INVESTIGATION OF DNA STABILITY IN *SACCHAROMYCES CEREVISIAE* MUTANTS DEFECTIVE IN THE REPAIR OF BROKEN CHROMOSOMES

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INVESTIGATION OF DNA STABILITY IN *SACCHAROMYCES CEREVISIAE* MUTANTS DEFECTIVE IN THE REPAIR OF BROKEN CHROMOSOMES

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This thesis is dedicated to the loving memory of my mom,

Thanh Thi Nguyen.

(1951-2008)

You are always in my heart.

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I can never adequately thank Dr. Kevin Lewis for all that he has done for me throughout my graduate career. His patience and dedication as an advisor, his thoughtful mentorship, and his intellectual depth have carried me through this journey. I feel deeply honored for having had the opportunity to study and work under his direction and could never have completed this without his encouragement and support. I will be forever grateful to him. I would also like to thank my family, who shared all the ups and downs of this process with me. Thank you for your unconditional love.

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CHAPTER I

INTRODUCTION

Chromosomal DNA within eukaryotic cells is constantly subjected to various sources of damage. Ionizing radiation such as X-rays and gamma rays, chemicals such as bleomycin and methyl methanesulfonate (MMS) as well as nuclease enzymes such as EcoRI can compromise chromosome integrity and cause multiple types of lesions to occur. Of particular interest are double-strand breaks (DSBs), which are considered the most difficult lesions to repair (1-5). Inefficient repair of these particular lesions can lead to mutations and chromosome rearrangements, increasing the risk of cell death and cancer (2-3, 6).

Cells have multiple mechanisms to repair broken DNA and attempt to preserve genomic stability. In eukaryotic cells, DSBs are repaired via two conserved pathways, nonhomologous end-joining (NHEJ) and homologous recombination (1-3). Repair by NHEJ in the model eukaryote *Saccharomyces cerevisiae* (budding yeast) involves the direct rejoining of broken ends utilizing three protein complexes: Yku70-Yku80, Mrx (Mre11-Rad50-Xrs2), and DNA Ligase IV (Dnl4-Lif1-Nej1) (Figure 1). In essence, the Yku70-Yku80 complex binds at the broken ends to protect them from degradation and to initiate NHEJ. The Mrx complex subsequently binds to the DNA-Yku structure to serve as a "bridging factor" between the broken DNA ends. DNA Ligase IV then binds to the break site in an ATP dependent manner. The ends are processed and the breaks are filled, allowing Dnl4 to ligate the ends (1, 3, 7).

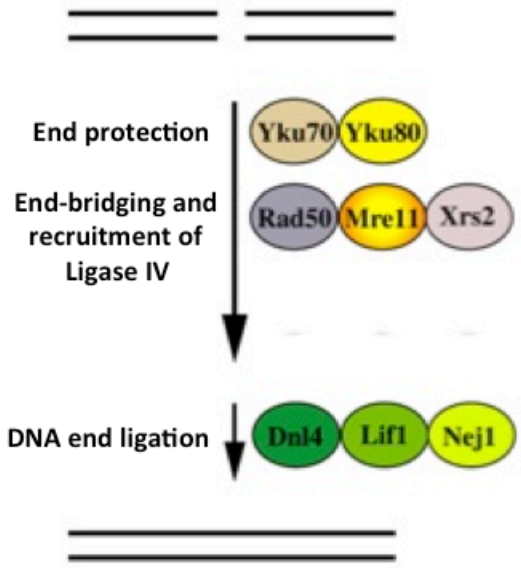


Figure 1. Illustration of the mechanism and proteins involved in the Nonhomologous End-Joining repair pathway.

The second pathway, homologous recombination, is considered the primary repair pathway for the budding yeast *Saccharomyces cerevisiae*, whereas it is the secondary pathway for humans (2, 7-8). Efficient repair of DSBs by this pathway involves an intricate, multi-step process requiring a large number of proteins (Figure 2). Initially, DSB ends are resected by the Mrx complex (Mre11-Rad50-Xrs2), producing long 3' tails. These overhangs function as substrates for several proteins such as Rad51, Rad52, and Rad54 which mediate strand invasion and exchange with another molecule such as a homologous chromosome or sister chromatid. Subsequent events involve DNA replication, branch migration of Holliday junction structures, and ultimately, resolution of the joined DNA strands (2, 9-10). Other important processes such as nucleosome

remodeling, sister chromatid cohesion, and damage-induced checkpoint signaling have also been implicated in this repair pathway (Figure 3) (2-3, 9, 46).

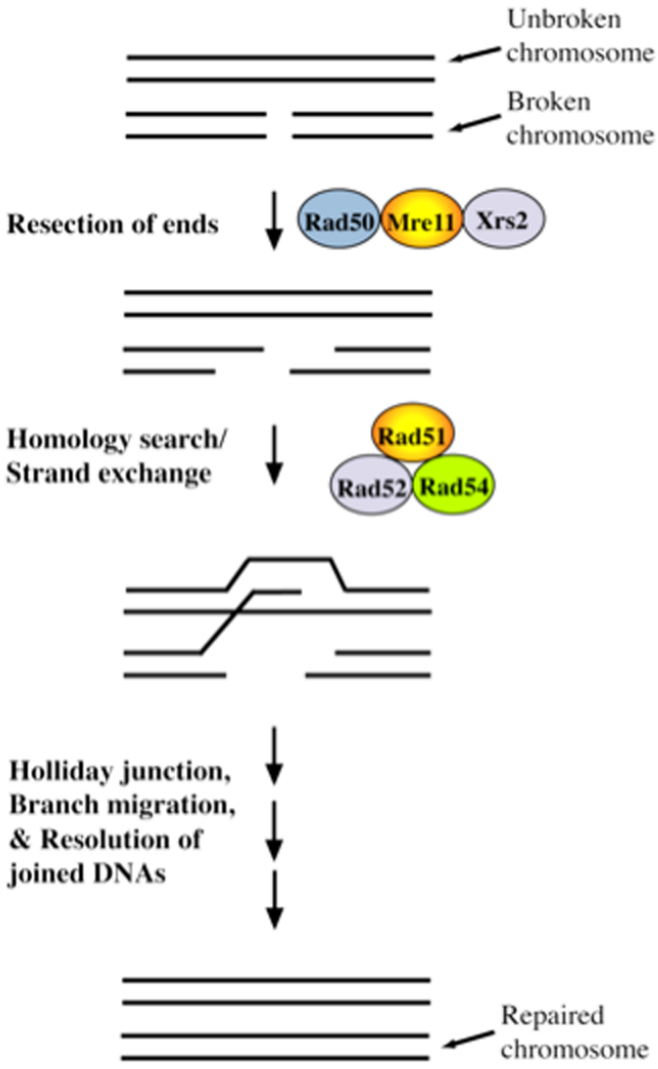


Figure 2. Illustration of the mechanism and proteins involved in the Homologous Recombination repair pathway.

When chromosomal DNA is damaged, a cell cycle checkpoint response occurs in which growth temporarily pauses in G_2 phase, allowing the DNA to be repaired before entering mitosis and completing the cell cycle. This checkpoint response involves a series of events that includes a large number of proteins. Essentially, once DSBs are detected, Mrx processes the broken ends allowing for replication protein A (RPA) to bind to the exposed ssDNA. The checkpoint protein Ddc2 then recognizes the damaged DNA by interacting with the Rpa-coated ssDNA. Ddc2 then associates and forms a complex with Mec1, a checkpoint kinase, which subsequently recognizes and interacts with the sites of DNA damage (11). Mec1, Tel1, and several other checkpoint proteins including Ddc1, Mec3, Rad17, and Rad24 phosphorylate and activate Rad53, a critical checkpoint kinase. Activation of Rad53 ultimately leads to cell cycle arrest and increased transcription of genes required for DNA synthesis and repair (11-13).

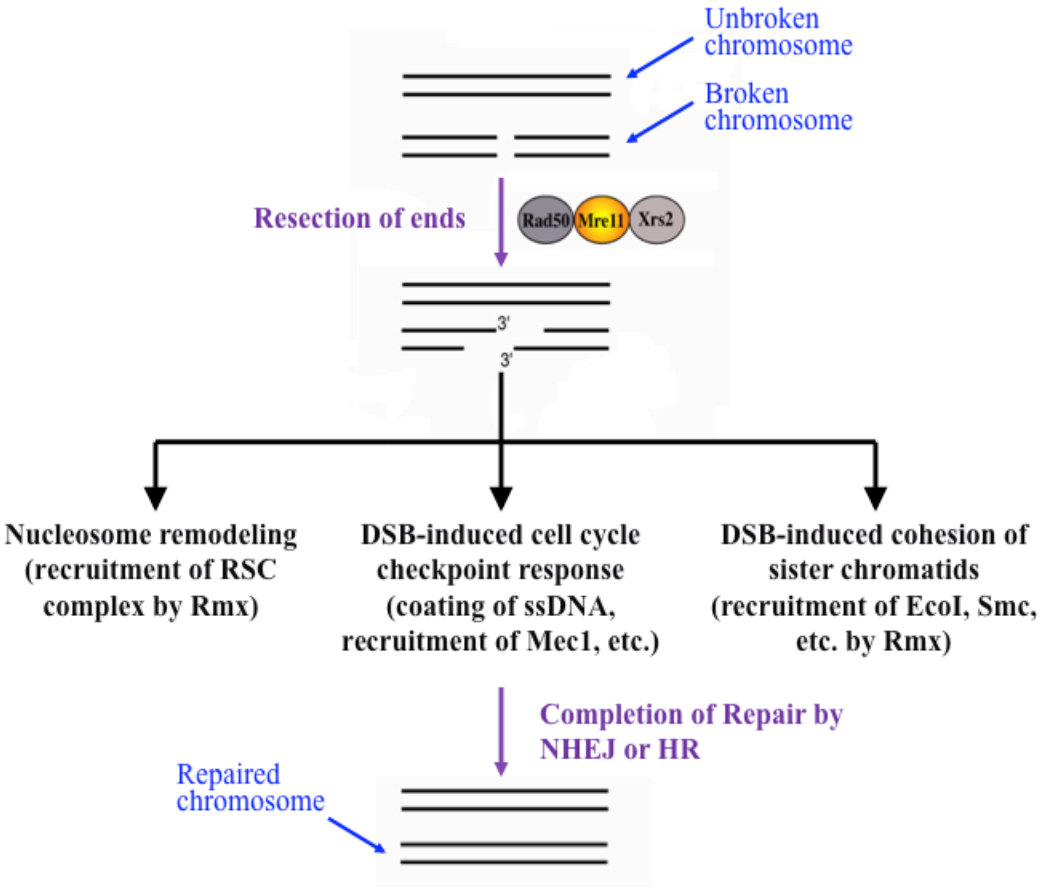


Figure 3: Schematic of processes that occur in response to a DSB.

