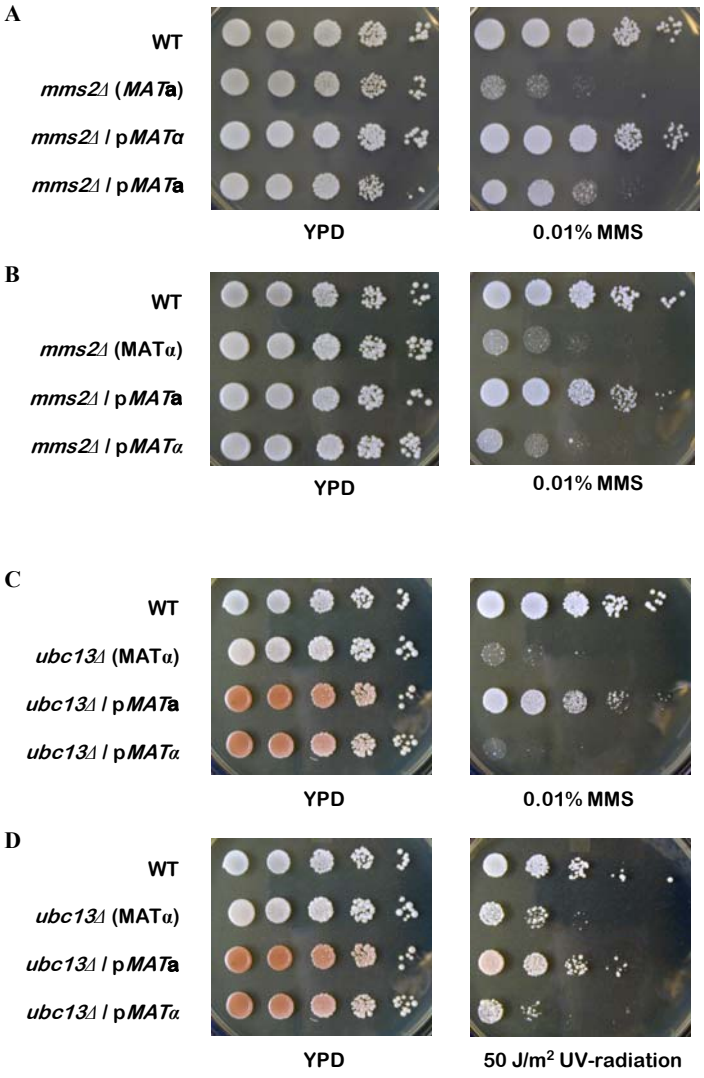

Figure 5.4. Sensitivity of *mms2Δ* or *ubc13Δ* mutants to MMS or UV. The cells were cultured in YPD at 30°C until they reached log-phase. The 10-fold serial dilutions of the cell suspension were spotted onto YPD plates or YPD plates containing the indicated concentration of MMS. Incubation was carried out at 30°C for 2 days before the plates were photographed. The strains were transformed with the YCp50, YCp50-*MAT***a** or YCp50-*MAT*α plasmids. **(A)** WXY901 (*mms2Δ*) sensitivity to 0.01% MMS; **(B)** WXY902 (*mms2Δ*) sensitivity to 0.01% MMS; **(C)** WXY906 (*ubc13Δ*) sensitivity to 0.01% MMS; **(D)** WXY906 (*ubc13Δ*) sensitivity to 50 J/m^2 UV-radiation.

sensitivity to MMS when expressing both mating type genes (Figure 5.5). This effect was opposite from genes with known roles in PRR. This result suggests that in the presence of both mating type genes, cells require a functional *SRS2* to prevent the channeling of inappropriate substrates into recombination at a point in the cell cycle when a homologous template is not available for repair.

