USE OF A NOVEL GENOMICS APPROACH TO IDENTIFY NEW DNA REPAIR GENES AND DEVELOPMENT OF IMPROVED REPAIR ASSAYS

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Jennifer Ann DeMars, B.S.

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USE OF A NOVEL GENOMICS APPROACH TO IDENTIFY NEW DNA REPAIR GENES AND DEVELOPMENT OF IMPROVED REPAIR ASSAYS

COMMITTEE MEMBERS APPROVED:

_____
L. Kevin Lewis, Chair

_____
Steven T. Whitten

_____
Wendi M. David

Approved:

_____
J. Michael Willoughby
Dean of the Graduate College

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I would like to begin by thanking my wonderful thesis advisor Dr. Kevin Lewis. Without his support and guidance, I would not have achieved this and be where I am today. I would also like to thank my thesis committee for their advice and comments. My amazing, caring family made me who I am. I am very blessed to have my family and I thank God everyday for them. Kyle Tripp, my loving and supportive fiancé, I offer my thanks and love to you most of all.

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CHAPTER I

INTRODUCTION

Deoxyribonucleic acid (DNA) is a double helix formed by bases attached to a sugar-phosphate backbone. The paired bases are adenine with thymine and cytosine with guanine. There are hydrogen bonds between the base-pairs, which along with base-stacking interactions create a high affinity between the strands of DNA. The two strands of the double helix are anti-parallel, with one strand running in the 5'-3' direction and the other strand running in the 3'-5' direction. DNA contains the genetic instructions used in the development and functioning of all living organisms. Inside cells, double-helical DNA may exist as long linear polymers or as circular molecules.

The DNA within cells may be exposed to many exogenous DNA damaging agents. Ionizing radiation such as X-rays and gamma rays cause many types of DNA damage, including DNA strand breaks. Chemicals such as bleomycin and MMS (methyl methanesulfonate) and enzymes like endonucleases and exonucleases can cause breakage to the strands of DNA as well. There are two types of ionizing radiation actions, direct and indirect (1). Direct actions result from the absorbance of radiation energy by DNA, which leads to the ionization of bases or sugars. Indirect actions result when DNA interacts with reactive oxygen species (e.g., hydroxyl radicals, superoxide anion radicals, etc.) formed in water by the radiation. Seventy percent of the actions of ionizing

radiation are indirect actions, while only thirty percent are direct actions. The DNA damage from radiation can occur on either a single strand or on both strands. There can be strand breaks and other lesions such as base change or loss. Also cross-links between DNA and bound proteins can be induced. Double-strand breaks (DSBs) are considered the most difficult lesions to repair and are the most lethal to cells (2).

Bleomycin is a chemical used as an antitumor drug that causes DNA DSBs and other lesions (3). It binds oxygen and iron (II) to form a complex that cleaves DNA. The chemical chelates metal ions, producing a pseudo-enzyme that reacts with oxygen to produce superoxide and hydroxide free radicals that cleave DNA (4). Bleomycin is made up of a metal binding site, where iron is usually located, a glycol-peptide part, where a sugar is attached and sits in the minor groove, and an end that intercalates into DNA. Methyl methanesulfonate (MMS) is another chemical that causes DNA double-strand breaks. It is an alkylating agent that attaches methyl groups to DNA bases, primarily 3meA and 7meG (5-7). These lesions can stall replication forks and cause difficulty because DSBs can form at damaged forks.

DNA nucleases catalyze the hydrolysis of phosphodiester linkages, inducing nicks or breaks within the strands of DNA. Exonucleases catalyze progressive hydrolysis of phosphodiester linkages from the ends of DNA. Endonucleases typically cut in the interior of DNA strands to produce either ends containing single-stranded DNA overhangs or blunt ends. The consequences of DSBs inside the cell are variable. They may lead to accurate repair, where the cell remains viable, or no repair or inaccurate repair and the cell dies or mutations develop that may compromise cell function.

When there is a DSB in DNA, there are two pathways for repair, Nonhomologous End-Joining (NHEJ) and Homologous Recombination. The NHEJ pathway, shown schematically in Figure 1, directly rejoins the broken ends of the DNA. This pathway is considered to be more error-prone and involves three essential complexes: Yku70/Yku80, Mrx (Mre11/Rad50/Xrs2), and DNA ligase IV (Dnl4/Lif1/Nej1). It is the secondary pathway in *Saccharomyces cerevisiae* (budding yeast), but is the primary pathway in humans.

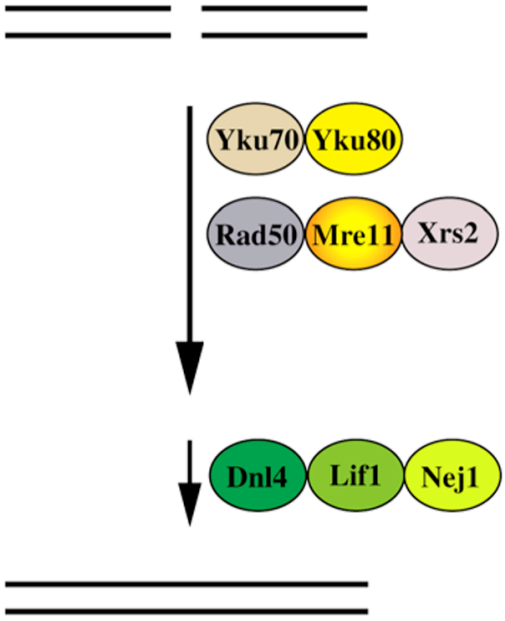

Figure 1. Illustration of the Nonhomologous End-Joining repair pathway.

Homologous recombination is a multi-step process that involves several proteins and homologous chromosomes. It requires substantial energy and is considered to be error free. This is the primary pathway in *Saccharomyces cerevisiae*. Homologous recombination involves initial resection of DSB ends by the Mrx complex (Mre11,

Rad50, Xrs2) that creates 3' tails or overhangs on both strands of DNA at break sites (Figure 2). Other proteins such as Rad51, Rad52, and Rad54 are involved in the following steps of homology search and strand exchange. These events are followed by double strand invasion with the formation of a Holliday junction followed by branch migration, and then finally the resolution of the joined DNAs where the final product is the repaired chromosome. Recent reports have suggested that the resolution step, which involves breakage of DNA strands in order to separate the DNA molecules, involves several enzymes including Mus8/Mms4, Yen1, Sgs1/Top3/Rmi1, and Slx1-Slx4 (9-11).

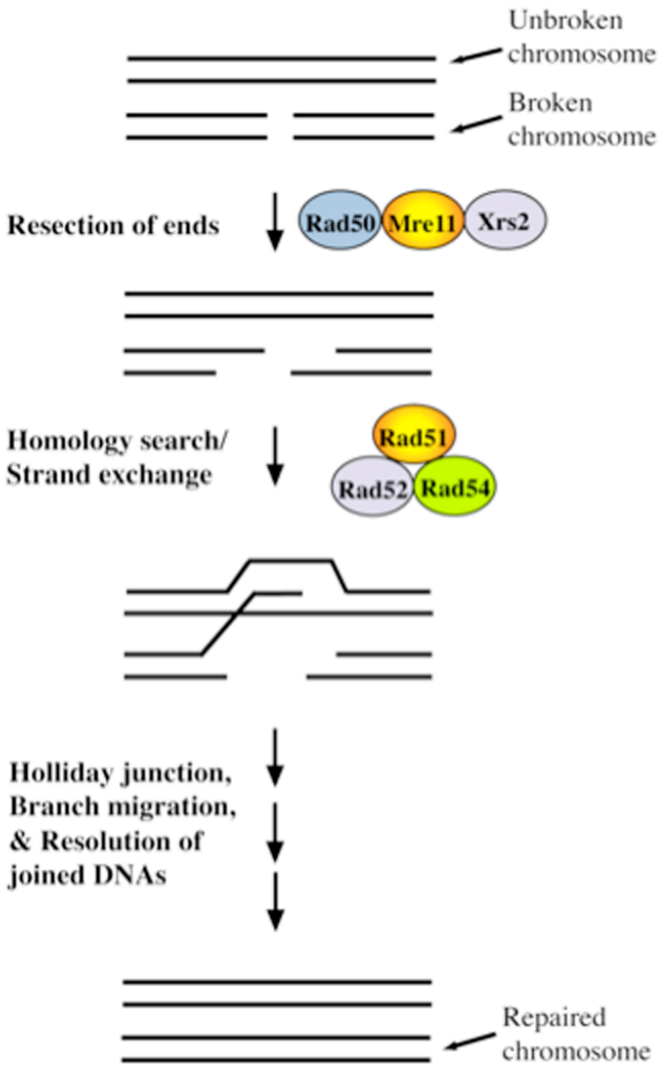

Figure 2. Illustration of the homologous recombination repair pathway.

There are other processes involved after the initial resection step. These include nucleosome remodeling (recruitment of the RSC complex by Mrx), DSB-induced cell cycle checkpoint responses which pause the cell cycle to allow more time for DNA repair, and DSB-induced cohesion of sister chromatids (recruitment of Eco1, the Smc complex, etc., by Mrx).

When genes that are involved in DSB repair by homologous recombination are inactivated cells become sensitive to *in vivo* expression of EcoRI. EcoRI is a bacterial restriction endonuclease consisting of two subunits that requires magnesium as a cofactor. It recognizes a specific sequence (GAATTC upper stand, CTTAAG lower strand) and then cleaves both strands of DNA to produce single-stranded overhangs (Figure 3).