In nucleosome remodeling, a large multi-subunit complex, Rsc, mobilizes nucleosomes at the chromosome ends to aid in the repair of DSBs (14). Additionally, proteins such as Eco1 and Smc mediate sister-chromatid cohesion in response to DSBs, allowing for a stable and tightly bound complex (15). This cohesion process has been postulated to promote homologous recombination by bringing sister chromatids together (16).

Studies have shown that mutant yeast cells defective in the recombination pathway, such as *RAD51* and *RAD52* cells, exhibit many common phenotypes including sensitivity to X-rays, gamma rays, and to chemicals such as bleomycin and MMS (1, 17-

18). These mutants exhibit increased mutation rates and are associated with several human genetic disorders (17, 19).

The chemicals bleomycin and MMS both induce DSBs, though by different mechanisms. Bleomycin, an antibiotic used for several cancer treatments, produces mutagenic lesions through a process that involves free radicals. Essentially, bleomycin binds to oxygen and metals, such as iron, resulting in the formation of a free radical complex that induces a variety of lesions to DNA (20-21). Methyl methanesulfonate (MMS), on the other hand, is a DNA alkylating agent that attaches methyl groups primarily to the DNA bases adenine and guanine. These bulky alkylated bases cause inhibition at DNA replication forks, which can ultimately lead to the formation of DSBs (22-23).

The restriction endonuclease enzyme EcoRI produces DSBs by recognizing and cutting the specific sequence G^AATTC to generate 5' - "sticky end" overhangs (Figure 4). While clastogens such as radiation, bleomycin, or MMS can produce many other types of DNA damage, EcoRI is believed to only produce DSBs (24-25).

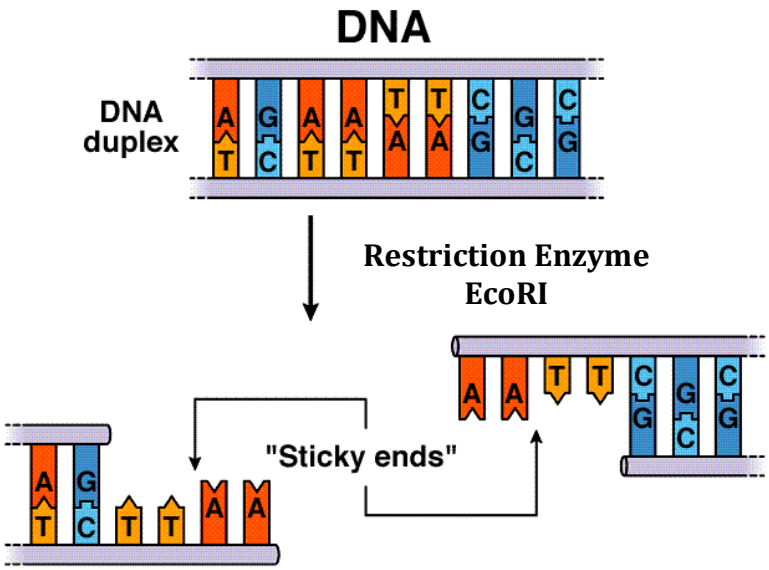


Figure 4: Diagram of EcoRI cleavage of double-stranded DNA.

Several years ago, characterization of yeast mutants sensitive to ionization radiation led to the identification of the RAD52 group of DSB repair genes along with several other genes. The RAD52 group includes *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11, XRS2,* and *RDH54*. Each of these genes is involved in homologous recombination repair of DSBs. Inactivation of all genes except *RAD59* and *RDH54* leads to strong sensitivity to ionizing radiation, MMS, and bleomycin. Mutations in several of the genes, e.g., *RAD50, RAD51, RAD52, RAD54*, etc., also cause strong sensitivity to *in vivo* expression of EcoRI endonuclease (6, 8).

In an effort to identify other mutants that display a gamma radiation sensitivity phenotype, yeast deletion strain libraries were screened that contained approximately 5000 mutant yeast strains. Each diploid mutant, having both copies of a different non-essential gene inactivated, was screened for sensitivity to gamma radiation (26-29). In total, 210 mutants were found to be gamma sensitive when using diploid yeast cells. To

identify genes specifically involved in DSB repair, graduate students Jennifer Summers, Sunaina Sethi, and Jennifer DeMars from the Lewis lab used mutant strains from *MAT*α and *MATa* haploid deletion strain libraries to test the roles of these 210 genes in resistance to EcoRI expression (30-32). An additional gene, *EXO1*, was also tested because haploid *exo1* mutants were known to be radiation sensitive, bringing the total to 211 genes. For these experiments, plasmids that could express EcoRI through the use of the galactose-regulatable promoter *GAL1* were transformed into each haploid strain. In total, 81 haploid mutants were found to be sensitive to EcoRI expression. Eight RAD52 group mutants (*rad50*, *rad51*, *rad52*, *rad54*, *rad55*, *rad57*, *mre11*, and *xrs2*) and 73 non-RAD52 group mutants were sensitive. The majority of these mutants also exhibited sensitivity to the clastogens bleomycin and MMS in subsequent tests, further implicating their involvement in DSB repair (32).

The primary goal of the current project was to define and further characterize the functions of the recently identified genes in the repair of DSBs. The new project investigated the relative sensitivities of additional mutants to EcoRI endonuclease, thereby completing the initial genetic screen. Moreover, testing of all 73 new EcoRI^s mutants to identify those that have high levels of unrepaired DNA damage was completed. Several additional experiments were performed to investigate the mutagen sensitivities and recombination proficiencies of selected mutants. These studies permitted identification of those new mutants that were most closely linked to the RAD52 epistasis group.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Ethylenediaminetetraacetic acid (EDTA), agarose and bleomycin were obtained from EMD Chemicals, Inc. (Darmstadt, Germany). A standard 1 Kb DNA ladder was purchased from New England Biolabs (Beverly, MA). Dimethyl sulfoxide (DMSO), ampicillin, RNase A, methylmethane sulfonate (MMS), lithium acetate, Hoechst 33258 and potassium chloride were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO). Ethidium bromide (EtBr) was obtained from Shelton Scientific, Incorporated (Shelton, CT). Sodium chloride, boric acid, and polyethylene glycol (PEG) 4000 were purchased from Mallinckrodt (Paris, Kentucky). Sonicated salmon sperm carrier DNA was purchased from Stratagene (La Jolla, CA). Tris base was purchased from VWR International (West Chester, PA). Dithiothreitol (DTT) was purchased from either Sigma-Aldrich or Gold Biochemistry (St. Louis, Missouri).

Molecular Biology Enzymes

Restriction enzymes were purchased from New England Biolabs (Beverly, MA).

Yeast and Bacteriological Media

Raffinose, D-(+)-galactose, D-(+)-glucose, and all amino acids were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bacto peptone, bacto yeast extract, bacto tryptone, bacto agar, yeast nitrogen base, and LB broth mix were purchased from Becton Dickinson Microbiological Systems (Sparks, MD).

Yeast Strains and Plasmids

The parent haploid yeast strain BY4742 has a genotype of *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (33*)*. BY4741 has a genotype of *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (33). The yeast deletion strain libraries were obtained from Open Biosystems (Huntsville, AL). The library was kept at -80 ^{o}C in 96-well microtiter dishes in which the cells were suspended in YPD broth + G418 (200 μg/ml) + 15% glycerol. Plasmids used in this study included pGALEcoRI (YCpGal::RIb) (*CEN/ARS URA3 GALp::EcoRI*) (34), pRS316 (*CEN/ARS URA3*) (35), pLKL37Y (*CEN/ARS HIS3 URA3*), pRS315 (*CEN/ARS LEU2*) (35), pRAD52Blast (*rad52Δ::hisG-URA3-hisG*) (8) and pGEM4Z S-H/URA (*yku70Δ::URA3*) (36).

Media and Cell Culture Solutions

For non-selective growth, yeast cells were grown on YPDA (rich) media (1% bacto yeast extract, 2% bacto peptone, 2% glucose, 2% bacto agar, 0.001% adenine). For mitochondrial function assessment, yeast cells were grown on YPG (1% bacto yeast extract, 2% bacto peptone, 2% bacto agar, 3% glycerol). YPDA liquid media was prepared as plate media without agar. Plasmid selection was determined when yeast cells

were grown on synthetic media with drop-out mix (0.17% yeast nitrogen base without amino acids, 2% glucose, 2% bacto agar, and all essential amino acids minus the amino acids used for selection). Raffinose (1%), raffinose plus galactose (1% and 3%, respectively) and galactose (2%) plates were made using synthetic media. Additionally, glucose complete plates were used. Plates with 0.2, 0.5, or 1 mM MMS were prepared using synthetic media with aliquots of a stock solution of 11.8 M MMS.

E. coli cells were grown in LB + ampicillin (Amp) broth (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 0.01% ampicillin) or on LB + Amp plates (as broth, with 1.5% agar).

II. METHODS

Chromosomal and Plasmid DNA Purification

Yeast chromosomal DNA was purified using the SDS/EDTA/Tris method (Lee, 2011). Plasmid DNA was purified using a Qiagen Spin Column Miniprep Kit (Maryland, VA).

Gel Electrophoresis

Gel electrophoresis was performed in a Life Technologies Horizon 10-14 gel rig using 0.7% agarose gels in 1X TAE (90 mM Tris-acetate, 2 mM EDTA) running buffer. The gels were run using a voltage of 140 V and were stained with ethidium bromide for 20 min. A Kodak IS440 CF Image Station instrument and Carestream imaging software were used to capture gel images.

Yeast Cell Transformations

Routine transformations of stationary phase yeast cells were performed using a modified version of the rapid DMSO-based transformation protocol of Soni *et al.* (38) involving pretreatment of cells with 500 μL of 0.1 M dithiothreitol (DTT) (32). Other experiments involving transformation of yeast cells were performed using the lithium acetate-based high efficiency protocol of Gietz *et al.* (37).