5.3.9. Cellular Tolerance to DNA Damage in Response to Mating Type is Specific to PRR

To determine the involvement of mating type with respect to DNA repair we surveyed genes involved in recombination repair and excision repair. Mutants in recombination, including *rad51Δ, rad52Δ* and *rad54Δ* (Figure 5.6 A,B,C) and the NER mutant *rad1Δ* (Figure 5.6 D) were transformed with the YCp50-MAT**a** and YCp50-MATα plasmids. The sensitivity of these mutants was determined on MMS, UV and γ-irradiation. There was no noteworthy difference in sensitivity to the DNA damaging agents when either recombination or excision repair mutants were heterozygous for mating type (Figure 5.6 and data not shown). These results suggest that the ploidy effect is specific to the PRR pathway.

5.4. Discussion

In the yeast *S. cerevisiae* the diploid is more resistant to γ-irradiation than their haploid counterparts, and this effect is partly due to the heterozygosity at the mating type locus. This has been well documented with the *rad18* diploids, where the

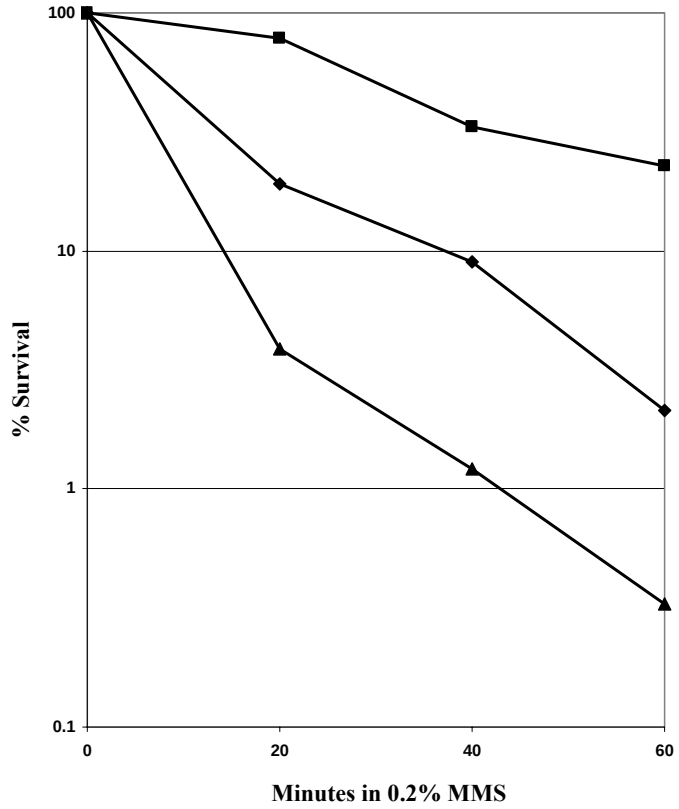

Figure 5.5. MMS sensitivity of *srs2Δ* haploids and pseudodiploids. The wild-type strain and its isogenic derivatives were treated with 0.2% MMS for the given time and plated onto YPD plates to score for cell survival and compared with untreated cells. These results show a typical experiment. Each experiment has been repeated at least three times. (■) HK578-10A (wild-type); (♦) HK590-1D (YCp50-*MAT***a,** *srs2Δ*); (▲) HK590-1D (YCp50-*MATα*, *srs2Δ*).