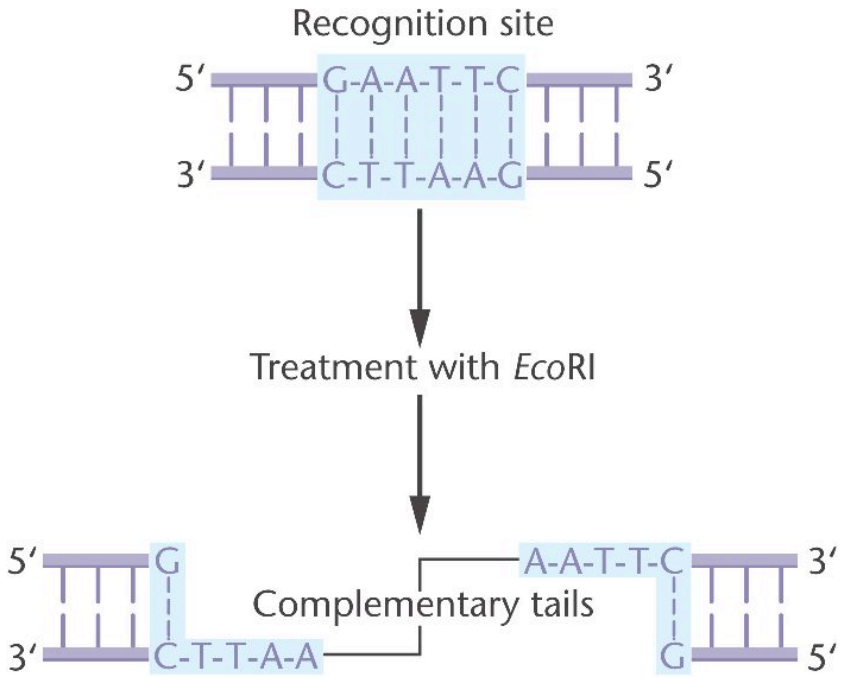

Figure 3. Schematic of EcoRI cleavage of double-stranded DNA (12).

Although it is a bacterial protein, it is possible to express EcoRI in eukaryotic cells. With EcoRI expressed inside the cell there are multiple DSBs produced in each chromosome. The advantage of the approach of using EcoRI to study genes and the roles

of genes involved in the repair of DSBs in DNA is that, unlike chemicals or radiation, only DSBs are generated in the DNA.

In normal cells, EcoRI expression is not lethal because both DSB repair pathways are active, but EcoRI is lethal in many DSB repair mutant cells. In a previous study conducted by Jennifer Summers in the Lewis lab, wildtype cells and four yeast mutants (*rad50, rad51, rad52, rad54)* were tested with EcoRI induced to measure sensitivity (13). Wildtype cells showed no killing with the expression of EcoRI, but all the yeast mutants showed killing. This result is consistent with previous studies showing that these mutants are hypersensitive to ionizing radiation and the strand-breaking chemicals MMS and bleomycin (5).

Yeast deletion strain libraries allow the testing of many mutants at once for a particular phenotype. Available libraries contain approximately five thousand yeast mutants (14, 15). Each of these mutants has one non-essential gene inactivated. In each strain, the coding sequence of the inactivated gene was deleted and replaced with a gene encoding a protein that makes cells resistant to the antibotic G418 (14). The mutants are stored in glycerol in ninety-six well microtiter dishes in a -80 ^{o}C degree freezer.

Promoters used for expression of proteins inside cells can either be constitutive or regulated. *GAL1* and *GAL10* are promoters that are tightly regulated by the galactose-glucose system and have been widely used for heterologous gene expression in yeast cells (5, 16). The *GAL1* promoter, a galactose-regulatable promoter in yeast cells, has previously been used to express EcoRI (5, 16). The expression of EcoRI is turned on in the presence of galactose and repressed, or turned off, in the presence of glucose.

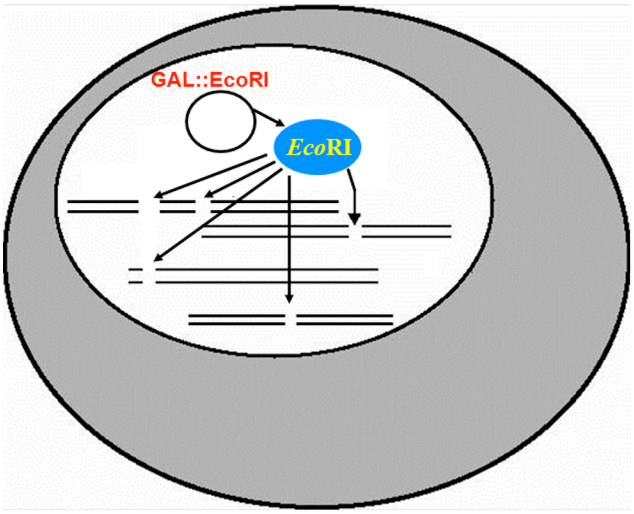

Figure 4. Use of a *GAL1* promoter to express EcoRI in yeast cells.

In the presence of the *GAL1* promoter inside the cell, EcoRI produces DSBs at its target sites in chromosomal DNA (Figure 4).

Two previous studies screened over four thousand seven hundred diploid yeast mutants for sensitivity to gamma radiation (14-18). The first study by Bennett *et al.* identified 190 non-RAD52 group genes as essential for normal resistance to radiation (14, 17). The second study identified 33 genes that caused increased sensitivity to ionizing radiation when they were inactivated in diploid cells (15, 18). Twenty-two of these genes were also identified by Bennett *et al.*, but 11 genes were not detected in the earlier study. The 190 genes identified in the first study plus the 11 new genes found in the second resulted in 201 non-RAD52 group genes total. In the Lewis lab, previous graduate students Jennifer Summers and Sunaina Sethi tested the abilities of the 190 mutants identified by Bennett *et al.* to repair DSBs induced by *in vivo* expression of EcoRI (13, 20). These experiments were performed using mutant strains from a *MAT*α haploid deletion strain library and were performed to identify which of the gamma-

sensitive mutants were specifically deficient in DSB repair. Sixty-one of the mutants displayed enhanced killing when EcoRI was expressed. Most of these mutants were subsequently found to also be sensitive to MMS and bleomycin. The results suggested that these seventy-six genes are important for double-strand break repair.

Many studies, like the EcoRI assays described above, require the transformation of yeast cells with plasmid DNAs. Transformation can be performed in stationary phase cells as well as in log phase cells. There are several common protocols that have been described for transformations and they all require most of the following additives. The first is polyethylene glycol (PEG), which is known to promote association of the DNA and the cells. Lithium acetate (LiAc) is another additive that disrupts cell membranes to allow the DNA inside the cell. Dithiothreitol (DTT) is a reducing agent that is frequently used to break disulfide bridges in cell surface proteins. This property of DTT shows great promise for allowing the transfer of plasmid DNA into cells, making it an easier task and one which will be used in depth in this project (21-23).

The primary goal of the current project was to identify and characterize new genes involved in double-strand break repair by expanding upon the earlier studies that employed EcoRI expression inside yeast cells. The new project has employed both MATα and MATa haploid cell libraries (the two mating types in which yeast cells exist) to investigate the relative sensitivities of additional mutants to EcoRI endonuclease, ionizing radiation, and the DNA strand breaking chemicals MMS and bleomycin, allowing classification of the mutants into specific groups. The combined results of this work and that of the earlier students have led to the identification of a total of 73 new non-RAD52 group genes that are required for efficient repair of DSBs. Furthermore,

several additional experiments were done that have led to development of an improved protocol for transfer of DNA molecules into yeast cells. These latter experiments were important because they have improved the lab's capability to perform several types of DSB repair assays with the new mutants that require transformation of cells with plasmids.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Dimethyl sulfoxide (DMSO), ampicillin, RNase A, methylmethane sulfonate (MMS), and potassium chloride were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Ethidium bromide (EtBr) was obtained from Shelton Scientific, Incorporated (Shelton, CT). Ethylenediaminetetraacetic acid (EDTA), agarose and bleomycin were obtained from EMD Chemicals, Inc. (Darmstadt, Germany). A standard 1 Kb DNA ladder were purchased from New England Biolabs (Beverly, MA). Lithium acetate, magnesium chloride, Hoechst 33258, glutamic acid (monopotassium salt) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Sodium dodecyl sulfate (SDS), 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). Sigma-Aldrich (St. Louis, Missouri) was where potassium chloride (KCl) and rubidium chloride (RBCl) were purchased. Dithiothreitol (DTT) was purchased from either Sigma-Aldrich or Gold Biochemistry (St. Louis, Missouri).

Yeast and bacteriological media

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

Yeast strains and plasmids

BY4742 was used as a wild type strain and has a genotype of *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (24). Several other yeast strain backgrounds were used: BWG1-7a, BY4741, S1, SK1, T334, and YPH102 (25, 26). BWGI-7a has a genotype of *MATa ura3-52 leu2-3,112 his4-519 ade1-100* (27). BY4741 has a genotype of *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (24). S1 has a genotype *MATα ura3-52 leu2-3,112 trp1-289 his7-2 ade5-1 lys::lnsE-4A* (25, 28). SK-1 has a genotype of *MATa ura3-52 leu2- Δ1 Δtrp1::hisG his1-7 lys2* (25, 29). The genotype for T334 is *MATα ura3-52 leu2-3,112 Δtrp1::hisG reg1-501* (25, 30). YPH102 has a genotype of *MATα ura3-52 lys2-801 ade2-101 leu2-Δ1 his3-Δ200* (26). The yeast deletion strain libraries were obtained from Open Biosystems (Huntsville, Al). Plasmids that were used were pGALEcoRI (YCpGalRIb) (*CEN/ARS URA3 GALp::EcoRI*) and pRS316 (*CEN/ARS URA3*) (13).

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. Glucose complete plates were also used. Plates with bleomycin were prepared using synthetic or YPDA media supplemented with aliquots of a stock solution of 0.5 mg/ml bleomycin to achieve varying final concentrations. Plates with MMS were also prepared using synthetic media with aliquots of a stock solution of 11.8 M MMS to prepare two different final concentrations (1 mM and 2 mM). *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

DNA purification

The purification of plasmid DNA was achieved using an alkaline lysis protocol (31).