Dilution Pronging Survival Assays

Yeast cells were initially grown on 1% Raff-Ura plates by allowing growth at 30 ^{o}C for 3 days or at RT for 4 days. The cells were then harvested and diluted 1/40 in H_2O, followed by brief sonication using a Vibra-cell sonicator supplied by Sonics and Materials Inc. (Newtown, CT). Cells were loaded onto a 0.1 mm deep Reichert Bright-Line hemocytometer (Buffalo, NY) and counted using a model M837T trinocular compound phase contrast microscope from Microscopenet.com (Hopewell Junction, NY). A total of 2 x 10^7 cells was added to H_2O in a sterile 96-well microtiter dish in a total volume of 220 μL per well. Five-fold serial dilutions were then made (40 μL into 160 μL H_2O) for a total of 6 columns across the length of the microtiter dish. The cells were pronged onto control plates containing 1% Raff-Ura and selective plates containing 1% Raff + 3% Gal-Ura or 2% Gal-Ura. After 3-4 days of growth at 30 °C, the plates were evaluated for sensitivity to EcoRI endonuclease killing. Images were taken of the plates using a Canon Powershot G3 digital camera and saved as JPEG files.

Yeast Strain Construction

rad52Δ::hisG-URA3-hisG double mutant constructs

BY4742-derived yeast strains YLKL898 (*mec3Δ::G418^r)* and YLKL935 (*rad17Δ::G418^r)* were transformed with SalI-HF digested plasmid pRAD52Blast to delete *rad52*. The cells were spread onto Glu-Ura plates and the resulting colonies were subsequently patched to fresh Glu-Ura plates. Isolates tested for *rad52* deletion were replica-plated to 0.2, 0.5, and 1 mM MMS plates and grown at 30°C for three days.

Cell Cycle Analysis

WT cells, mutants previously determined to be EcoRI-sensitive, checkpoint mutants, and a select few other repair pathway mutants were harvested, sonicated, and counted with a hemacytometer as previously described. The cells were then diluted into four YPDA cultures at $3x10^6$ cells/ml for each mutant. The cultures (1 mL) were then vigorously shaken for 3.5 - 4 hrs at 30 °C in a microfuge tube shaker inside an incubator. Each culture was sonicated and then analyzed with a hemacytometer to determine the fraction of cells that were unbudded, small-budded, or large-budded. A total of 100 cells were counted for each culture. Large-budded cells were defined as cells in which the size of the bud was > 50% of the size of the mother (8).

Single Exposure Bleomycin Survival Assays

Four EcoRI sensitive mutants that were also gamma sensitive and four mutants that were gamma resistant, as well as WT cells, were exposed to a single brief dose of bleomycin. Thirty μL of overnight cultures grown in YPDA broth (3 mL) were diluted in

600 μL YPDA broth with four cultures for each mutant. The cultures were shaken vigorously for 2 hrs at 30 °C. Cells from each culture were then exposed to 1.0 - 10 μg/mL of bleomycin for 20 - 40 min at RT (20 °C). The cultures were microcentrifuged for 20 s at RT (20 °C), the supernatant removed and pellets were resuspended in 600 μL H_2O. Ten-fold serial dilutions were made and appropriate volumes of the diluted cultures were spread onto YPDA plates and incubated at 30 °C for 3-4 days. The plates were evaluated for survival by counting colonies. In the final optimized version of the protocol, cells were treated for 30 min with 10 μg/mL bleomycin.

Continuous Exposure Bleomycin Survival Assays

The above mutants were similarly exposed to bleomycin continuously. Overnight cultures in YPDA broth (3 mL) were diluted by adding 10 μL of cells into 800 μL fresh YPDA broth, with four cultures for each mutant. The diluted cultures were grown to log phase by vigorously shaking at 30 ^{o}C for 2 hrs. Cultures were then inoculated with 0.1 - 2.0 μg/mL of bleomycin and incubated at 30 ^{o}C for approximately 18 hrs. Ten-fold serial dilutions were made and appropriate volumes of the diluted cultures were spread onto YPDA plates and incubated at 30 °C for 3-4 days.

Homologous Recombination Efficiency Assays Using Integrating Plasmids

Preparation of plasmid DNA to be transformed into cells

Plasmid pLKL37Y was digested with BseRI to create a DSB in the *HIS3* gene. For the digestion of pLKL37Y the following were added to a 1.5 ml microcentrifuge

tube: 80 μL pLKL37Y DNA, 230 μL ddH_2O, 80 μL 5X KGB Buffer, and 10 μL BseRI (40 units). The reaction mixture was incubated for ≤ 2 hrs at 37 ^oC and the enzyme subsequently heat inactivated at 65 ^oC for 20 min. Samples were run on a 0.7% agarose gel to verify complete digestion.

Transformation

The ten recently identified EcoRI-sensitive mutants that are also MMS, bleomycin, and gamma sensitive (32) were transformed using a modified version of the high efficiency protocol of Gietz *et al.* (37). These mutants, which contain a mutant *HIS3* gene, were transformed with the plasmid BseRI-pLKL37Y (800 - 1400 ng per transformation) to allow for selection of recombinant cells that have integrated the entire plasmid into *his3-Δ1* and been converted from His^- Ura^- to His^+ Ura^+. All strains were also transformed with the uncut control vector pRS315 (*CEN/ARS LEU2*) (56 - 109 ng per transformation) to serve as a control for differences in transformation efficiencies among strains.

The modified version of the high efficiency protocol by Gietz *et al.* (37) is as follows:

1. Grow 5.0 ml YPDA overnight culture of cells.
2. In the morning, dilute 0.5 - 0.7 ml cells into 5.0 ml fresh YPDA broth and shake at 30 ^oC for 2.5 - 4 hrs.
3. Pellet 1.5 mL of cells in a microcentrifuge for 15 s and discard the supernantant.
4. Remove supernatant and add 0.5 ml ddH_2O. Vortex and microcentrifuge for 15 s.
5. Remove supernatant and add 0.4 ml 0.1 M LiAc. Vortex and microcentrifuge for 15 s.

6. Add to the pellet 240 μL PEG 3350 (50 % w/v), 36 μL LiAc (1.0 M), 5 μL single-stranded carrier DNA (10 mg / mL), 19 μL 2 M DTT (0.11 M final) and 43 μL of sterile ddH_2O and plasmid DNA (up to 10 μL), in the order given, and mix the pellet by vortex mixing briskly until resuspended. If performing multiple transformations, the chemicals and DNA can be premixed and added as a single 351 μL aliquot to the cell pellet. The cell pellets were resuspended by vortex mixing.

7. Shake the tube at 30 ^{o}C for 20 min.

8. Incubate the tube in a water bath at 42 ^{o}C for 30 min.

9. Microcentrifuge the transformation tube for 15 s at room temperature (20 ^{o}C) and remove the supernatant.

10. Resuspend the pellet in 1.0 mL of YPDA broth and incubate for 30 min at 30 ^{o}C.

11. Microcentrifuge the transformation tube for 30 s at room temperature (20 ^{o}C) and remove the supernatant.

12. Pipette 250 μL of sterile water into the transformation tube. Stir the pellet with a sterile micropipette tip to break up the cell pellet and vortex thoroughly to resuspend.

13. Pipette appropriate volumes of the cell suspension onto a plate containing synthetic complete drop-out selection medium. Typically, plate 100 or 200 μL samples. Allow the spread liquid to absorb into the plates by incubation at room temperature.

14. Incubate the plates at 30 ^{o}C. Transformants can be identified after 3 to 4 days.

CHAPTER III

RESULTS AND DISCUSSION

The primary focus of this research project was to complete and expand upon prior studies in the Lewis lab that implicated new yeast genes in the repair of DNA DSBs. Previous studies by Bennett *et al*. and Game *et al*. screened approximately 5000 diploid yeast mutants for sensitivity to gamma radiation and identified 210 genes as being required for normal resistance to ionizing radiation (27-29). Subsequent studies by graduate students Jennifer Summers, Sunaina Sethi, and Jennifer DeMars from the Lewis lab utilized the restriction endonuclease EcoRI to home in on genes specifically involved in the repair of DSBs. Using mutant yeast strains from haploid deletion strain libraries, the roles of the previously identified 210 genes, plus an additional gene, *EXO1*, which was known to be radiation sensitive in its haploid mutant form, were tested for resistance to EcoRI expression. In total, 81 of the haploid mutants including 8 RAD52 group mutants were found to be sensitive (Figure 5) (30-32). The first goal of this project was to complete the genetic screen by investigating the relative sensitivity of an additional mutant, *ycl007c*, to EcoRI endonuclease, which inadvertently did not get tested in the earlier work.

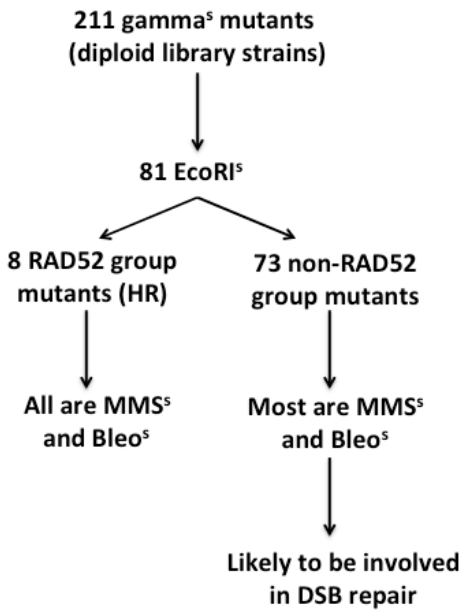


Figure 5. Outline of findings determined from the current genetic screenings.

EcoRI, though naturally existing from *E. coli* bacterial cells, can be expressed in a controlled manner in eukaryotic cells; in yeast cells, the galactose-regulatable *GAL1* promoter has previously been used to express EcoRI (Figure 6) (1, 34, 39). The expression of EcoRI is positively regulated in the presence of galactose and strongly repressed in the presence of glucose. By transforming mutant cells with a plasmid containing a *GAL1p::EcoRI* promoter fusion, sensitivity to EcoRI can be determined by testing survival after galactose induction of EcoRI expression inside the cells. Cells are often grown on raffinose just before transfer to galactose media. There is less glucose repression of the *GAL1* promoter in raffinose and this leads to faster induction kinetics in galactose.

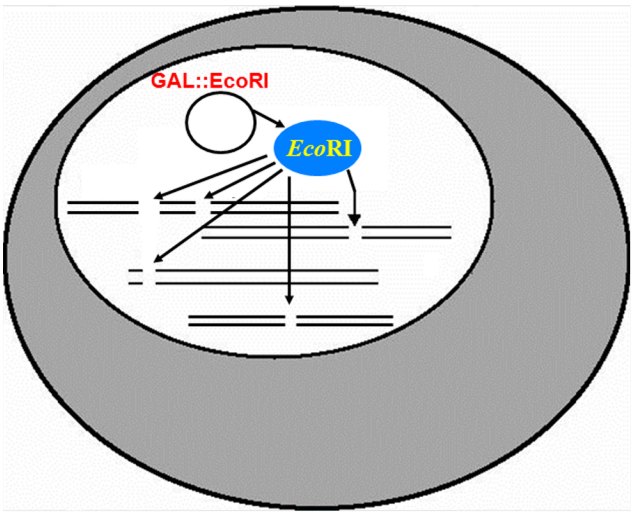


Figure 6. EcoRI expression in yeast cells through the use of a *GAL1* promoter.