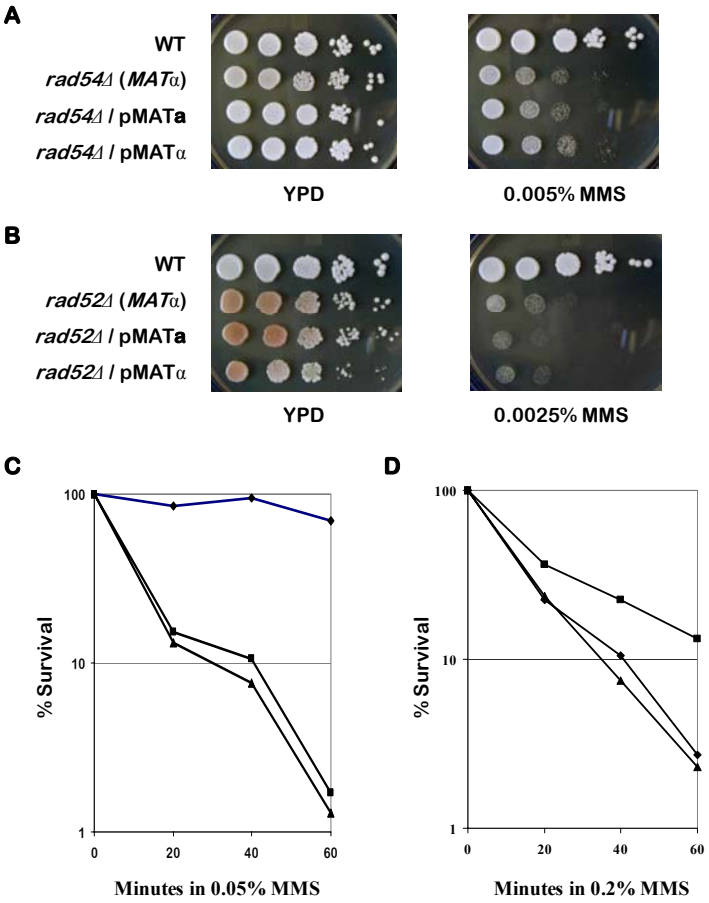

Figure 5.6. Sensitivity of recombination and NER mutants to MMS. For the plate assay, cells were cultured in YPD at 30°C until they reached log-phase. The 10-fold serial dilutions of the cell suspension were spotted onto YPD plates or YPD plates containing the indicated concentration of MMS. Incubation was carried out at 30°C for 2 days before the plates were photographed. For the MMS-induced liquid killing, the wild-type strain and its isogenic derivatives were treated with MMS for the given time and plated onto YPD plates to score for cell survival and compared with untreated cells. These results show a typical experiment. Each experiment has been repeated at least three times. **(A)** LSY404 (*rad54Δ*) with YCp50, YCp50-*MAT***a** or YCp50-*MATα*; **(B)** LSY387 (*rad52Δ*) with YCp50, YCp50-*MAT***a** or YCp50-*MATα*; **(C)** (♦) LSY390 (wild-type), (■) LSY401 (*MAT***a** *rad51Δ*), (▲) LSY401 (YCp50-*MATα, rad51Δ*); **(D)** (♦) HK578-10A (wild-type), (■) HK615-1A (*MATα*, *rad1Δ*), (▲) HK615-1A (YCp50-*MAT***a,** *rad1Δ*)**.**

sensitivity to both UV and γ-rays is increased when the cells are homozygous for mating type (Heude and Fabre, 1993). The coexpression of *MAT***a** and *MAT*α in *rad18* haploids also suppresses the sensitivity to γ-irradiation, but only in the G2 subpopulation of cells. Also, mating type heterozygosity in RAD^{+} haploids reduces the UV-induced mutagenesis in G2 cells (Heude and Fabre, 1993). These results led to the proposal that the **a**1-α2 complex promotes channeling of some DNA structures from a mutagenic DNA repair pathway into the recombinational repair process.

We recovered two suppressors in the SLM-9 synthetic lethal strain which led us to expand the investigation of mating type heterozygosity in DNA repair mechanisms. From the genomic library screen, both the *MAT*α2 and a truncated *SIR3* gene were shown to cause an increased survival in the *mms2Δ* synthetic lethal mutant. To ensure that the complementation of MMS in the SLM-9 strain is a result of mating type heterozygosity, both the *MAT***a** and *MAT*α genes were expressed in *mms2Δ* mutants expressing either *MAT***a** or *MAT*α at the mating type locus. Complementation of the MMS sensitivity was only observed when the *mms2Δ* mutant expressed both mating type genes. Since deletion of the mating type locus did not result in a synthetic lethal phenotype with *mms2Δ*, it was concluded that mating type heterozygosity is required for the increased resistance to DNA damaging agents.