Yeast transformations

Routine transformation of yeast cells was performed using the lithium acetate-based high efficiency protocol of Gietz *et al.* (22). Routine transformations of stationary phase yeast cells were performed using the rapid DMSO-based transformation protocol of Soni *et al.* (21).

Pre-treatment of cells with dithiothreitol (DTT) for yeast transformations

Typically, ~ 700 ng of plasmid DNA was used for transformations involving either pretreatment with DTT or inclusion of DTT in the PEG/Li mix. Highest efficiencies achieved using DTT in these studies were > 50,000 transformants per μg. Cells from overnight cultures grown in YPDA broth (1.5 mL) were centrifuged at 16,000 x g for 15-30 seconds, the supernatant was removed, and 500 μL of 0.1 M dithiothreitol (DTT) was added. After mixing to re-suspend cells, they were incubated at 42 ^{o}C for 20 minutes before proceeding onto the rapid DMSO-based transformation protocol of Soni *et al.* (21).

The modified Soni *et al.* protocol used here was as follows:

1. Spin ~ 1 x 10^8 cells for 0.5 min in a microcentrifuge and pull off the supernatant.
   (Typically use 1 ml of overnight liquid culture; can be 150 μl of more concentrated culture)
2. Add 500 μL of 0.1 M DTT, re-suspend and incubate at 42 ^{o}C for 20 min. Spin down cells for 0.5 min in a microcentrifuge and pull off the supernatant.
3. Add 5 μL of 10 mg/ml carrier DNA (sonicated [=sheared] salmon sperm DNA) + 1-10 μL of plasmid DNA (~ 200-300 ng)
4. Vortex briefly and add 500 μL PEG/LiAc solution
   - To make PEG+LiAc+Tris+EDTA, mix 800 μL 50% PEG + 100 μL 1 M LiAc + 20 μL 50 mM EDTA + 10 μL 1 M Tris (pH 7.5) + 70 μL H_2O. Make more than is needed.
   - Note: if transforming several tubes, open all their lids first and add 500 μL to all the tubes using one P-1000 tip and without touching the recipient tube (get a new tip if tube is touched accidentally). Leave the lids open and go to step 4 and add the DMSO with a P-200 without having to change the tip.

5. Add 1/10th volume of DMSO (usually 56 μL) and either scrape tube on bottom of a metal test tube rack or vortex tube to mix.
6. Incubate at RT for 15 min (30 ^{o}C has also been used but unknown if it's better)
7. Transfer to a 42 ^{o}C waterbath for 15 min (can microwave water in a beaker if needed)
8. Spin 0.5 min and remove supernatant
9. Re-suspend cells in 200 μL H_2O, scrape on test tube rack to mix well, and spread 10 ul and 100 μL onto selective plates. When spreading less than 50 μL, spot 50-100 μL of H_2O in the center of the plate and pipette the cells directly into the liquid before spreading the plate.

(Note: Soni et al. re-suspended in TE, mixed, spun, pulled off the TE, and then re-suspended in H_2O before spreading, but this is not normally done in the Lewis lab)

10. Incubate the plates at 30 ^{o}C for ~ 3 days or at RT for 4-5 days.

Other stationary phase protocols that were used for transformation of yeast cells

I. The quick and easy transformation protocol by Gietz *et al.* (22) is as follows:

1. Pellet 1.5 mL of cells in a microcentifuge for 30 s and discard the supernantant.
2. Add to the pellet 240 μL PEG 3350 (50 % w/v), 36 μL LiAc (1.0 M), 50 μL single-stranded carrier DNA (2.0 mg mL^{-1}) and 34 μL of sterile water plus and plasmid DNA (up to 1 μL), in the order given, and mix the pellet by vortex mixing briskly until resuspended. If performing multiple transformations, the transformation mix can be premixed and added as a single 360 μL aliquot to the cell pellet. The cell pellets are resuspended by vortex mixing.

3. Incubate the tube in a water bath at 42 ^{o}C for 20-180 min. Many yeast strains will produce more transformants if the incubation time is increased beyond 20 min; however, this is strain-specific and should be tested.

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

5. Pipette 1.0 mL of sterile water into the transformation tube. Stir the pellet with a sterile micropipette tip to break up the cell pellet and vortex mix thoroughly resuspend. Alternatively, the pellet can be resuspended in smaller volumes such as 400 μL of sterile water.

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

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

II. The one-step transformation of yeast in stationary phase protocol by Chen *et al.* (23) was used and is as follows:

1. Spin down yeast cells in stationary phase (density up to 2.5 x 10^{8} cells/mL) from YPDA broth (1 % yeast extract, 2 % bactopeptone, 2 % dextrose) into a tube.

2. Vortex, with ONE-STEP buffer [lithium acetate, 0.2 N; polyethylene glycol (PEG) 3350, 40 %; pH 5.0; dithiothreitol (DTT), 100 mM], 50 ng-1 μg of plasmid DNA and 50 μg of single-stranded carrier DNA in a total volume of 100 μL with a density of 5 x 10^{8} cells/mL.

3. After incubation at 45 ^{o}C for 30 min, suspensions are then plated directly onto selective medium and incubated at 30 ^{o}C for 3-4 days.

Pre-treatment of cells with various salts for yeast transformations

Yeast cells (1.5 mL of overnight cultures in YPDA broth) were pelleted and re-suspended in 500 μL of each 0.1 M salt. Lithium acetate, potassium chloride, and rubidium chloride were the three salts that were tested. The pre-treatments were conducted for five minutes at room temperature or at 42 ^{o}C. After the five minutes of pre-treatment, the rapid DMSO-based transformation protocol of Soni *et al.* (21) was completed.

Gel electrophoresis

Gel electrophoresis was performed using 0.7% agarose gels in 1X TBE (90 mM Tris-borate, 2 mM EDTA) running buffer in a Life Technologies Horizon 10-14 gel rig. The gels were run using a voltage of 145 V and were stained with EtBr for 15 minutes. A Kodak IS440 CF Image Station instrument and Carestream imaging software were used to capture gel images.

Gamma irradiation of yeast cells

Arrays of yeast cells were irradiated using the ^{137}Cs source at the National Institute of Environmental Health Sciences (NIEHS) as part of a collaboration with Michael Resnick and James Westmoreland. Log phase yeast cells were diluted five-fold and pronged onto YPDA plates where they were irradiated at different doses (0, 30, and 60 krads).

Replica-plating

Replica-plating was used for colony and patch transfers. This involved making an imprint of the colonies or patches cultured on a solid nutrient surface, using a velvet-cloth-covered cylinder, and transferring the imprint to one or more fresh plates for subsequent growth. Prior to replica-plating, a locking ring was used to help secure the sterile velvet-cloth onto the cylinder.

Double imprint replica-plating

To test EcoRI sensitivity, mutants and WT cells containing plasmids were patched onto 2% Glu-Ura synthetic plates, grown for 2-3 days at 30 ^{o}C and replica-plated onto 1% Raff-Ura, 1% Raff + 2% Gal-Ura, and 2% Gal-Ura synthetic plates. The 1% Raff-Ura plate was immediately used as a new master plate to replica-plate cells to other plates. The double imprints were grown at 30 ^{o}C for 2-3 days. WT cells were resistant to killing by EcoRI, but several mutants showed moderate to strong sensitivity when grown on media that turned on expression of EcoRI.

Dilution pronging survival assays

All mutants that were found to be sensitive to EcoRI endonuclease using replica-plating were then further quantitatively tested using survival pronging assays. Both mutants containing pGALEcoRI and mutants containing the control vector pRS316 were initially cultivated on 1% Raff-Ura plates by allowing growth at 30 ^{o}C for 3 days or at RT for 4 days. Then the cells were 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 then loaded onto a 0.1 mm deep Reichert Bright-Line hemacytometer (Buffalo, NY) and counted using a Lomo HT-30.01 microscope (St. Petersburg, Russia).

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. Finally, 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.

A similar procedure was implemented to test for MMS and bleomycin sensitivity. The EcoRI sensitive mutants and WT cells were initially streaked onto YPDA plate media. Then they were patched to glucose complete synthetic plates and grown for 2-3 days at 30 ^oC. The cells were then harvested for the pronging assays. Once the cells were diluted, sonicated, counted, and properly placed into the microtiter dish, they were pronged onto synthetic glucose complete plates with varying concentrations of MMS or bleomycin. Three to four days later, cell survival was assessed and each mutant's level of sensitivity was evaluated. Gamma radiation sensitivity assays were also completed with this protocol.

Analysis of homologies between yeast proteins and human, rat and mouse proteins

Protein sequences from each of the genes conferring EcoRI-sensitivity when mutated were analyzed against human, mouse, and rat proteins utilizing the *Saccharomyces* Genome Database and the Basic Local Alignment Search Tool (BLAST) application at the National Center for Biotechnology Information. Each protein sequence was initially retrieved from the *Saccharomyces* Genome Database. That sequence was then copied into the BLAST search engine, where it was used in searches against each of the three genomes, human (*homo sapiens*), mouse (*mus*), and rat (*rattus*).