To quantitatively assess sensitivity to EcoRI, dilution pronging was utilized, as illustrated in Figure 7. Transformed cells containing either the control plasmid vector pRS316 or the plasmid *GAL1p::EcoRI* were harvested into water, counted on a hemacytometer using a phase contrast microscope, and 2×10^7 cells in a total volume of 220 uL were placed into a microtiter dish well and serially diluted five-fold. A metal pronger was then placed in the wells of the microtiter dish and subsequently spotted onto an agar plate surface, leaving an imprint of cells. The cells grew at 30 ^{o}C for two to three days and were then evaluated for sensitivity to EcoRI relative to wildtype cells expressing EcoRI. Relative sensitivities to EcoRI were classified as follows: resistant (R), moderately sensitive (S), or strongly sensitive (SS). Resistant mutants exhibited < 2-fold killing relative to wildtype cells. Moderately sensitive mutants exhibited > 2-fold but < 25-fold killing relative to wildtype cells on plates with galactose only. This means that growth was reduced by up to 2 columns relative to wildtype cells containing the EcoRI

plasmid. Strongly sensitive mutants exhibited ≥ 25-fold killing on plates with galactose only (equal to or more than two full columns of growth less than in wildtype cells).

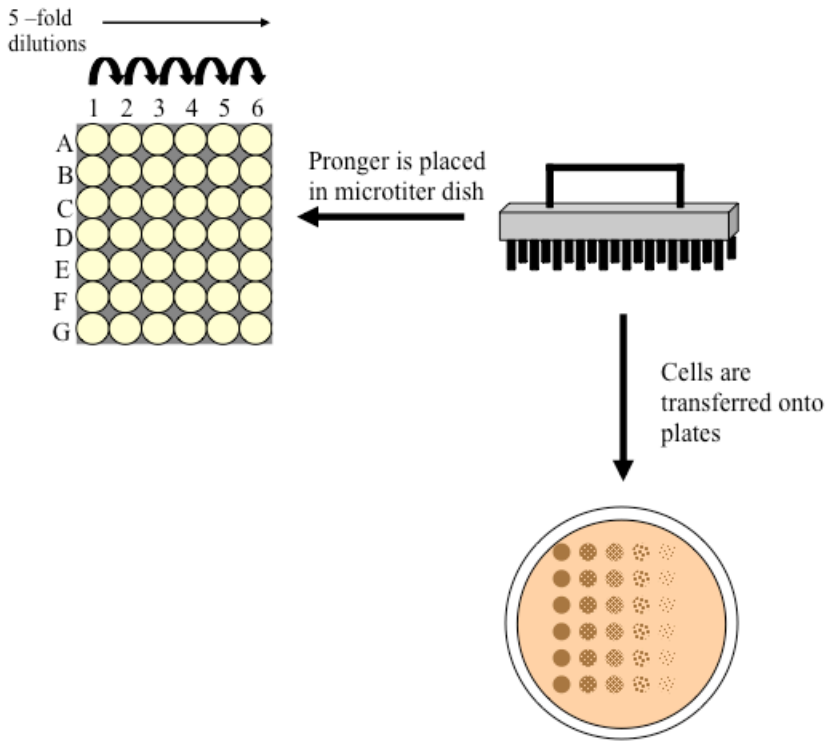


Figure 7. Pronging assays used 96 well microtiter dishes, a pronging device, and Petri dishes (30).

The dilution pronging survival assay to determine the sensitivity of mutant *ycl007c* cells to EcoRI is shown in Figure 8. Two different types of sugar media, 1% raffinose and 2% galactose, were used to regulate EcoRI expression. The first two rows consist of WT cells with the control vector and the *GAL1p::EcoRI* plasmid. The subsequent rows show mutant *ycl007c* cells containing the control vector and the *GAL1p::EcoRI* plasmid. Note that growth of WT cells expressing EcoRI was slower than cells containing the vector only (resulting in smaller colonies), but survival was high

(measured as the total number of colonies in the 4th and 5th columns). It was determined that *ycl007c* was not sensitive to EcoRI, as it showed < 2-fold killing relative to the wildtype cells (~1.5-fold in this assay).

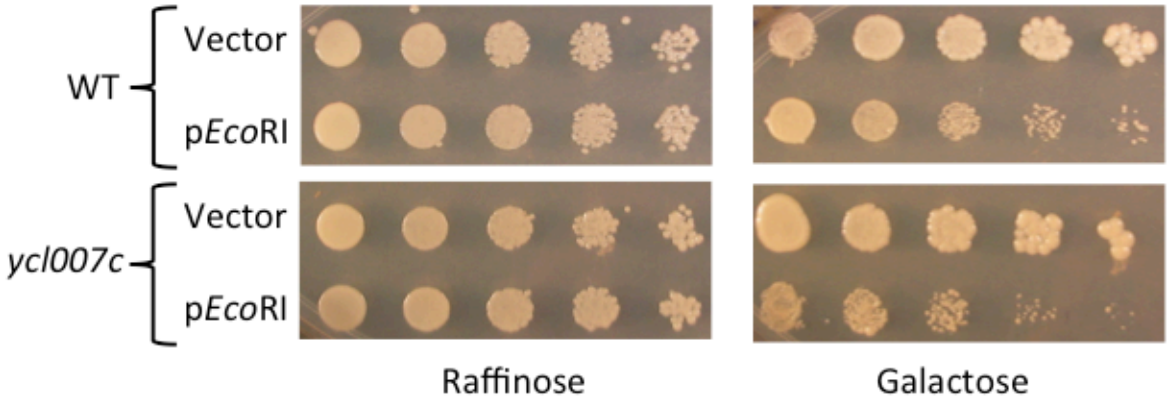


Figure 8. Pronging of wildtype and *ycl007c* cells containing a control vector (pRS316) or the *GAL1p::EcoRI* plasmid (pEcoRI).

The testing of mutant *ycl007c* cells thereby completed the genetic testing of the 211 genes for EcoRI sensitivity. Table 1 shows the collective results for all of the mutants tested in both *MATα* and *MATa* haploid deletion strain libraries, combining the previous work of J. Summers, S. Sethi, and J. DeMars with the current project. In total, 73 non-RAD52 group genes were determined to be required for EcoRI resistance in *MAT*α cells, *MAT*a cells, or in both cell types.

Table 1. Survival of EcoRI^s mutants after exposure to MMS, bleomycin or gamma radiation.

	MMS (mM)		bleo (μg/ml)		Γ (krads)			MMS (mM)		bleo (μg/ml)		Γ (krads)	
Mutant	1	2	2	4	30	60	Mutant	1	2	2	4	30	60
RAD52 group:													
mre11	SS	SS	S	SS	SS	SS	*rad54*	SS	SS	S	SS	SS	SS
rad50	SS	SS	S	SS	SS	SS	*rad55*	SS	SS	R	SS	SS	SS
rad51	SS	SS	S	SS	SS	SS	*rad57*	SS	SS	R	SS	SS	SS
rad52	SS	SS	S	SS	SS	SS	*xrs2*	SS	SS	S	SS	SS	SS
New EcoRI^s mutants:													
adk1	R	R	SS	SS	R	R	*mrps35*	R	S	R	S	R	R
ado1	S	S	SS	SS	R	S	*not5*	R	R	R	R	R	R
akr1	R	R	R	R	R	R	*nup84*	R	S	S	SS	R	R
apq13	S	SS	S	SS	R	R	*och1*	R	S	S	SS	R	R
arp5	R	S	R	R	R	S	*psy1*	R	R	R	R	R	R
atp2	S	SS	S	S	R	R	*rad5*	SS	SS	S	SS	R	R
bck1	S	S	R	R	R	S	*rem50*	R	SS	R	S	R	R
bik1	R	R	S	R	R	R	*rpb9*	R	SS	S	SS	S	SS
bud19	S	S	SS	SS	R	R	*rpl31a*	R	R	R	R	R	R
bud30	R	R	S	S	S	S	*rsm7*	R	R	S	SS	R	R
bud32	R	R	SS	SS	R	S	*rtf1*	R	S	R	SS	S	SS
bur2	R	S	R	S	R	R	*rvs161*	R	R	R	SS	R	R
cax4	R	R	SS	SS	S	S	*sae2*	R	S	R	R	R	R
ccr4	R	R	S	SS	R	R	*sam37*	S	SS	S	SS	R	R
cdc40	SS	SS	SS	SS	SS	SS	*sco1*	R	R	R	R	R	R
cgi121	R	R	S	S	SS	SS	*sfp1*	R	R	R	S	R	R
cis3	S	SS	R	S	R	R	*slm4*	S	SS	S	SS	R	R
cnm67	R	S	R	SS	R	S	*spt10*	R	S	SS	SS	S	SS
ctf4	SS	SS	R	S	R	R	*spt20*	R	R	S	SS	R	R
ctf8	R	SS	R	R	R	R	*srv2*	R	R	R	R	R	R
dcc1	S	SS	R	R	R	R	*taf14*	S	SS	R	S	R	R
ddc1	R	S	R	R	R	R	*trm9*	S	S	S	S	R	R
eaf1	R	S	R	S	R	R	*tsr2*	R	R	R	S	R	R
exo1	R	R	R	R	S	SS	*ubp8*	R	R	R	R	R	R
gcn5	R	SS	R	S	R	R	*ubr1*	S	S	R	S	SS	SS
gnd1	S	S	R	SS	R	R	*ume6*	S	S	R	R	R	R
hsp150	R	R	R	R	R	R	*vma7*	S	S	SS	SS	R	S
htl1	R	S	SS	SS	S	SS	*vph2*	R	R	SS	SS	R	R
ids2	R	R	R	R	R	R	*ybr099c*	S	SS	R	R	R	R
img2	R	R	R	S	R	R	*ydr417c*	S	SS	S	SS	R	R
lip5	R	R	R	S	R	S	*ydr433w*	R	SS	SS	SS	R	R
lrp1	R	R	R	S	R	R	*ygl218w*	R	SS	R	R	R	R
lsm7	S	S	R	S	R	R	*ylr235c*	SS	SS	R	S	R	R
mct1	S	SS	R	S	R	SS	*yml009w-b*	S	SS	R	S	R	R
mms2	S	SS	R	R	R	R	*yml012c-a*	S	SS	R	S	R	R
mms22	SS	SS	R	S	R	R	*ynr068c*	SS	SS	S	SS	R	R
mms4	S	SS	R	R	R	R							

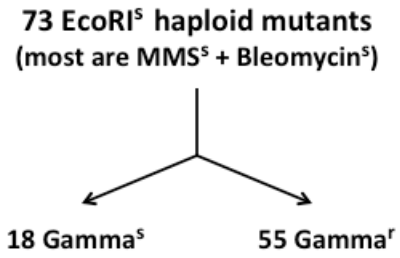
[a] Mutants were ranked as resistant (R), sensitive (S), or strongly sensitive (SS). Mutants classified as SS exhibited ≥ 25-fold higher killing than wildtype cells using semi-quantitative dilution pronging assays. Γ, gamma radiation.