The second suppressor, a truncated *SIR3* gene, displayed a similar phenotype in the SLM-9 strain. Since the *SIR* genes are required for silencing at the *HML* and *HMR* loci, it is not surprising that a truncated Sir3 protein would allow for some derepression of the silencing at the *HML* and *HMR* loci resulting in mating type heterozygosity. Indeed, deletion of *SIR3* in either the *mms2Δ* mutant or SLM-9 did not result in a

synthetic lethal phenotype; instead the deletion of the *sir3* gene in these strains results in a similar phenotype as expression of both mating type genes, which suggests that deletion of the *SIR* genes act as suppressors to the synthetic lethal mutation. The above results indicate a role for *sir3* in rescuing PRR mutants via mating type heterozygosity. However, the complexity of mating type silencing at *HML* and *HMR* and the a1-α2 repression of haploid-specific gene has prevented us from identifying the synthetic lethal mutant. It is unlikely that genes involved in either mating or sporulation are directly involved in the conditional synthetic lethality in SLM-9 due to the ability of the cell to undergo both processes.

DNA repair pathways require the synthesis of new DNA, which must then be reassembled into chromatin. Hence, the stable repair of damaged DNA might be sensitive to defects in chromatin assembly (Green and Almouzni, 2002). Also, proteins such as yKu70 and yKu80, the Sir proteins, DNA damage checkpoint proteins Mec1, Mec3 and Rad53, and the histone chaperones Cac1 and Asf1, have all been shown to affect DNA damage repair (Tsukamoto et al., 1997). Genes that are required for the maintenance and stable inheritance of the silenced state are not likely candidates for the synthetic lethal mutation. Mutations in these genes should result in a derepression of the *HM* genes and thus would again result in the suppressor phenotype displayed with *MAT*α and the truncated *SIR3*. However, haploid specific genes, which are suppressed by expression of both **a**1 and α2 may be potential candidates. It is unlikely that the mutation in SLM-9 is a simple loss-of-function mutation and as a result may be resulting in a dominant negative phenotype. Repression of this type of mutation after expression of both mating type genes may account for the suppression seen in

heterozygous *MAT* haploids. Needless to say, the synthetic lethal mutation is suppressed by mating type heterozygosity, thus signaling the cell to use a HR repair pathway, and thus the elusive mutation in SLM-9 is no longer a factor under these conditions. A couple of different approaches may be used to attempt to identify this mutation. By crossing the strain with a wild-type parental strain and back cross for only the synthetic lethal mutation, you could then transform with a library to find the mutation. This will eliminate the PRR defect in these strains and hopefully eliminate the suppressors. However, the synthetic lethal mutation displays a low sensitivity to DNA damaging agents. This would make it difficult to screen based on the MMS sensitivity. A second option might be to disrupt *SIR3* in SLM-9, and carry out a library screen on this mutant. It still shows a high degree of sensitivity on MMS that could be used for complementation. This should also eliminate the suppressors recovered in the original library screen.

Our results indicate that the phenomenon of mating type heterozygosity is specific to the PRR pathway. The genes tested within the PRR pathway displayed an increase in resistance to MMS and UV. In contrast, the *srs2Δ* mutation, which is responsible for channeling repair intermediates into PRR and away from recombination, displayed an increased sensitivity to DNA damaging agents when both mating type genes were expressed. This is most likely a result of channeling inappropriate intermediates into a recombination repair mechanism when a sister chromatid is not available. The same result was not observed when both mating type genes were expressed in recombination or NER mutants. This suggests that the increased resistance observed due to the mating type heterozygosity is specific to the PRR pathway.