CHAPTER III

RESULTS AND DISCUSSION

This project's major goal was to complete earlier studies done in the Lewis lab that led to the identification of new yeast genes involved in DNA DSB repair. Bennett *et al.* tested 4,746 diploid yeast mutants for sensitivity to gamma radiation (14, 17) identifying several RAD52 group mutants (*rad50, rad51, rad52,* etc.) plus one hundred and ninety other genes required for normal resistance to ionizing radiation in diploids. Graduate students Jennifer Summers and Sunaina Sethi tested all 190 non-RAD52 group genes conferring gamma sensitivity as diploids to see their effects on DSB repair in haploid cells. This was accomplished by transforming each haploid mutant cell with a plasmid containing a *GAL1p::EcoRI* promoter fusion and testing survival after galactose induction of EcoRI expression inside the cells. Unlike gamma radiation, EcoRI only generates DSBs in DNA and therefore is a more specific assay for mutants unable to repair DSBs. As controls they also tested seven of the nine known RAD52 group mutants, which include *rad50, rad51, rad52, rad54, rad55, rad57, rad59*, *mre11*, and *xrs2*. All of these control mutants were tested in their screens except *mre11* and *xrs2*.

J. Summers and S. Sethi tested the EcoRI sensitivities ($EcoRI^s$) of the mutants using a haploid *MATα* deletion strain library constructed in strain BY4742 (13, 20). They identified 61 genes that confer $EcoRI^s$ when inactivated in haploid *MATα* cells.

These genes are listed in Table 1. Gene names containing a forward slash refer to strains in which the inactivating gene mutation potentially affected more than one gene. In each mutant in the library, the coding region of a gene was deleted and replaced with a new gene encoding resistance to the antibiotic G418 (14). At some chromosomal loci, the deletion removed the coding region of one gene plus a portion of the coding region of another putative gene that overlaps the first gene. This phenomenon will be explained diagrammatically later in the chapter.

The first goal of the current project was to re-test each of these 61 genes using a different mutant library that was prepared using *MATa* haploid cells rather than *MATα* cells. Yeast cells can exist in three forms: diploids, haploid *MATα* cells and haploid *MATa* cells. Deletion mutant libraries have been constructed in all three types of cells. It is possible that some of the 61 *MATα* mutants found to be EcoRI^s are actually sensitive because of an uncharacterized secondary mutation in another gene. Re-testing all 61 genes that affected DSB repair in the original *MATα* library strains using a separate *MATa* strain library permits confirmation of the original findings and also serves as a test of the quality of the libraries. If a large number of the 61 mutants fail to demonstrate EcoRI sensitivity using the second library, then this will suggest that secondary mutations are abundant. If almost all of the 61 mutants reproduce the EcoRI^s phenotype, then this will indicate that library quality is high.

Table 1. All 61 genes producing sensitivity to EcoRI in MATα library strains[a]

adk1	*htl1*	*sam37*
ado1	*ids2*	*sfp1*
akr1	*img2*	*slm4*
apq13/net1[b]	*lip5*	*spt10*
atp2	*lrp1*	*spt20*
bck1	*lsm7*	*srv2*
bud19/rpl39[b]	*mct1*	*taf14*
bur2	*mms2*	*trm9*
cax4	*mms22*	*tsr2/ylr434c*[b]
cgi121	*mms4/ybr099c*[b]	*ubr1*
cis3	*mrps35*	*ume6*
cnm67	*not5*	*vma7*
ctf4	*nup84*	*vph2/ykl118w*[b]
ctf8	*och1*	*ybr099c/mms4*[b]
dcc1	*rad5*	*ydr417c/rpl12b*[b]
ddc1	*rem50*	*ydr433-w/npl3*[b]
eaf1/opi7[b]	*rpb9*	*ylr235c/top3*[b]
exo1	*rpl31a*	*yml009w-b/spt5*[b]
gcn5	*rvs161*	*yml012c-a/ubx2*[b]
gnd1/yhr182c-a[b]	*sae2*	*ynr068c*
hsp150		

[a] From Jennifer Summers and Sunaina Sethi (13, 20).
[b] Gene names separated by a forward slash indicate deletions affecting two overlapping open reading frames. The coding region of the first gene listed was precisely deleted in these strains.

In the experiments by Summer and Sethi, the students were unable to test all of the 190 *MATα* library mutants because a few did not grow well enough on galactose or raffinose plates to be tested using the *GAL1p::EcoRI* system. In addition, some mutants could not be tested because of some other problem. For example, the *cdc40* mutant was found to be $URA3^{+}$, though it should have been $ura3^{-}$, and therefore it could not be transformed with the pGALEcoRI (*URA3)* plasmid used for the EcoRI sensitivity assays. These *MATα* deletion library strains and the reasons that they could not be tested are listed in Table 2.

Table 2a. Mutants not tested in previous screens for EcoRI sensitivity because of growth problems of the *MATα* library strains

ada2	*mdm10*
ade12[a]	*mdm20*
arp5	*pre9*
asm4	*rsm7/yjr114w*[e]
bik1	*rtf1*
bud30/rpc53[e]	*rvs167*[d]
bud32	*sac6*
ccr4	*sco1*
cdc40[b]	*xrs2*[d]
clc1	*mtc7*
cwh36/vma9[c,e]	*ygl218w/mdm34*[e]
gon7	
hfi1	

[a] *MATα ade12* mutants did not produce Ura$^+$ colonies after repeated transformations with the plasmid pGALEcoRI (YCpGAL::RIb).
[b] *MATα cdc40* cells were already *URA3*$^+$ and could not be transformed with pGALEcoRI.
[c] *cwh36* is not a gene. It is known to be a partial deletion of *VMA9*.
[d] These gene mutants were not present in the haploid *MATα* library collection. All other mutants in the table were not tested due to poor growth on raffinose and/or galactose media.
[e] Gene names separated by a forward slash indicate deletions within two overlapping open reading frames. The coding region of the first gene listed was precisely deleted in these strains.

Summers and Sethi attempted to test all genes producing gamma-sensitivity in diploids when inactivated that were identified by Bennett *et al.* (14, 17), but they did not analyze the genes identified in another genome screen published later by Game and Brown (15). In this later screen a total of 33 diploid mutants were identified that were gamma^s. Interestingly, 22 of the 33 mutants were also identified as gamma^s by Bennett *et al*., but 11 of the genes were not detected by Bennett. Thus, the total number of new gamma^s mutants identified in both projects was 190 (Bennett) + 11 (Game and Brown), or 201 total (not including the 9 RAD52 group mutants). The 11 new mutants identified by Game *et al.* but not analyzed by Summers and Sethi are listed in Table 2b.

Table 2b[a]. Gamma-sensitive mutants identified by Game *et al*. (15) that were not previously tested for EcoRI[s] in the Lewis lab

gpx2	*rpl20a*
idp1	*she1*
irc4	*tda5*
nab6	*thr1*
psy1/ykl075c[b]	*ubp8*
rkm1	

[a] Game *et al*. (15) identified 33 mutants in a diploid deletion strain library that were gamma radiation-sensitive. Twenty-two of the 33 mutants were also identified in the two Bennett studies (14, 17), but the 11 genes shown in the table were not detected by Bennett *et al*. and were therefore added to the current study.
[b] Gene names separated by a forward slash indicate deletions within two overlapping open reading frames. The coding region of *psy1* was deleted in the strain.

The second major goal of the current project was to test the mutants in Table 2a and 2b for EcoRI sensitivity to complete the screen of previously identified genes required for radiation resistance. For the mutants in Table 2a that exhibited poor growth as *MATα* cells, this was accomplished by obtaining a new *MATa* haploid library and attempting to test these mutants for EcoRI sensitivity. The logic here is that library strains sometimes have secondary mutations in other genes that cause them to have growth problems, but these secondary mutations are unlikely to be the same in equivalent mutants within a different library.

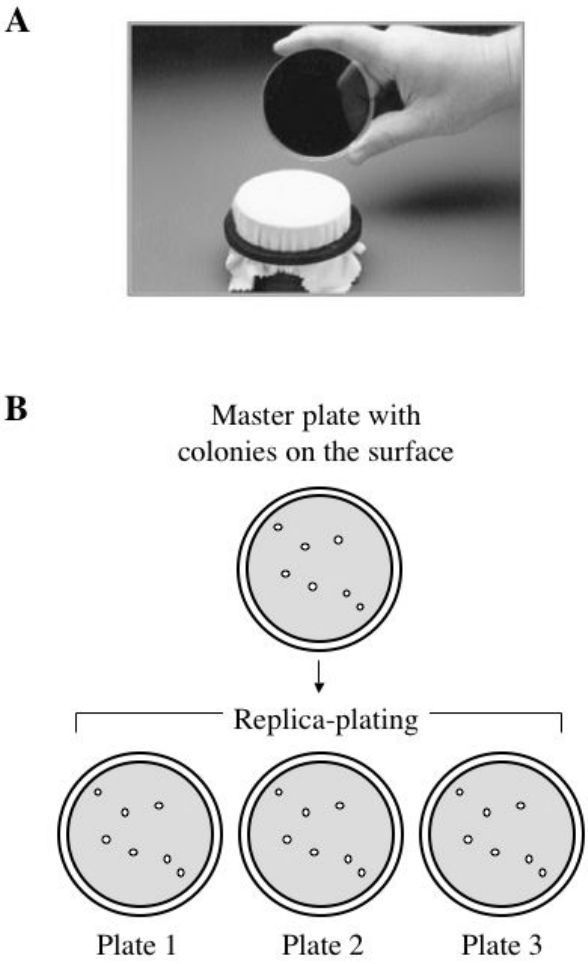

Figure 5a. Schematic representation of a replica-plating assay (13). A master plate with the colonies on it is pressed onto a velvet material. Different plates are then pressed on top of the velvet. The orientation of the colonies/patches is reproduced on the new various plates.

One of the first assays used in this project to test EcoRI sensitivity is called replica-plating (Figure 5a). This assay was accomplished by taking plasmid-containing cells grown on agar media and pressing the colonies or patches onto a velvet material. An imprint of the orientation of the colonies/patches from the plate was left on the

material. Other plates were then placed onto the material so that the same orientation of cells would appear on them. Agar with different sugars was used to inhibit or induce expression of EcoRI. Once the colonies or patches grew at 30 ^{o}C for about three days, the amount of resistance was categorized relative to wildtype cells. An example of this assay is shown below in Figure 5b. The transformants in this example were streaked in patch formation to show definitive results of the resistance or sensitivity to EcoRI. Two mutants, *bur2* and *mct1*, were shown to be sensitive in the assay.