Interestingly, most of the 73 EcoRI^s haploid mutants were also sensitive to the chemical clastogens MMS and bleomycin, but only 18 were sensitive to ionizing radiation (Table 1 and Figure 9A). In the next portion of this project, experiments were performed in an effort to explore the incongruity of these findings. In these particular studies, the survival of cells to ionizing radiation was determined after a single exposure to gamma radiation lasting for approximately 30 minutes. The cells were then grown on agar plates in which they were no longer exposed to the radiation, until the surviving cells grew into colonies and could be counted. In the EcoRI, MMS, and bleomycin studies, however, cells were exposed continuously to these clastogens as they grew on agar plates for three to four days (30-32). In essence, these cells constantly went through cell cycles while continuously being exposed to DSBs (shown schematically in Figure 9B). Cells are particularly sensitive to DNA damage in certain parts of the cell cycle, such as during mitosis (41). It was thereby postulated that some genes were needed for survival during constant exposure to DSBs, but not in cells that experienced damage only once in a single phase of the cycle.

Bleomycin damages DNA directly by entering cells, forming a free radical complex, and binding to DNA in the nucleus. Cells can be exposed to this clastogen continuously by adding it to liquid or plate cell growth media. It is also possible to perform a single exposure experiment by adding the bleomycin to cells for a brief time, e.g., 30 minutes, and then washing the cells several times to remove the drug before spreading onto plates to determine survival. Most of the new mutants identified here were killed by continuous exposure to bleomycin in plates, but not by a single brief exposure

to gamma radiation. We hypothesized that these mutants would be similarly resistant to a brief exposure to bleomycin if continuous exposure was the key to their sensitivity.

A



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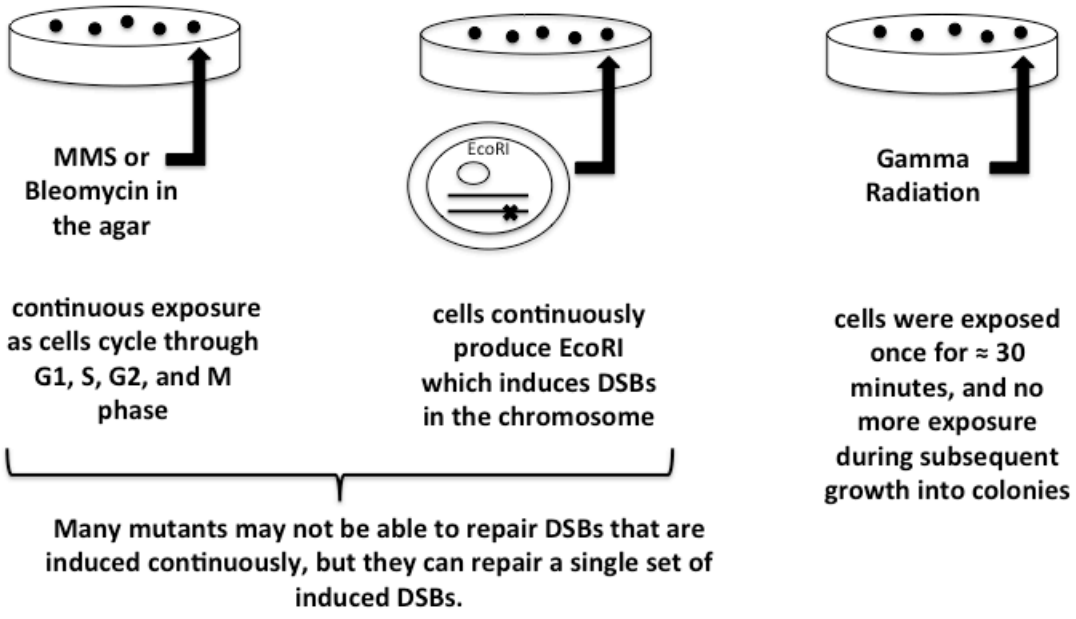


Figure 9. (A) Surprisingly, most of the 73 EcoRI^{S} mutants were also sensitive to MMS and bleomycin, but not ionizing radiation. (B) Diagram depicting cell exposure to MMS, bleomycin, EcoRI, and gamma radiation.

To assess survival after a single exposure to bleomycin, cells were initially harvested from patches on plates and inoculated into YPDA broth and grown to log phase. Cells were then exposed to 1.0 - 10 μg/mL bleomycin for 20 - 40 min at RT (20 °C). Ten-fold serial dilutions were made and appropriate volumes of the diluted cultures were spread onto YPDA plates and incubated at 30 °C for three to four days. The plates were then evaluated for survival. The optimum conditions determined for these experiments are presented in the Materials and Methods.

In addition to the single exposure assays, experiments to assess continuous exposure to bleomycin in liquid cultures were performed in which cells were instead inoculated with low concentrations of bleomycin in YPDA broth and incubated at 30 ^{o}C for approximately 18 hours to allow growth in the presence of the drug. Serial dilutions and plating were performed as above. The results of these continuous exposure liquid assays were unusually variable, likely due to resistant "jackpot" mutants and/or unknown characteristics of the liquid culture. Additionally, it was possible that there was a certain variability in the rate of occurrence of lethal lesions induced by bleomycin. Consequently, this method of assessing survival after continuous exposure in liquid media was not pursued.

Initially, four mutants resistant to a brief exposure to gamma were tested for their sensitivities to a brief treatment with bleomycin with the expectation that they would also be resistant. Table 2 summarizes the results of these experiments. The relative sensitivities of the three controls used in these studies are listed in Table 2A. Control RAD52 group mutants *rad57* and *rad52* exhibited strongly reduced levels of survival upon a single exposure to bleomycin, coinciding with their previously characterized

phenotypes as part of the RAD52 group. Surprisingly, each of the gamma resistant mutants exhibited sensitivity to a single exposure of bleomycin, with survival reduced by 7.6 - 100 fold (Table 2B). This finding appeared to disprove the original hypothesis. We then reasoned that perhaps the gamma resistant mutants are sensitive to brief bleomycin exposure, but mutants that are gamma sensitive would be *more* sensitive to the drug. However, initial testing of four gamma sensitive mutants showed sensitivities that were similar to those of the gamma resistant mutants (8.1 - 43.5 fold reductions in survival) (Table 2C). The chief findings from these studies indicate that a single exposure to bleomycin does not correlate with a single exposure to gamma radiation, thus disproving the original hypothesis. It is possible, however, that the mutants displayed different results with bleomycin for technical reasons. For example, the drug may have persisted inside the cells for several hours, extending the length of time and the number of cell cycle phases in which DNA damage occurred.

Table 2. Relative survival of gamma resistant and gamma sensitive mutants after single and continuous exposure to bleomycin. R, S, and SS sensitivity scores are taken from Table 1. Averages ± standard deviations are shown.

	Strain	Single Exposure Gamma	Single Exposure Bleomycin (10 μg/mL)	Fold Reduction in survival	Continuous Exposure Bleomycin (pronging) (4 μg/mL)
A	WT	R	100%		R
	rad57	S	0.8% ± 0.3%	125x	SS
	rad52	S	0.04% ± 0.007%	2500x	SS
B	*nup84*	R	13.2% ± 1.2%	7.6x	SS
	rem50	R	8.4% ± 2.5%	11.9x	S
	mms22	R	5.1% ± 2.0%	19.6x	S
	ctf4	R	1.0% ± 0.7%	100x	S
C	*cnm67*	S	12.3% ± 2.9%	8.1x	SS
	rpb9	SS	6.3% ± 1.1%	15.9x	SS
	htl1	SS	2.9% ± 0.3%	34.5x	SS
	ubr1	SS	2.3% ± 1.0%	43.5x	S

A summary of the sensitivities and resistances of the 81 mutants to the four different clastogens tested are listed in Figure 10. Of the 73 non-RAD52 group mutants, ten were found to be sensitive to all four DNA damaging agents, consistent with the phenotype of RAD52 group mutants (shown in boldface print in the upper right box in Figure 10). It can therefore be suggested that these ten mutants are the most critical in the repair of DNA DSBs and are likely to affect the recombination pathway, as seen with RAD52 group mutants.

EcoRI^S	MMS^S	Bleo^S	Gamma^S	***rad50, rad51, rad52, rad54, rad55, rad57, mre11, xrs2,*** *cdc40, cnm67, htl1, vma7, ado1, mct1, rpb9, rtf1, spt10, ubr1*
			Gamma^R	*taf14, atp2, apq13/net1, bud19/rpl39, cis3, ctf4, eaf1/opi7, gcn5, gnd1/yhr182c-a, lsm7, sam37, bur2, mms22, mrps35, nup84, och1, rad5, rtt109(rem50), slm4, trm9, ydr417c/rpl12b, ylr235c/top3, yml012c-a/ubx2, ynr068c, yml009w-b/spt5, ydr433w/npl3*
		Bleo^R	Gamma^S	*arp5, bck1*
			Gamma^R	*dcc1, mms2,mms4/ybr099c, ume6, ddc1, sae2, ctf8, ygl218w/mdm34*
	MMS^R	Bleo^S	Gamma^S	*bud30/rpc53, bud32, cax4, cgi121, lip5*
			Gamma^R	*adk1, ccr4, img2, lrp1, rsm7/yjr114w, rvs161, sfp1, spt20, tsr2/ylr434c, vph2/ykl118w*
		Bleo^R	Gamma^S	*exo1*
			Gamma^R	*akr1, bik1, hsp150, ids2, not5, psy1/ykl075c, rpl31a, sco1, srv2, ubp8*

Figure 10. Resistance of EcoRI^s mutants to physical and chemical DNA damaging agents. Gene names separated by a forward slash indicate deletions within overlapping open reading frames. The first gene listed was deleted in the indicated mutant, except for *mms4/ybr099c*, in which both gene deletions were tested independently.