The resistant effect in **a**/α diploids is reproducible in G2 haploids expressing both mating type genes, indicating that the mating status, and hence the expression of both the **a**1 and α2 products, provides an intracellular signal for HR. The results from this study suggest that several factors determine the most appropriate repair mechanism to use. Expression of both mating type genes is indicative of a 2n content of DNA and would suggest the presence of a sister chromatid for use in a recombinational repair mechanism. This is also substantiated by the evidence that only the PRR pathway, which is proposed to use either a TLS or SCE repair mechanism, depending on the use of the error-prone or error-free branch respectively, is influenced by mating type heterozygosity. It can be further argued that since G1 haploid cells have no homolog with which to repair damage through recombination mechanisms, whereas diploid cells contain homologous chromosomes, cells may sense their mating status to favor HR in diploid cells while favoring PRR in G1-S haploid cells. This is demonstrated in the increase in sensitivity to DNA damaging agents seen in *srs2* mutants heterozygous for mating type. These mutants would cause the early channeling of inappropriate intermediates into a recombination pathway leading to lethal intermediates. These results together suggest cells contain several mechanisms to ensure that the appropriate type of repair is carried out during the cell cycle. For example, in G1 the cell would benefit from an excision repair mechanism, whereas during late S-phase and G2 the cells would benefit from either PRR or recombination, depending on the availability of a sister chromatid as a homologous template.

Chapter Six – Discussion and Conclusions

6.1. Regulation of DNA Damage Repair Pathways

The synthetic lethal screen uses a colony-sectoring assay to identify novel genes functioning in an alternative pathway to the gene of interest. This method demands an appropriate level of stability of the plasmid carrying the gene of interest. The high-level stability of YCp plasmids results in a large number of false positives that must be further characterized. By regulating the plasmid stability and copy number by placing a yeast centromere sequence under the control of an inducible promoter we were able to improve the original synthetic lethal screen protocol. Hence, altering the culture conditions under which yeast cells carrying the plasmid P_{GAL1}-CEN4 allowed us to develop a method that eliminated virtually 100% of false positives and drastically reduced the time required to carry out a synthetic lethal screen. In the eukaryotic organism, *S. cerevisiae*, a synthetic lethal screen was used to reveal mutations synergistic with cells containing a deletion in the error-free PRR pathway gene *MMS2*. The *MMS2* gene encodes a ubiquitin-conjugating enzyme variant protein belonging to the *RAD6* repair pathway. A mutation in *MMS2* does not result in severe sensitivity to DNA damaging agents; however, in combination with a deletion from error-prone PRR, a synergistic phenotype is produced. By taking advantage of the synergism between error-free PRR and mutagenesis pathway mutations, a conditional synthetic lethal

screen was used to identify novel genes genetically involved in PRR. We isolated several mutants, with most containing a mutation in the *REV3* and *REV1* genes. Of the conditional synthetic lethal strains recovered, two were utilized for further characterization and revealed a function for both checkpoints and mating type heterozygosity in regulating PRR.

Cell-cycle checkpoints monitor the integrity of the eukaryotic genome and ensure that cell cycle progression is deferred until chromosome damage is repaired. Although it is clear that checkpoints and DNA damage repair are essential mechanisms to maintain genome integrity, it is still not well defined how checkpoints are activated and coordinated with DNA damage and repair at the molecular level. Although several proteins involved in the signaling of DNA damage have been identified, it is uncertain which proteins directly detect the DNA damage, or if the primary signal is the DNA strand break. For example, at the molecular level, damage to the DNA duplex can result in several different damage intermediates, and each type of lesion may be repaired by a specialized mechanism that directly recognizes the damage intermediate. Alternatively, the ssDNA produced by resecting the ends by a 5' to 3' exonuclease would result in a subset of lesions that are then recognized by the cell. It has also yet to be elucidated whether the DNA damage and replication checkpoints or the repair machinery directly sense the damage, or if cooperation between the two mechanisms results in the cell cycle delay and repair of the damage. One must keep in mind that the same sensor/transducer kinase *MEC1* is often activated in response to various genotoxic insults, yet the cellular results of these insults show high specificity and very different outcomes. It is plausible that the damage-sensing components of the DNA damage