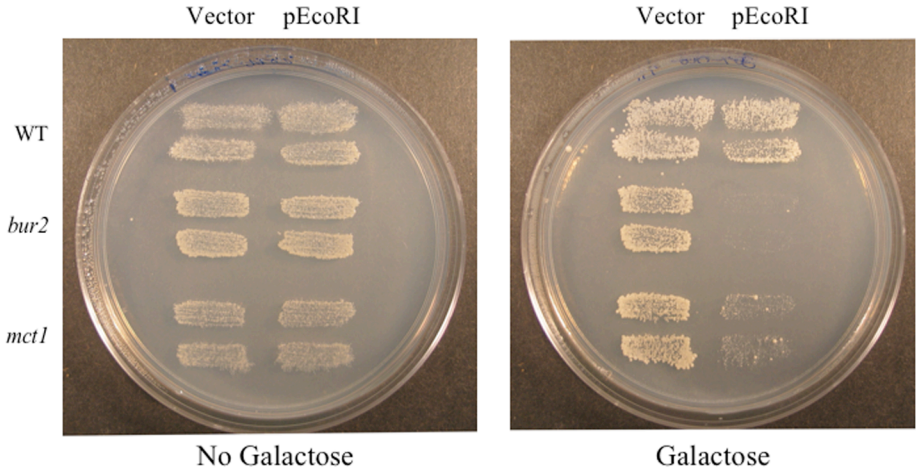

Figure 5b. Replica-plates with EcoRI expression being induced with galactose. Patches of cells containing either the vector pRS316 or pGALEcoRI were replica-plated to glucose and galactose plates.

Pronging was another assay used for more quantitative results showing sensitivity to EcoRI. Figure 6a shows the main points of how cells were pronged onto different plate media. Transformed cells were first diluted, sonicated and then loaded onto hemocytometers, where they were counted using a phase contrast microscope. After calculations, 1-2 x 10^{7} cells were loaded into a microtiter dish well. These cells were then serially diluted five fold to where a metal pronger would be placed in these wells.

The pronger was then placed onto the plate surface leaving an imprint of cells in droplets from the pronger. The cells grew at 30 ^{o}C for two to three days, when they were evaluated for sensitivity to EcoRI relative to wildtype cells. Mutants were classified as resistant to EcoRI (R), moderately sensitive (S), or strongly sensitive (SS). Moderately sensitive mutants exhibited < 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).

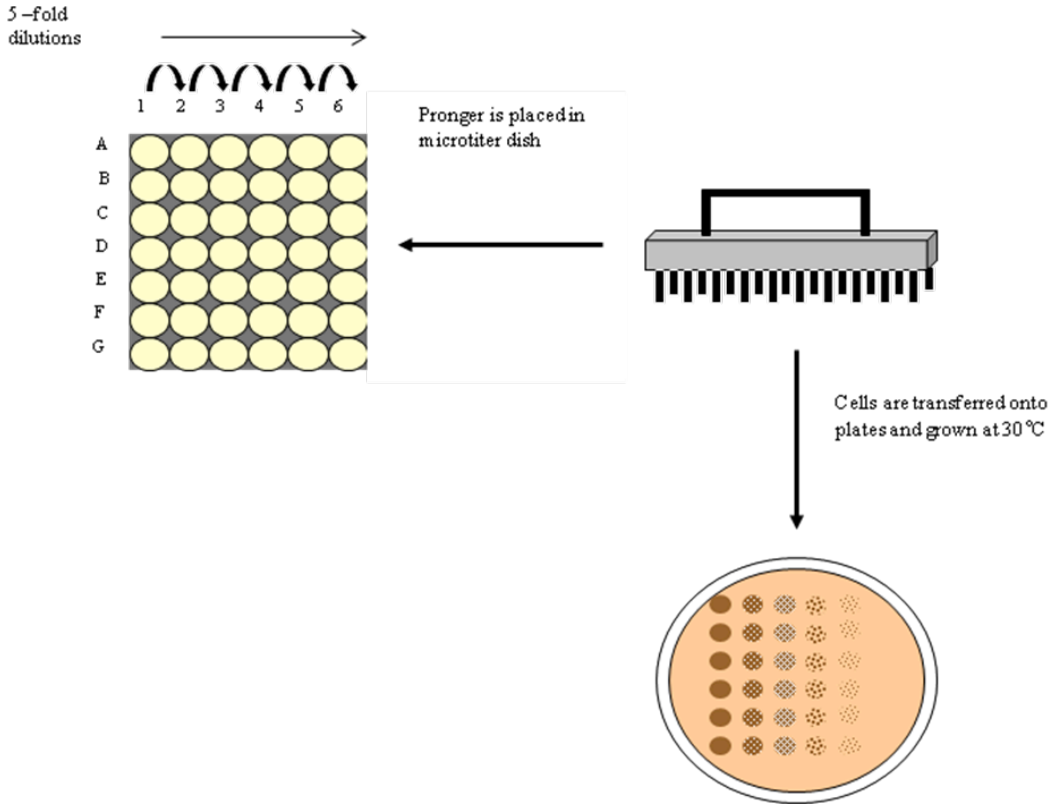

Figure 6a. Pronging assays using 96 well microtiter dishes and petri dishes (13). A metal pronger is placed in a 96 well micotiter dish where it is then pressed onto the surface of an agar plate.

Figure 6b is an example of a pronging series using three different types of sugar media to regulate EcoRI. The GAL1p::EcoRI fusion is fully induced in galactose media and is expressed at intermediate levels in Raff + Gal media. Every two rows there is a mutant transformed with vector and a mutant transformed with EcoRI. *rad52,* a control RAD52 group mutant, and *sae2* are the two mutants that displayed sensitivity when EcoRI was expressed on galactose. Rows five and six indicated that *rad5* mutants show wildtype resistance to EcoRI.

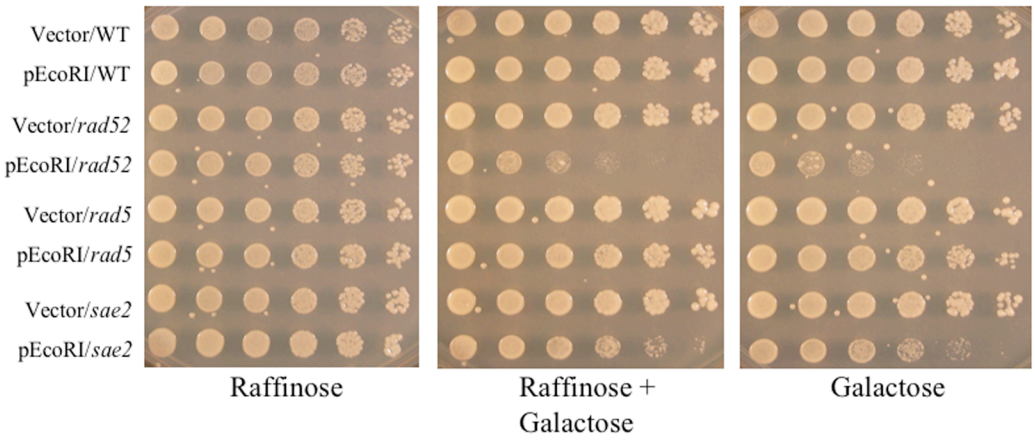

Figure 6b. Pronging of wildtype cells and several mutants containing a control vector (pRS316) or the GAL1p::EcoRI plasmid.

Table 3 shows the results of testing survival in each of the 61 *MATa* equivalents of the mutants that originally produced sensitivity to EcoRI in the *MATα* deletion library. Interestingly, *rad5* and *spt20* mutants displayed wildtype EcoRI resistance, which was not the case when cells with these genes inactivated were tested in the *MATα* library. Survival in seven of the 61 mutants was not determined (N/D) due to the *MATa* library strains not being able to grow on galactose media. Therefore, they could not be tested by our *GALIp::EcoRI*-based assay. Since the equivalent mutants in the *MATα* library were

able to grow using galactose as carbon source, the results suggest that the *MATa* versions may have secondary mutations. The other 52 mutants were confirmed as producing sensitivity to EcoRI in both libraries (Table 3).

Table 3. Results of testing *MATa* versions of the 61 mutants that produced sensitivity to EcoRI in the original *MATα* library screen in the Lewis lab

Mutant	EcoRI$^{S/R}$	Mutant	EcoRI$^{S/R}$
adk1	S	*mrps35*[b]	N/D
ado1	S	*not5*	SS
akr1	S	*nup84*	SS
apq13/net1[a]	S	*och1*[b]	N/D
atp2	S	*rad5*	R
bck1	S	*rem50*	S
bud19/rpl39[a]	SS	*rpb9*	SS
bur2	SS	*rpl31a*	S
cax4	SS	*rvs161*	SS
cgi121	SS	*sae2*	S
cis3	S	*sam37*	SS
cnm67	S	*sfp1*	S
ctf4	SS	*slm4*	S
ctf8	S	*spt10*	S
dcc1	SS	*spt20*	R
ddc1	S	*srv2*	S
eaf1/opi7[a,b]	N/D	*taf14*	S
exo1	S	*trm9*	SS
gcn5	S	*tsr2/ylr434c*[a]	S
gnd1/yhr182c-a[a]	SS	*ubr1*[b]	N/D
hsp150	S	*ume6*	S
htl1	SS	*vma7*	S
ids2	SS	*vph2/ykl118w*[a]	S
img2[b]	N/D	*ybr099c/mms4*[a]	S
lip5	S	*ydr417c/rpl12b*[a,b]	N/D
lrp1	S	*ydr433-w/npl3*[a,b]	N/D
lsm7	SS	*ylr235c/top3*[a]	S
mct1	SS	*yml009w-b/spt5*[a]	S
mms2	S	*yml012c-a/ubx2*[a]	S
mms22	SS	*ynr068c*	S
mms4/ybr099c[a]	SS		

[a] Gene names separated by a forward slash indicate deletions within two overlapping open reading frames. The coding region of the first gene listed was deleted in these strains.
[b] N/D, the *MATa* library mutant was unable to grow on galactose plates and therefore could not be tested.