In an effort to further characterize the roles of these particular ten most sensitive mutants, recombination proficiency studies were performed. Gene targeting assays were developed previously by graduate student Rachel Roberts to assess recombinational repair of site-specific DSBs by utilizing a DNA plasmid containing a single DSB within the *HIS3* gene. The BY4742 strain used to construct the yeast deletion strain library, which contains a mutation in the same gene, is transformed with the plasmid to stimulate homologous recombination repair. As shown in Figure 11, the plasmid DNA containing a DSB in the *HIS3* gene is transformed and integrated into yeast chromosome XV, converting cells from His^- Ura^- to His^+ Ura^+. Recombination decreases ranged from 3-fold to 170-fold with the RAD52 group mutants (41-42).

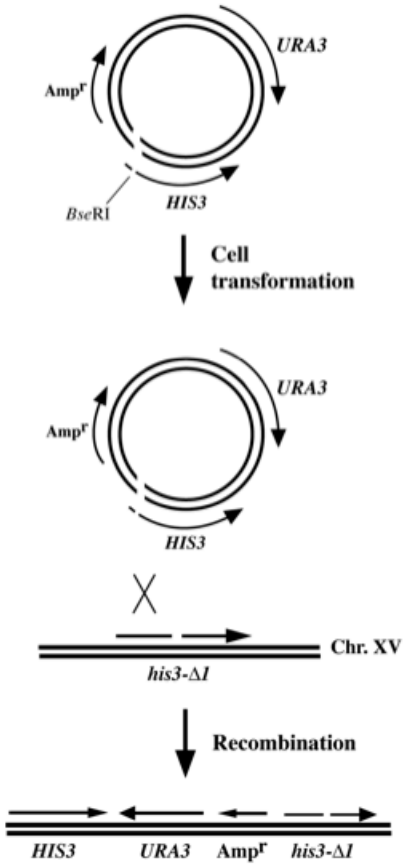


Figure 11. Representation of the recombination assay used to monitor repair efficiency between a plasmid containing a DSB and an intracellular homologous chromosome.

Recombination proficiency assays were performed by transforming 800 - 1400 ng of BseRI-cut pLKL37Y DNA into WT and mutant cells and counting the number of His^+ Ura^+ colonies forming on plates lacking histidine and uracil. In these experiments, cells were also transformed with 56 - 109 ng of the uncut control vector pRS315 (*CEN/ARS LEU2*) and aliquots were spread onto plates lacking leucine. To account for possible variations in transformation efficiencies, the number of transformant colonies achieved per μg of DNA with the broken plasmids (formed by recombination) was normalized to the number achieved with pRS315 (formed by simple transformation).

As seen in Table 3, five of the ten mutants had repair efficiencies near that of WT cells. Three mutants, *ado1, ubr1,* and *spt10*, exhibited reduced levels of repair, which suggests that these mutants are most closely linked to the RAD52 epistasis group, though their fold reductions were modest. Interestingly, two additional mutants, *rtf1* and *vma7*, displayed elevated levels of repair. This "hyperrecombination" phenotype has been seen in other mutants previously. For example *mrx* mutants have elevated levels of recombination between homologous chromosomes in diploid mutants (1). Also, *sgs1* and *fen1* (*rad27*) mutants exhibit high levels of spontaneous recombination between chromosomes (1, 43). Only three of the new mutants showed reduced recombinational repair in this assay, despite the fact that each of the ten mutants were sensitive to all DSB-inducing agents tested. It should also be noted that assays for two of the mutants, *cnm67* and *vma7*, were performed three times using four cultures for each trial in which standard deviations were consistently high. The results shown in Table 2 represent averages of all three trials. This initial recombination assay focused on repair of a plasmid DNA DSB; further experiments will be needed to determine if these mutants may have defects in other types of recombination, such as between nucleosome-covered chromosomal DNAs.

Table 3. Repair efficiencies of ten most sensitive mutants.

Mutant	EcoRI	MMS 2 mM	bleomycin 4 μg/ml	Γ 60 krads	Recombination Proficiency[a]	Fold Change
WT					100% ± 23.0%	
Reduced						
ado1	S	S	SS	S	27.8% ± 8.6%	-3.6x
ubr1	S	S	S	SS	51.3% ± 11.9%	-2.0x
spt10	S	S	SS	SS	52.6% ± 20.0%	-1.9x
Increased						
rtf1	SS	S	SS	SS	173% ± 27.4%	+1.7x
vma7	SS	S	SS	S	748% ± 413%	+7.5x
Near WT[b]						
htl1	SS	S	SS	SS	61.5% ± 16.2%	-1.6x
rpb9	SS	SS	SS	SS	84.2% ± 57.9%	-1.2x
cdc40	SS	SS	SS	SS	140% ± 35.6%	+1.4x
mct1	SS	SS	S	SS	158% ± 100%	+1.6x
cnm67	SS	S	SS	S	245% ± 133%	+2.5x

[a] Numbers shown represent averages ± standard deviations
[b] Standard deviations for these mutants overlapped standard deviations for WT cells.

The eukaryotic cell cycle consists of four phases: G_1, S, G_2, and M. As yeast cells progress through the cycle, they undergo distinctive changes in shape (Figure 12a). As soon as a new cell is released from its mother, the daughter cell appears with a slightly elliptical form (G_1 phase). The cell eventually begins to develop a small "bud" as it progresses through the cell cycle (S phase). Ultimately, the bud grows until it reaches a size approximately equal to the size of the "mother" cell from which it arose (G_2 phase). The bud is then released from the mother during M phase and both cells start the cell cycle process again.

A

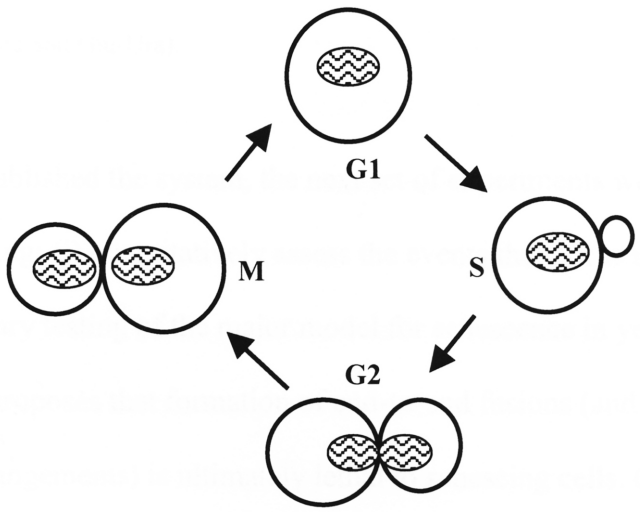


Figure 12a. The mitotic cell cycle in yeast. *S. cerevisiae* cells divide by budding.

It is possible to monitor cell cycle progression in yeast by observing the cells in a phase contrast microscope (Figure 12b). Unbudded cells are in G_1 phase, small-budded cells with a bud < 50% of the size of the parent are in S phase, and large-budded cells with a bud > 50% of the size of the parent are in the G_2/M phase of the cell cycle. (Cells in G_2 cannot be distinguished from M phase cells using light microscopy, however, the majority of large budded-cells are in G_2.)

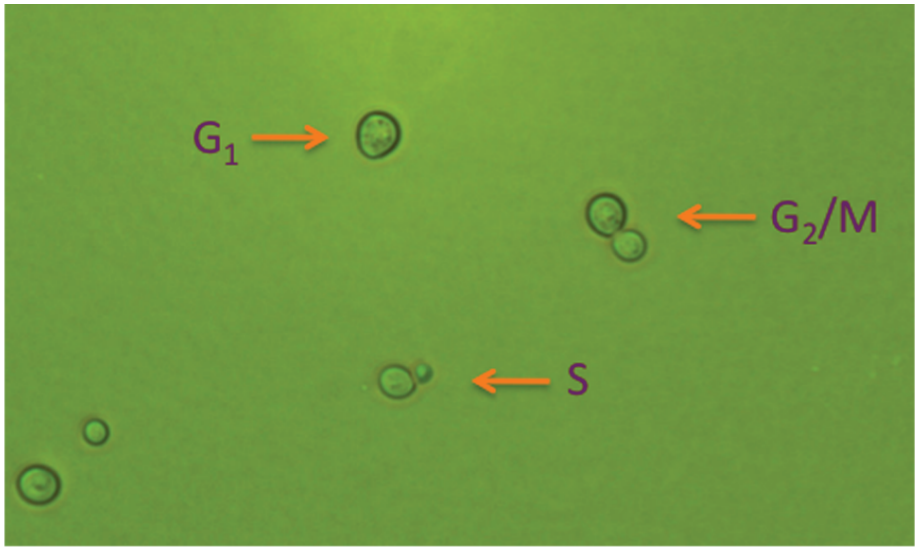
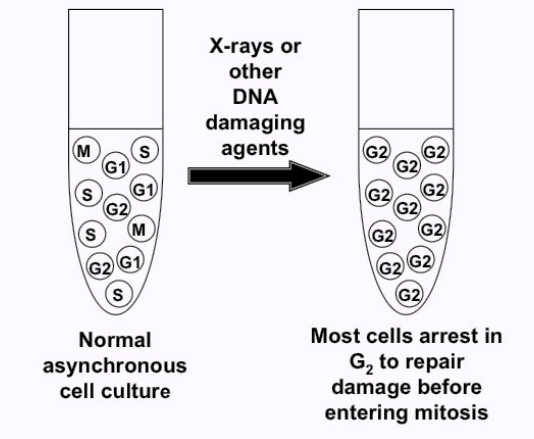


Figure 12b. Representation of cells and bud size for each phase of the cell cycle, as observed using a phase contrast microscope.

When a normal, asynchronous cell culture with a distribution of cells in all phases of the cell cycle is treated with a DNA damaging agent, most cells will arrest in G_2, pausing to repair the damage before entering mitosis (Figure 13A). On the other hand, a mutant cell that is defective in DNA repair has a constant increased level of DNA damage in their chromosomes during normal growth (6, 41, 44). In yeast cells, this can result in an increased fraction of cells that are in G_2 phase during normal, log phase growth (Figure 13B). The damage signals the cells to pause growth in G_2 phase to allow time to repair the damage before chromosomes are attached to the spindle and pulled apart during M phase. In these cells, G_2 is elongated but other phases are not (Figure 13C).