checkpoint gain access to DNA lesions through their interactions with DNA repair proteins. Checkpoint deficiencies might sensitize cells to DNA damage and replication blocks not only by failing to arrest the cell cycle and failure to induce important proteins but also by failure to activate and/or optimally recruit the DNA damage repair machinery to the site of damage. This hypothesis is supported by the failure to fully suppress the DNA damage sensitivity in *S. cerevisiae rad9* (Weinert and Hartwell, 1988) and *rad53* (Allen et al., 1994) mutants by an artificial cell cycle arrest. This suggests that mechanisms other than cell cycle arrest contribute to the increased sensitivity observed in these mutants.

The above proposal is further supported by the recovery of the *RAD9* gene in one of the synthetic lethal mutations, which implies a genetic interaction between checkpoint genes and the PRR pathway. The synergistic interactions of several checkpoint genes with both the error-free and error-prone branch of PRR, as well as the epistatic relationship between *rad18* and *rad9* imply that a checkpoint response is important in PRR in order to help maintain viability. Moreover, we found that a chemical induction of a delay at the G2 phase was unable to fully recover the DNA damage sensitivity seen in our PRR checkpoint double mutants. We further demonstrated that in response to MMS, a cell cycle delay has no effect on cell survival when the PRR pathway is inactivated. It is plausible that upon sensing DNA damage the cell uses a variety of signals to determine the best mechanism to repair the damage. It is plausible that during S-phase, DNA damage triggers the phosphorylation of Srs2, which would thus disrupt the Rad51 nucleofilament and prepare the substrate for a DNA polymerase most likely mediated by the *RAD6*-dependent PRR pathway. Binding

of the lesion by the Rad6-Rad18 complex would recruit Rad5-Mms2-Ubc13, leading to the polyubiquitination of PCNA (Hoege et al., 2002). The ubiquitination of PCNA may itself be the signal for checkpoint delay, or alternatively may convey a signal to the Rad9 checkpoint protein to trigger the slowing of cell cycle progression. In the absence of PRR the cell would use an alternative repair mechanism and thus not require the delay at this point in cell cycle progression. It will be important to determine if mutations at K164R in *POL30* prevent genetic interactions with *RAD9*. If the ubiquitination of PCNA is required to signal checkpoints, then disruption of this ubiquitination process via the K164R mutation in *POL30* will eliminate the requirement for *RAD9* in PRR.

In the absence of PRR the cells would use a *RAD51*-dependent recombination repair mechanism. For successful recombination, the cell must be equipped with a means to establish the stage of cell cycle progression with proper DNA content. This is important in determining the appropriate type of repair, as several different substrates can be used as recombination intermediates. For instance, nicked, gapped, or broken, replicating or nonreplicating intermediates can all produce intermediates that are proficient recombination substrates. It is likely that multiple modes of recombination exist, dependent possibly on the cell cycle (G1, S or G2), cell mating type and type of DNA substrates used.

The recovery of mating type genes in one of the synthetic lethal strains suggests a role for mating type heterozygosity in determining the mode or repair used by the cell. Heterozygosity of mating type is indicative of 2n DNA content and would preferentially channel lesions into a recombination repair pathway. Although both the *MATα2* and a