The twenty-four *MATa* mutants that grew too poorly or had other problems preventing them from being tested in the previous studies by students J. Summers and S. Sethi using *MATα* library mutants were analyzed next. Interestingly, all of the *MATa* versions of these mutants were able to grow on galactose-complete plates (not shown). However, two mutants had characteristics that prevented them from being tested for EcoRI sensitivity by our assay. *ade12* cells in both libraries could not be transformed with the *CEN/ARS* plasmid used for our assay, i.e, Ura^+ colonies were never produced, even after repeated attempts at DNA transformations. *hfi1* cells could not be tested because the *MATa* library strain did not grow on YPDA (glucose) media. Eleven of the 22 mutants that could be tested were found to be EcoRI-sensitive (Table 4). One mutant listed in the table, *xrs2*, was not present in the original *MATα* library, but is a well known member of the RAD52 group and was expected to be EcoRI sensitive.

Table 4. Results obtained after testing *MATa* versions of the *MATα* mutants from Table 2a that could not be tested previously[a]

Mutant	EcoR1$^{S/R}$
ada2	R
arp5	**S**
asm4	R
bik1	**S**
bud30/rpc53[e]	**SS**
bud32	**S**
cdc40	**SS**
ccr4	**S**
clc1	R
gon7	R
mdm10	R
mdm20	R
myo4	R
pre9	R
rsm7/yjr114w[e]	**S**
rtf1	**SS**
rvs167	R
sac6	R
sco1	**SS**
xrs2[d]	**SS**
mtc7	R
ygl218w/mdm34[e]	**SS**
ade12[b]	N/A
hfi1[c]	N/A

[a] R, cells exhibited wildtype resistance to EcoRI exposure; S, cells were moderately EcoRI^s (survival was reduced by < 25 fold in dilution pronging survival assays); SS, strongly EcoRI^s (≥ 25 fold reduction in survival using pronging assays).
[b] *ade12* strains from neither *MATα* nor *MATa* libraries could be transformed with the *CEN/ARS* plasmids used in this study.
[c] *hfi1* could not be tested because the *MATa* strain did not grow on YPDA (glucose) plates.
[d] *xrs2* is an established member of the RAD52 group of recombination genes known to be EcoRI^s but was not present in the original *MATα* library (32).
[e] Gene names separated by a forward slash indicate deletions affecting two overlapping open reading frames. The coding regions of *BUD30, RSM7,* and *YGL218W* were deleted in these strains.

The 11 new mutants from Game's gamma sensitivity screen were tested using the *MATα* library in the same way that J. Summers and S. Sethi did using this library. The mutants were examined as haploid strains for sensitivity to expression of EcoRI. Two of the deletion mutants, *psy1* and *ubp8*, exhibited modest sensitivity to EcoRI (Table 5).

Table 5. Two of the 11 mutants identified by Game *et al*., but not detected by Bennett *et al.*, were found to be EcoRI[s] as haploid *MATα* cells[a]

Mutant	EcoR1[S/R]
gpx2	R
idp1	R
irc4	R
nab6	R
psy1/ykl075c[b]	**S**
rkm1	R
rpl20a	R
she1	R
tda5	R
thr1	R
ubp8	**S**

[a] These 11 were identified as gamma radiation sensitive by Game *et al.*, but were not detected in the screen by Bennett *et al.* They were not previously tested by J. Summers and S. Sethi.
[b] Gene names separated by a forward slash indicate deletions within two overlapping open reading frames. The coding region of *PSY1* was deleted in this strain.

psy1 and *ubp8* cells were then tested in the second haploid deletion strain library, the *MATa* library. When these two mutants were transformed with plasmids and tested by pronging transformants onto galactose to induce EcoRI, only one mutant showed consistent sensitivity to EcoRI, *ubp8*. The table below, Table 6, shows the cumulative results for all of the mutants tested in both haploid deletion strain libraries, combining the data from the current project with that of J. Summers and S. Sethi. Eight of the nine RAD52 group genes were tested during this work and their sensitivities are shown at the top of the table. A total of 73 non-RAD52 group genes were found to be required for EcoRI resistance in *MATα* cells, and *MATa* cells, or in both cell types.

Table 6. List of all mutants identified as EcoRI-sensitive in *MATα* cells, *MATa* cells, or both *MATα* and *MATa* strains[a]

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

[a] The 73 EcoRI[s] mutants include 61 *MATα* strains identified in work done by J. Summers and S. Sethi plus the 12 non-RAD52 group mutants identified as EcoRI[s] in the current study.
[b] Control RAD52 group mutants that were also tested.
[c] N/D, usually indicates that the *MATα* library strain or the *MATa* library mutant was unable to grow on galactose plates. Some genes could not be tested for other reasons, e.g., *MATα cdc40* cells were Ura$^+$ (see text).
[d] These are the 2 mutants from Game et al. found to be EcoRI[s] in the current work.
[e] Gene names separated by a forward slash indicate deletions within two overlapping open reading frames. The coding region of the first gene listed was deleted in each strain.
[f] *yml009w-b* overlaps the verified gene *spt5* and another uncharacterized open reading frame called *yml009c-a*.

The twelve newly determined EcoRI^s mutants identified in the current study were also tested for sensitivity to two chemical clastogens, methyl methanesulfonate (MMS), and bleomycin. In addition, the mutants were exposed to gamma radiation, a known source for exogenous DNA damage. In Figure 7, mutant sensitivity to MMS was compared to wildtype. As depicted in the figure, the mutants showed resistance to MMS, as their growth is comparable to wildtype cells. Rows three and four plus rows seven and eight contained mutants *ubp8* and *psy1*. With *rad52* and wildtype cells used as controls, both mutants did not show killing, in contrast to control *rad52* cells.

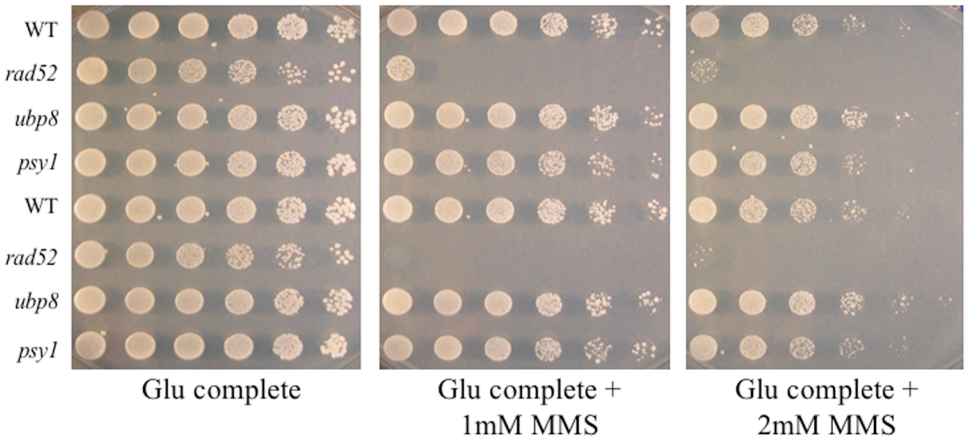

Figure 7. Pronging of EcoRI^s mutants to measure MMS sensitivities.

Figure 8 shows an example of pronging onto agar media containing different amounts of bleomycin. This series showed that *ubp8* and *psy1* mutants were resistant to bleomycin when compared to wildtype cells. *rad52* and wildtype cells were also used as controls.

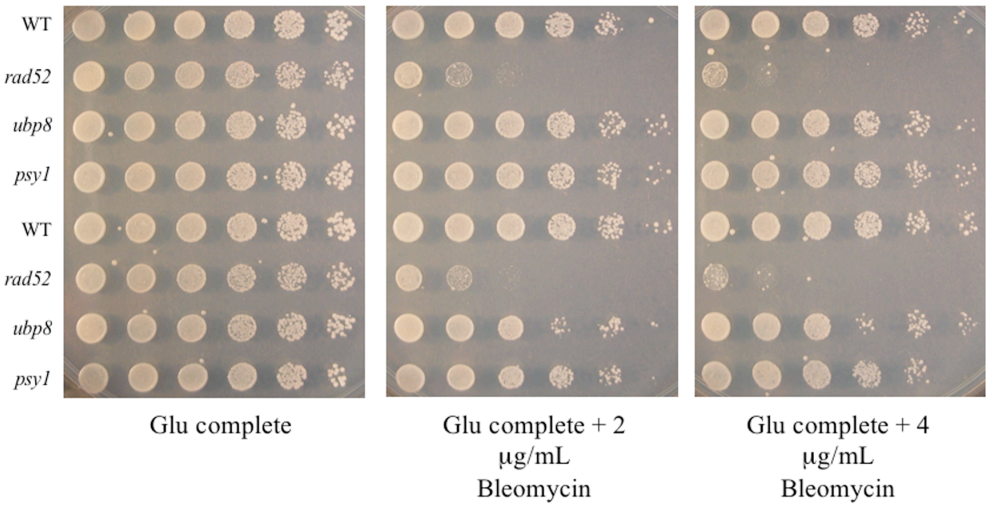

Figure 8. Pronging of EcoRI^s mutants to assess bleomycin sensitivity.