A



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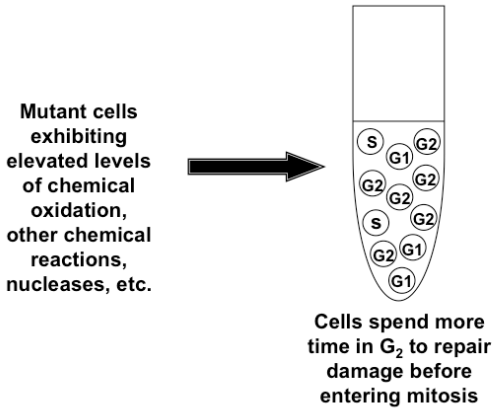

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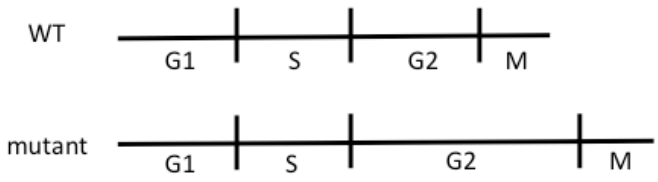
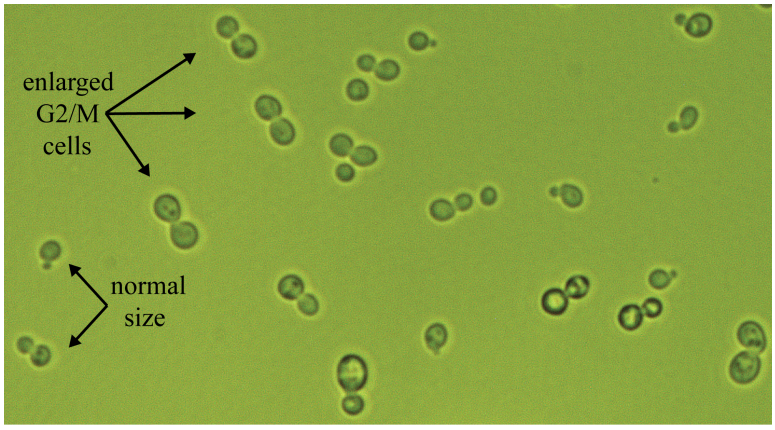


Figure 13. (A) Normal cells that incur DNA damage from exposure to clastogens transiently arrest growth in G_2 phase before they resume cycling. (B) Repair-defective mutants have a constant increased level of DNA damage in their chromosomes, resulting in an increased fraction of cells that are in G_2 phase. (C) Cells spend more time in G_2 phase to allow for repair of the damage, but other phases are not strongly affected.

To screen for potential repair mutants with constant unrepaired damage, cells were harvested from plates and inoculated into YPDA liquid cultures at a starting concentration of 1 x 10^6 cells/mL. Cells were then grown for 3.5 - 4 hours in a shaker at 30 °C to reach mid-log growth phase. Four cultures of each strain were counted using a phase contrast light microscope and the percentage of G_2/M cells was averaged.

In earlier studies done by graduate student J. Summers, several known homologous recombination mutants (*rad50-rad59* and *mre11*) were found to have high levels of spontaneous cell cycle arrest in G_2 phase (30). In the current work, the mutant *xrs2*, another well-known member of the RAD52 group, was also analyzed. These mutants consistently displayed a similar G_2 arrest phenotype, with many of the large-budded cells appearing greatly enlarged (Figure 14A). Combining the previous work with the new results revealed that all RAD52 group mutants tested exhibited higher spontaneous levels of G_2/M cells than WT (Figure 14B), suggesting high levels of unrepaired DNA damage. Results obtained for each mutant were statistically significant, as standard deviations for WT and mutant cells did not overlap.

A



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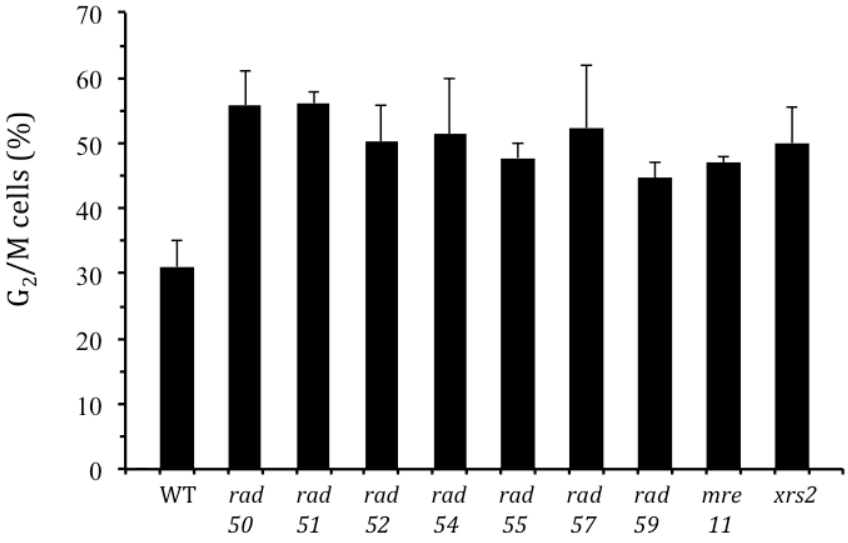

Figure 14. (A) Representation of enlarged G_2/M cells, as observed using a phase contrast microscope. (B) Percentages of G_2/M cells in log phase cultures of the RAD52 group mutants. Error bars indicate standard deviations.

In the previous work of J. Summers, S. Sethi, and J. DeMars, 73 non-RAD52 group mutants were found to be EcoRI sensitive. Sixty-one of these mutants were screened to measure spontaneous cell cycle arrest in G_2 phase. In total, 13 of the 61 mutants were found to have elevated levels. In this current work, the twelve remaining mutants found to be EcoRI sensitive were screened, as described above, to measure spontaneous cell cycle arrest in G_2 phase.

Three of the thirteen mutants tested consistently exhibited higher levels of G_2/M cells than WT (with non-overlapping standard deviations) during normal growth. In Figure 15, the average percentages for WT cells and the mutant cells that displayed high levels of G_2/M cells from both the previous and current works are shown. Sixteen mutants were found to have higher levels of G_2/M cells than WT cells. It can therefore be suggested that these mutants are likely to have high levels of spontaneous unrepaired DNA damage, resulting in constantly activated checkpoints. This suggestion is supported by the fact that some of these mutants have previously been linked to DNA repair, e.g., *rad5, rem50, ctf4*, and *ctf8* (10, 46).

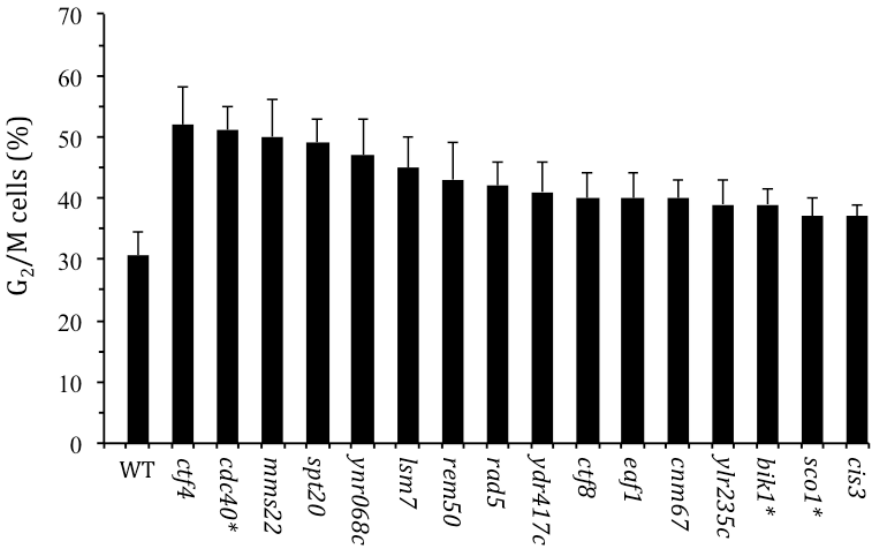

Figure 15. Characterization of spontaneous levels of G_2/M cells in mutants that were sensitive to EcoRI. Genes marked with an asterisk (*) were identified in this work. Error bars indicate standard deviations.

Having established that recombination-deficient RAD52 group mutants have elevated G_2/M cells, several known mutants defective in NHEJ were also analyzed, as described above, to measure spontaneous cell cycle arrest in G_2 phase. Both *MATα* and *MATa* haploid versions of the mutants were tested. As seen in Figure 16, two mutants, *yku70* and *yku80*, exhibited higher levels of G_2/M cells than WT cells in both the *MATα* and *MATa* versions. The *dnl4* mutant consistently had G_2/M cell levels near that of WT cells. *nej1* and *lif1* cells displayed variable levels of G_2/M cells, but were also similar to that of WT cells. *MATa lif1* mutants were an exception, as they were consistently modestly elevated (Figure 16). These results suggest that *yku70* and *yku80* mutants may also have high levels of unrepaired DNA damage. *yku70* and *yku80* mutants have shorter telomeres than normal, in part because the Ku proteins are needed to protect chromosome ends from nucleases and to help recruit telomerase to the ends (47). The other NHEJ mutants do not have short telomeres. Thus, it is possible that the *ku* mutants have higher G_2/M cells because the short telomeres provoke a DNA damage response, not because of a general increase in damage.

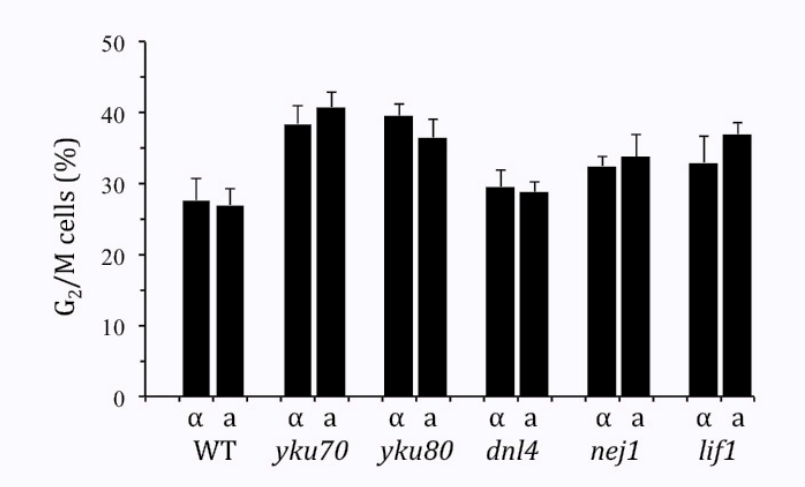

Figure 16. Characterization of G_2/M cells in mutant strains that are defective in NHEJ.

DNA damage-induced cell cycle checkpoint arrest is mediated by several proteins, including Mec1, Mec3, Rad9, Rad17, Rad24, and several others. Inactivation of the genes encoding these proteins leads to reduced ability to pause in G_2 after exposure to agents such as gamma radiation of UV light. In an effort to see whether the high levels of G_2/M cells were due to a checkpoint response, *rad52 mec3* and *rad52 rad17* double mutants were constructed. The idea behind this assay was that mutants known to exhibit high levels of G_2/M cells, such as *rad52*, would exhibit much lower levels when *MEC3* and *RAD17* are absent.