truncated *SIR3* gene are suppressor mutations recovered from the library screen, these genes give valuable insight into how the cell regulates DNA repair. In a haploid cell, the *SRS2* gene is responsible for switching the cell between PRR and recombination by means of disrupting the Rad51 nucleofilament. In circumstances where inappropriate recombination would result in deleterious results (i.e. no available homologous template), Srs2 can prevent recombination by removing recombination proteins and stabilizing the ssDNA (Krejci et al., 2003). During late S and G2-phase of the cell cycle, Srs2 activity may be down-regulated, possibly by dephosphorylation, and thus signals that a homologous template is available for recombination. In diploid cells a new set of factors, such as expression of both mating type genes, would be responsible for indicating a 2n content of DNA and thus a homologous template is always available for repair. Expression of both mating type genes would therefore bypass the need for PRR when a recombination substrate is available for use in DNA repair. This hypothesis is supported by our findings that haploids expressing both mating type genes are more resistant to DNA damaging agents, a phenomenon specific to the PRR pathway. As mentioned above, the same ssDNA intermediate can be used for either PRR or recombination. In the G1 haploid cells there is no homolog with which to repair the damage through recombination, but cells heterozygous for mating type would send an incorrect signal for recombination repair. This is seen in haploid *srs2* mutants heterozygous for mating type which show an increased sensitivity to DNA damaging agents, likely a result of cells committing inappropriate substrates into recombination when a homologous template is not available. In contrast to *srs2Δ*, cells deleted for PRR genes become more resistant to DNA damaging agents when expressing both

mating type genes. Although Srs2 would still be available in these cells and would signal for a PRR pathway, which is no longer available, the signal received from the mating type heterozygosity would override Srs2 and transfer the intermediate into HR.

The rescuing effect seen in PRR mutants heterozygous for mating type is reminiscent of the rescuing obtained from deleting *SRS2* in the PRR mutants. In both cases, the cells channel lesions into a recombination repair pathway and away from a non-operational PRR pathway. Also, deletion of *SIR3* partially rescues PRR mutants, most likely by disrupting silencing at the *HML* and *HMR* loci allowing expression of both mating type genes. Expression of the **a**1/α2 complex may directly or indirectly be responsible for stabilizing the recombination intermediate for use in HR, or alternatively may down regulate genes responsible for the activation of Srs2 and thus prevent the disruption of Rad51 nucleofilaments. On the other hand, the **a**1/α2 complex may be acting on an unidentified regulator of the PRR pathway. For example, Tup1, Ssn6 and Mcm1 form a complex and act as a repressor for haploid-specific genes (Wahi and Johnson, 1995). Tup1 has also been shown to interact with Hug1 in a two-hybrid assay (Uetz et al., 2000), and Hug1 is involved in the Mec1-mediated checkpoint pathway (Basrai et al., 1999). The complexity of mating type heterozygosity, and the limited information on the downstream proteins involved in response to the **a**1/α2 complex, along with the large amount of proteins involved with Tup1 and Ssn6 makes it difficult to determine exactly how mating type and deletion of *SIR3* play a role in DNA damage response. Further elucidation of the subset of genes regulated by mating type heterozygosity may help us understand how the cell regulates DNA repair. It is also important to determine how the cell is dealing with damage intermediates in the *srs2Δ*

pseudodiploids. One would expect that if the expression of mating type heterozygosity in *srs2Δ* is resulting in early and inappropriate recombination the recombination rates in these cells would be elevated above that of the *srs2Δ* mutants expressing a single mating type. It is also important to determine if the rescuing effect seen by disruption of the *SIR3* gene is simply a result of expression of both mating type genes, or a more complex regulation of DNA repair, possibly involving the activation of checkpoints. The importance of the checkpoints within PRR has been demonstrated, but to what extent are they required for efficient PRR? Is it possible to override the requirement for the checkpoints in PRR mutants by expressing mating type heterozygosity? By addressing these questions we may gain a better insight into the regulation of PRR and HR by both *SRS2* and mating type heterozygosity.