The final DNA damaging agent that the EcoRI^s mutants were exposed to was gamma radiation. Figure 9 shows a pronging series of 0, 30, and 60 krads of gamma rays. In this example, only two mutants showed sensitivity to gamma radiation, *cdc40* and *rtf1*. The *xrs2* cells in the 2nd row served as a RAD52 group control and exhibited strong killing at both 30 and 60 krads.

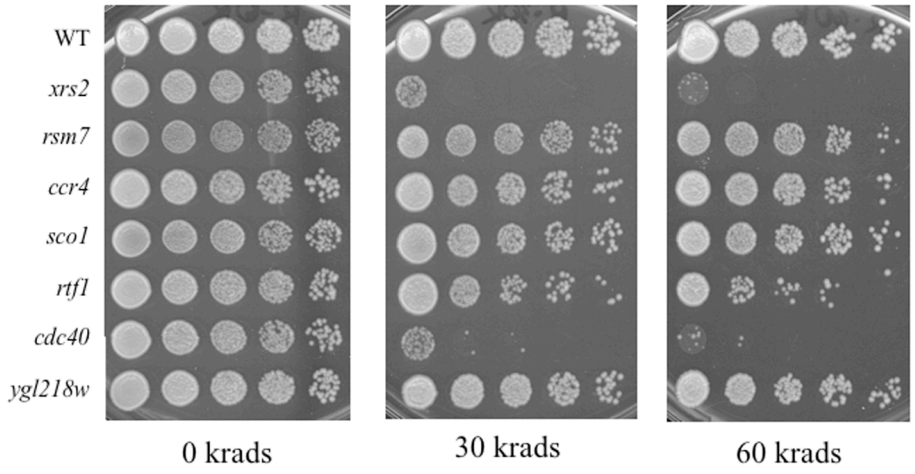

Figure 9. Pronging of EcoRI^s mutants to test gamma radiation sensitivity.

Table 7 summarizes the results of testing the twelve new EcoRI^s mutants for sensitivity to the exogenous DNA damaging agents MMS, bleomycin, and gamma radiation. Out of the twelve mutants tested, many of them showed differing results. Some mutants such as *sco1* and *ubp8* were resistant to all three DNA damaging agents. Others were extremely sensitive to every dose of chemicals or radiation, *cdc40* cells for example. The majority of the mutants had sensitivities that lie in between, being sensitive to MMS but not to bleomycin for example.

Table 7. Resistance of EcoRI^s Mutants identified in this study to MMS, Bleomycin, and Gamma Radiation[a]

	MMS		Bleomycin		Gamma Radiation	
Mutant	1 mM	2 mM	2 ug/mL	4 ug/mL	30 krads	60 krads
BY4742	R	R	R	R	R	R
rad52	SS	SS	SS	SS	SS	SS
arp5	R	S	R	R	R	S
bik1	R	R	S	R	R	R
bud30/rpc53[b]	R	R	S	S	S	S
bud32	R	R	SS	SS	R	S
ccr4	R	R	S	SS	R	R
cdc40	SS	SS	SS	SS	SS	SS
psy1/ykl075c[b]	R	R	R	R	R	R
rsm7/yjr114w[b]	R	R	S	SS	R	R
rtf1	R	S	R	SS	S	SS
sco1	R	R	R	R	R	R
ubp8	R	R	R	R	R	R
ygl218w/mdm34[b]	R	SS	R	R	R	R

[a] All the thirteen above mutants are EcoRI^S.
[b] Gene names separated by a forward slash indicate deletions within two overlapping open reading frames. The coding region of the first gene listed was deleted in these strains.

Table 8 is a summary of all 73 mutants tested in this study and in the work of J. Summers and S. Sethi, grouped based on their sensitivities and resistances in all four tests. Ten of the 73 mutants are sensitive to all four DNA damaging agents, which is consistent with the phenotype of RAD52 group mutants shown in boldface type in Table 8. This suggests that these ten mutants are most important in DNA DSB repair. It is important to note that less than half of the 73 haploid mutants were found to be sensitive to gamma radiation, but all of them exhibited gamma sensitivity as diploids. It is not clear why these differences in haploid vs. diploid sensitivity were observed, but some mutants, such as *srs2* cells, are known to exhibit this phenotype (43).

Table 8. Resistance of EcoRI^S mutants to physical and chemical DNA damaging agents[*]

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*
	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, ygl218w/mdm34*

[*] 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*, which both gene deletions were tested independently.

Game *et al.* identified *YDR014W* as a gene needed for gamma radiation resistance in diploids and haploids that was not required for resistance to ultraviolet light. These phenotypes are also found in RAD52 group mutants and Game *et al.* renamed *YDR014W* as *RAD61* (33). *rad61* mutants were quantitatively tested in the current study because

they display RAD52 group phenotypes. *rad61* cells were tested in both *MATa* and *MATα* haploid deletion library strains for EcoRI sensitivity. Figure 10 shows the resistance of *rad61* mutants to EcoRI when pronged onto galactose. These mutants displayed the same resistance to EcoRI as in wildtype cells (compare the 4th and 6th rows to the 2nd row). This level of resistance was not seen with other members of the RAD52 group, and these results are therefore not consistent with *RAD61* being a part of this group.

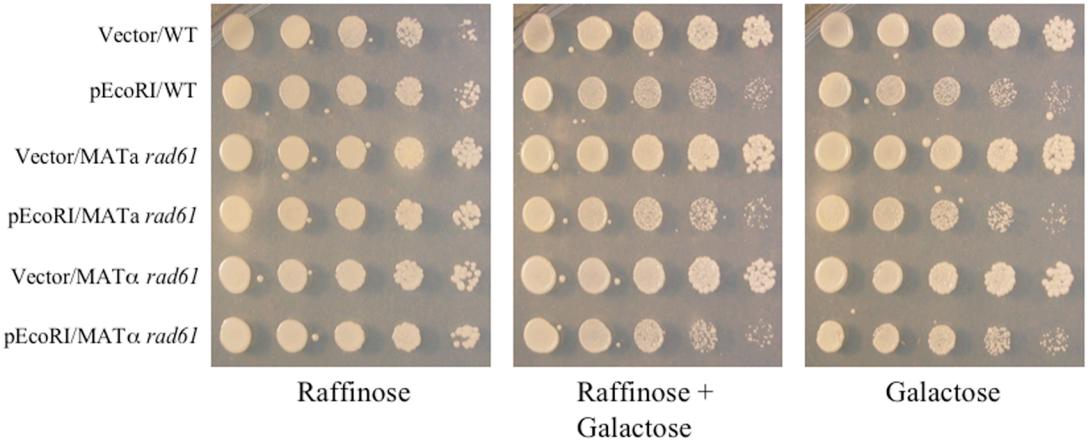

Figure 10. Pronging of *rad61* mutants to determine EcoRI sensitivity.

Using information compiled at the Saccharomyces Genome Database, 60 of the 73 mutants were classified into ten categories depicting various types of functions and processes (Table 9). Of the 60, a large number (14) were previously linked to transcription regulation. Many others are known to be involved in DNA metabolism, affecting sister chromatid cohesion, histone and chromatin structure, and chromosome segregation (Table 9). Seven of them are known to encode mitochondrial proteins. Atp2 for example, is a beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase. This

is a large, conserved, enzyme complex that is required for the synthesis of ATP. At this time it is unclear how inactivation of genes affecting mitochondria or cell membranes (Table 9) affect resistance to EcoRI and the chemical clastogens MMS and bleomycin. Some of the gene names are separated by a forward slash; this is to indicate deletions affecting two overlapping open reading frames. The gene name that is listed first is the protein product that was used to describe their functions and processes.

Table 9. EcoRI-sensitive mutants and their functions

Function or process	Genes[a]
Sister chromatid cohesion	*CTF4, CTF8, DCC1, HTL1*
Histone modification	*EAF1/OPI7, GCN5, RTT109(REM50)*[b]*, SPT10, UBP8*
Nuclease processing of DNA	*EXO1, MMS4/YBR099C, SAE2, TOP3/YLR235C*
Chromatin-associated proteins	*ARP5, MMS2, RAD5*
Chromosome stability/segregation	*BIK1, CGI121, CNM67, DDC1, MMS22*
Transcription regulation	*NET1/APQ13, BUD32, BUR2, CCR4, NOT5, NUP84, RPB9, RPC53/BUD30, RTF1, SFP1, SPT20, TAF14, UME6, SPT5/YML009W*
RNA processing/modification	*CDC40*, *LRP1, LSM7, TRM9, TSR2/YLR434C, NPL3/YDR433W*
Cell membrane/cell wall	*CIS3, HSP150, RVS161, SAM37, VMA7, VPH2/YKL118W*
Protein posttranslational modification	*AKR1, BCK1, CAX4, OCH1, UBR1, UBX2/YML012C*
Mitochondrial proteins	*ATP2, IMG2, MCT1, MDM34/YGL218W, MRPS35, RSM7/YJR114W, SCO1*

[a] Gene names separated by a forward slash, e.g., *MMS4/YBR099C*, indicate deletions within two overlapping open reading frames. Functions and processes are described for the protein product of the first gene listed.
[b] The *RTT109* gene is frequently referred to as *REM50* in the literature.

Analysis of the 73 genes and their chromosomal locations on the 16 yeast chromosomes revealed an interesting phenomenon; a few of them lie directly adjacent to each other. For example, *HSP150* and *CIS3* are 575 bp apart on chromosome X (Figure

11a). Due to their chomosomal orientation, polar effects on transcription could possibly result in their sensitivity to different DNA damaging agents. It is possible that deletion of one gene may affect transcription of the other. The *UBP8* gene coding region is 445 bp from that of the well-studied RAD52 group gene *MRE11* (Figure 11a, part B). It is possible that *ubp8* deletion mutants are not sensitive to EcoRI because of the loss of Ubp8 protein, but rather it may be due to polar effects that reduce transcription or mRNA stability of *MRE11*. Part C of Figure 11a depicts an example of two adjacent genes (*TRM9* and *UBX2*) and also the overlapping of two genes (*UBX2* and *YML012C-A)*. *YML012C-A* has the size and characteristics of a normal gene, but it has not yet been shown to produce protein products and it may not be functional.