The pRAD52Blast gene disruption plasmid was used to delete the *RAD52* gene in *mec3* and *rad17* cells. This plasmid contains the *URA3* marker gene, which is flanked on both sides with DNA sequences homologous to the ends of the *RAD52* gene. The *URA3* region was removed from the plasmid via restriction digestion with SalI and was then transformed into the cells, resulting in the replacement of the *RAD52* gene with the *URA3* gene (Figure 17). These cells were then able to grow on agar plates lacking uracil. To confirm *rad52* deletion, isolates were replica-plated to 0.2 M and 0.5 M MMS plates and grown at 30 °C for three days.

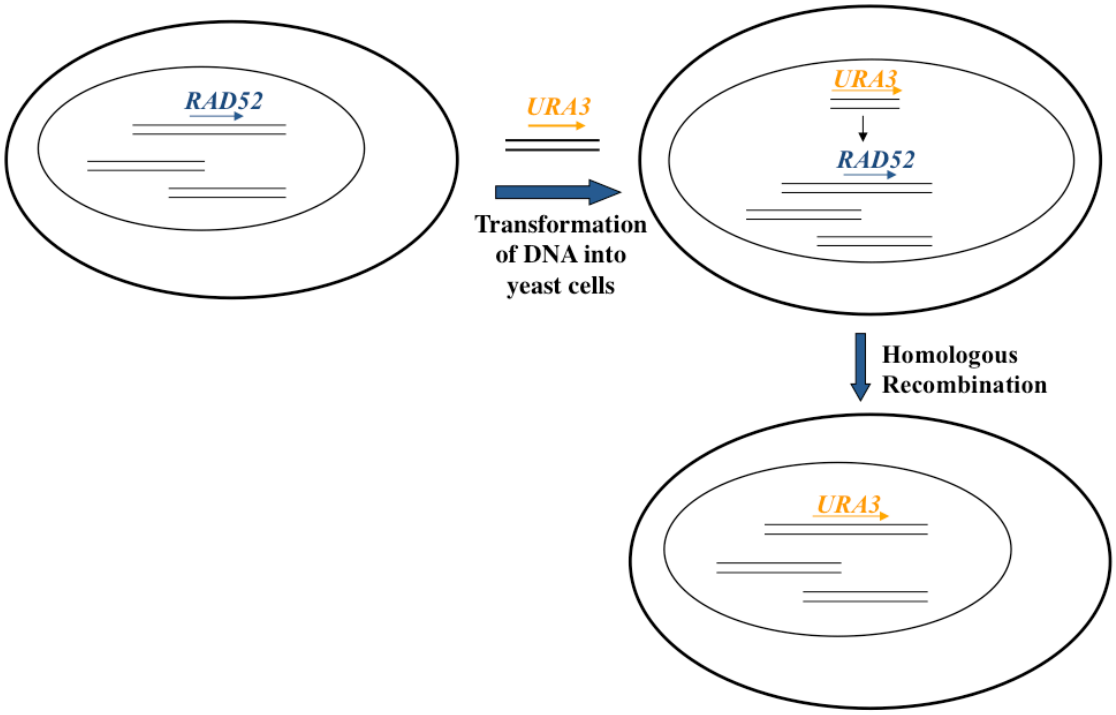

Figure 17. Schematic representation of the *rad52Δ::hisG-URA3-hisG* gene disruption procedure.

Several control mutants as well as the double mutant constructs were screened, as described above, to measure spontaneous cell cycle arrest during log phase growth. In Figure 18, the average percentages are shown. As expected, the *mec3* and *rad17* checkpoint mutants exhibited levels of G_2/M cells near that of WT at approximately 29%. *rad52* cells exhibited high levels of G_2/M cells (~ 45%). The *rad52 mec3* and *rad52 rad17* double mutants had significantly reduced levels of G_2/M cells relative to the *rad52* mutant (32% and 33% vs. 45%). This result strongly suggests that the high levels of G_2/M cells were due to a DNA damage checkpoint response.

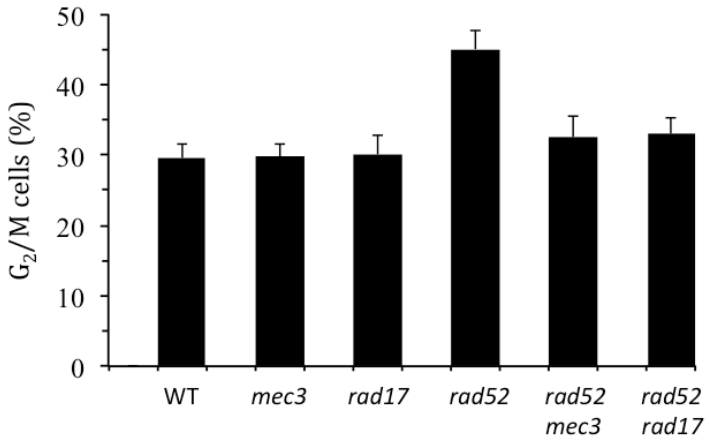

Figure 18. Characterization of G_2/M cells in checkpoint mutants and double mutants. Error bars indicate standard deviations.

Summary and Conclusions

The damage of DNA via DSBs can lead to mutations and chromosome rearrangements, increasing the risk of cell death and cancer. Several genes involved in the most complex pathway of DSB repair, homologous recombination, have been identified, but many genes and steps remain uncharacterized.

A previous genome-wide screen of approximately 5000 yeast mutants identified 210 genes that were required for resistance to gamma radiation. In the combined work of J. Summers, S. Sethi, and J. DeMars, 81 haploid mutants were found to be sensitive to EcoRI expression, eight of which were members of the RAD52 group. In this current study, the initial genetic screen was completed by testing the final gamma sensitive mutant, *ycl007c,* for its relative sensitivity to EcoRI. This mutant was found to be EcoRI resistant, thereby leaving the original findings of 81 EcoRI sensitive haploid mutants unchanged.

Surprisingly, most of the 73 non-RAD52 group haploid mutants were also sensitive to the clastogens MMS and bleomycin, but only 18 strains showed sensitivity to gamma radiation. The experiments determining the resistance of cells to ionizing radiation used a procedure that exposed the cells to a single dose of gamma radiation lasting for approximately 30 minutes. The cells were then grown on agar plates in which they were no longer exposed to the damage. In the EcoRI, MMS, and bleomycin studies, however, cells were exposed continuously to these particular clastogens as they grew on agar plates for three to four days. In the second portion of this study, several gamma sensitive and gamma resistant mutants were tested with a single exposure to bleomycin. It was determined that single exposures to bleomycin did not correlate with single

exposures to gamma radiation. At present, it is unclear why these differences occurred. It is possible that brief exposure to bleomycin differs from radiation studies in that the drug may persist inside the cells for a time, even after repeated washing to remove it. The half-life of the chemical inside the cells is not known, but if it lasts for hours rather than minutes, then the drug could remain active for one or more cell cycles (the yeast cell cycle in cells grown on plates is ~ 2 hours).

In the studies by Bennett *et al.*, diploid mutants were screened and categorized as gamma sensitive if there was (a) reduced survival in dilution pronging assays and (b) a slow recovery from gamma-induced damage (causing very small colonies) even if the number of surviving colonies was similar to WT cells (26-27). In the Game *et al*. screenings, an unusual method was utilized which involved irradiating pools of diploid deletion library strains, purifying and amplifying DNA from the surviving cells, and identifying those mutants that had reduced abundance due to killing of the cells (28-29). The current study differed in that haploids were used instead of diploids. Also, mutants were only scored as sensitive if survival was reduced, i.e., the number of surviving colonies on pronged plates was reproducibly decreased by > 2-fold. Additionally, haploids and diploid mutants sometimes differ in mutagen sensitivities. Both *SRS2* and *RDH54* have been found to produce radiation sensitivity when inactivated in diploids but not in haploid cells (48-49). These factors may explain why so many more of the diploid strains were classified as gamma-sensitive in previous studies than the haploids in the current study.

Of the 73 non-RAD52 group mutants, ten were found to be sensitive to EcoRI, MMS, bleomycin, and gamma radiation, consistent with the phenotypes of RAD52 group

mutants. Because these mutants were likely to be the most critical for DNA DSB repair, they were analyzed for recombination proficiency. Five of the ten mutants had repair efficiencies near that of WT cells. Three mutants, *ado1, ubr1,* and *spt10*, exhibited modestly reduced levels of repair, which suggests that these mutants are most closely linked to the RAD52 epistasis group. Two other mutants, *rtf1* and *vma7*, displayed elevated levels of repair. Understanding why these mutants are unable to repair DSBs will require additional experiments that assess other types of recombinational repair. Future studies will involve testing these mutants for intrachromosomal recombination between DNA repeat sequences and recombination between homologous chromosomes.

Several mutants determined to be EcoRI-sensitive were tested for spontaneous cell-cycle arrest in G_2 phase to screen for phenotypes that are associated with high levels of unrepaired DNA damage. The combined work of the current study with the prior studies by J. Summers, S. Sethi, and J. DeMars found that all HR-deficient RAD52 group mutants tested had high spontaneous G_2 cell arrest during log phase growth. Sixteen of the new EcoRI-sensitive mutants also exhibited this phenotype, suggesting that elevated levels of spontaneous DNA damage were causing them to spend more time in G_2. Interestingly, ten of the sixteen mutants have previously been associated with processes affecting DNA or RNA metabolism in the nucleus. The functions include sister chromatid cohesion, mitotic nuclear migration, and nuclease processing of DNA, all of which are processes that may affect DNA repair efficiency. Additionally, cell cycle analysis experiments were performed on several NHEJ deficient mutants in both their *MATα* and *MATa* versions. Two of the mutants, *yku70* and *yku80,* exhibited higher levels of G_2/M cells than WT cells in both the *MATα* and *MATa* versions, suggesting either high

levels of DNA damage or checkpoint activation due to the shortened telomeres in these mutants. Other NHEJ repair mutants had G_2/M cell levels near that of WT.

In an effort to determine whether the high levels of G_2/M cells were truly due to a checkpoint response, cell cycle analysis experiments were performed using *rad52 mec3* and *rad52 rad17* double mutants. The double mutant constructs exhibited significantly reduced levels of G_2/M cells relative to the *rad52* mutant, therefore reinforcing the idea that high levels of G_2/M cells were due to activation of a DNA damage-inducible checkpoint response. Future studies investigating the phosphorylation of specific checkpoint proteins such as Rad53 may offer additional confirmation of these results.

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