6.2. Regulation of PRR and HR in Higher Eukaryotes

All eukaryotic cells, except certain embryonic cells, possess checkpoints to monitor cellular integrity (Hartwell and Weinert, 1989). The importance of DNA checkpoints in cancer pathology is now well established. In the past few years, studies in organisms from budding yeast, Drosophila, mouse and mammalian systems have enhanced our understanding of how checkpoints maintain genome stability, and how cells lacking functional checkpoints display genomic instability due to a failure to properly respond to DNA damage. The checkpoint response has been highly conserved from budding yeast to mammalian cells. For example, the phospho-inositide kinase (PIK)-related protein Mec1/Tel1 is homologous to ATM and ATR in mammals. In budding yeast the Rad24 and the Rad17-Mec3-Ddi1 complex is homologous to *S.*

pombe Rad17 and Rad9-Rad1-Hus1 complex respectively (reviewed in (Zhou and Elledge, 2000)). Human homologs of *S. pombe* Rad1, Rad9 and Hus1 have also been shown to form a complex (Volkmer and Karnitz, 1999), and *S. pombe* Rad17 shares homology with all five subunits of RFC (O'Connell et al., 2000). Given that checkpoints are highly conserved in eukaryotes, studies in *S. cerevisiae* will help contribute to the understanding of human diseases associated with the DNA damage checkpoint response.

However, the role of negative regulation in cell-type-specific gene expression is just beginning to be appreciated in mammalian cells. Negative regulation occurs in yeast cells by the direct binding of the α2 protein to two DNA sequence elements flanking the binding site of the transcriptional activator MCM-1. The expression of a mammalian major histocompatibility complex (MHC) class I gene is in part regulated by a silencer DNA sequence element which binds a complex of silencer factors. This MHC class I silencer DNA element shows modest DNA sequence homology with the yeast α2 operator, and the negative regulatory system is strikingly similar to the yeast α2 mating type repression system. (Weissman and Singer, 1991). The MHC class I silencer specifically binds to the yeast α2 operator DNA and interacts with a yeast α2-binding protein. The α2 operator can function as a silencer element in mammalian cells when placed upstream of a MHC class I promoter (Weissman and Singer, 1991). Although the genes regulated by α2 are those which determine mating type and mating type recognition in yeast cells, the system is a self-nonself recognition system analogous to the MHC organization in mammalian cells. It is feasible that the two

regulatory systems derived from a common evolutionary origin and the molecular mechanisms which regulate their expression have been conserved.

A striking difference between unicellular and multicellular eukaryotic organisms is cell identity established by the mating type locus in yeast compared to the specialized sex chromosomes that govern differentiation and gamete production in multicellular eukaryotes. Although the mating type loci of fungi and the sex chromosomes of mammals appear quite divergent, recent studies of mating type loci have revealed features shared with the complex sex chromosomes of algae, plants and animals. The mating type loci and sex chromosomes have evolved to coordinately control gamete production and the formation of zygotes, the establishment of the diploid or dikaryotic state and the uniparental inheritance of organelles (Fraser and Heitman, 2004). In addition, in female mammals one of the X chromosomes is silenced to compensate for the gene dosage difference between males and females (Lyon, 1961). X inactivation occurs early in female embryogenesis when one of the X chromosomes undergoes heterochromatinization. The inactivation is controlled by a region on the X chromosome termed the X-inactivation centre (Lee et al., 1996; Rastan, 1983; Willard, 1996) and may be similar to the silenced loci of *HML* and *HMR*.

The accurate repair of dsDNA breaks is performed through gene conversion. This cellular mechanism serves to protect the genetic information in the cell from damage. Meiotic crossing over is supposed to create genetic diversity by producing new combinations of the alleles derived from parents. Although the genetic diversity may help cells to adapt to unfavorable conditions, the significance of meiotic gene conversion is not well understood. Gene conversion between nonallelilc genes can also

potentially generate a gene with novel functions by shuffling parts of the parental ORF with aligned coding frames. This type of gene conversion is the mechanism used to create immunoglobulin diversity in some mammals (Weill and Reynaud, 1987). A good model system to study gene conversion is the mating type switching observed in homothallic mitotic haploid cells of *S. cerevisiae*. Such advances in this area will provide us with a greater understanding of the operation and molecular mechanisms of HR, as well as improve our knowledge of the regulatory mechanisms that control it.

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