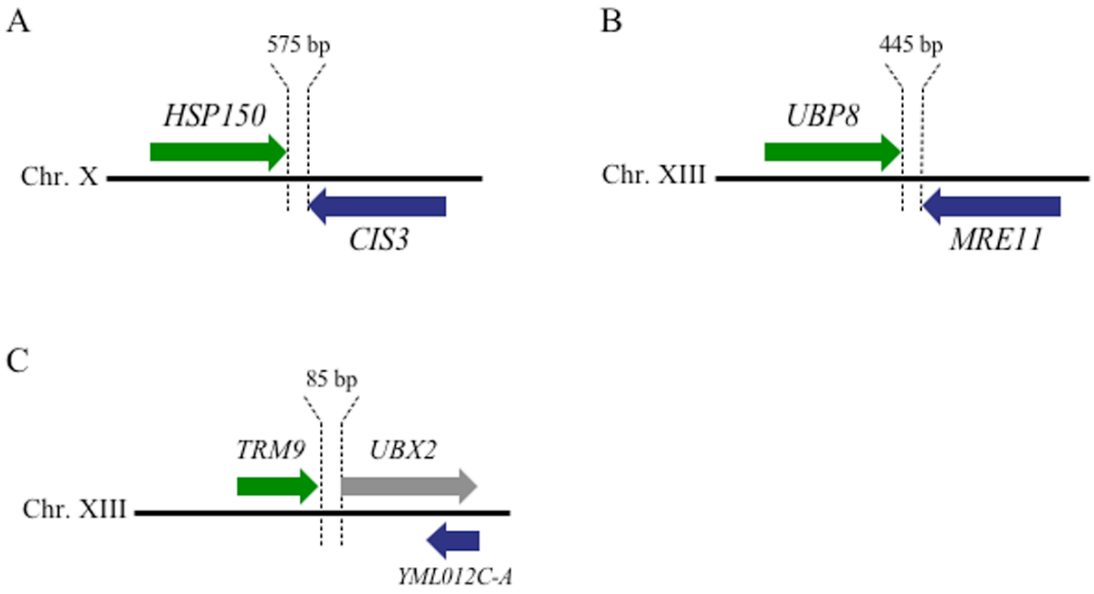

Figure 11a. Schematic representation of adjacent genes required for resistance to EcoRI. A, B and C show three different arrangements in which genes affecting EcoRI sensitivity lie adjacent to each other.

Interestingly, overlapping of two open reading frames is a characteristic of 16 of the loci identified in this work. When overlapping occurs, it is not completely certain that the gene that was precisely deleted from its start codon to its stop codon has caused the observed phenotype. Examples of this arrangement are shown in Figure 11b. Parts A-D in the figure illustrate four different orientations of overlapping genes. The two or more putative genes are sometimes transcribed in the same direction on the chromosome but they may also go in opposite directions. In most cases, one of the overlapping genes has been shown to produce a protein *in vivo* but the other one has not been proven and is likely to be nonfunctional.

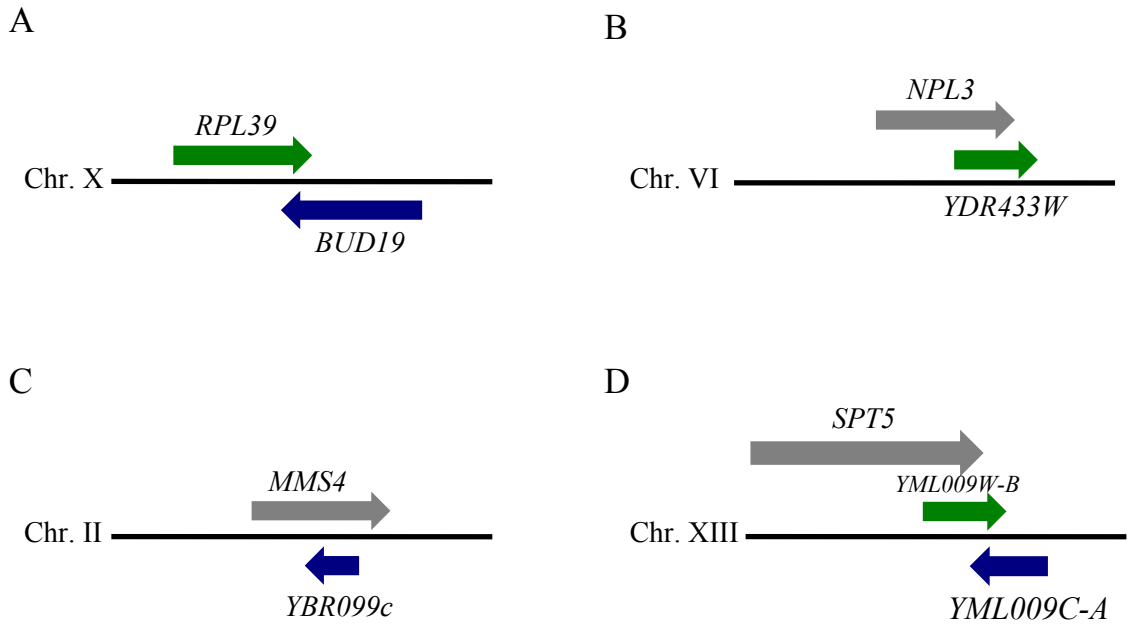

Figure 11b. Schematic representation of overlapping genes. A-D depict four different arrangements of overlapping genes / open reading frames. In most cases, only one of the genes is believed to be functional *in vivo*.

Detailed analysis of the properties of the genes and proteins identified in this work at the Saccharomyces Genome Database revealed that 40 of the proteins display

interactions with other proteins that are required for efficient repair of DSBs induced by EcoRI. Many of the proteins were found to interact with each other and also with other proteins that were previously linked to DSB repair. All forty proteins are listed in Table 10 along with proteins that have been shown to interact physically with them. Gcn5 is a protein that not only interacts with 6 other proteins identified in this study, but it also interacts with Rad59, a protein known to be involved in the repair of DSBs in DNA. Similarly, Spt5 has been shown to physically interact with 4 proteins encoded by genes identified in the current work.

Table 10. Interactions among proteins required for efficient repair of EcoRI-induced DSBs*

Name	Interacting proteins	Name	Interacting proteins
Adk1	Bck1, Sir2	Net1	Cac2, Rad53, Sir2
Akr1	Gcn5, Dun1	Not5	Ccr4
Arp5	Taf14	Npl3	Bur2, Spt5
Bck1	Adk1, Lip5	Rad5	Pol30, Rad18, Rev1, Srs2
Bud32	Cgi121	Rem50	Asf1, Pol30
Bur2	Npl3, Rad52, Rpa1	Rpb9	Spt5, Taf14
Ccr4	Not5, Ubr1	Rpl12b	Rpl31a, Rpl39
Cgi121	Bud32, Srs2	Rpl31a	Rpl12b, Rpl39
Cnm67	Mlp2	Rpl39	Rp12b, Rpl31a
Ctf4	Mms22	Rsm7	Mrps35
Ctf8	Dcc1	Rtf1	Spt5
Dcc1	Ctf8	Rvs161	Gcn5
Ddc1	Mec3, Rad17, Rad52, Rad53, Rev7	Sae2	Sir3, Srs2
Gcn5	Akr1, Mms4, Rvs161, Srv2, Spt20	Sfp1	Asf1
	Ubp8, Rad59	Spt5	Npl3, Rpb9, Rtf1, Taf14
Gnd1	Ubr1	Spt20	Gcn5, Ubp8
Lip5	Bck1	Srv2	Gcn5
Mms2	Pol30	Taf14	Arp5, Rpb9, Spt5, Mus81
Mms4	Gcn5, Mus81, Rad27	Top3	Dna2, Sgs1
Mms22	Ctf4, Mms1	Ubp8	Gcn5, Spt20, Sir3
Mrps35	Rsm7	Ubr1	Ccr4, Gnd1, Rad6, Srs2

* Includes proteins interacting physically and proteins that are shared components of a multisubunit complex. Underlined names indicate proteins identified in the current study. Other proteins listed in the table have previously been linked to DSB repair. Associations were taken from the Saccharomyces Genome Database (34).

The protein products of all known and putative genes affecting EcoRI sensitivity were analyzed for homology across three main genomes: *Homo sapiens* (human), *Mus musculus* (mouse), and *Rattus* (rat). Forty-four proteins showed strong homology to proteins listed for the three genomes corresponding to 60.3 % of the 73 loci identified in this study. In the following table (Table 11), all 44 proteins were ranked based on their BLAST program e-values (homology score) starting with the RAD52 group members. Rad51 had the strongest homology to proteins in all three genomes with e-values of $2e^{-170}$ to $5e^{-170}$. All the other RAD52 group members also had strong homologies with the exception of Xrs2. Of the 44 new proteins, Gnd1 had the highest homology score, $5e^{-158}$. Gnd1 is known to be part of basic sugar metabolism in the pentose phosphate pathway (34). Top3 is another protein that had a strong homology score of $4e^{-149}$. It is known to be DNA topoisomerase III, which relaxes single-stranded negatively supercoiled DNA and is important for transcription and DNA replication. These proteins are likely to be well conserved because they are involved in major metabolic pathways common to all organisms.

Table 11. Many of the proteins linked to DSB repair in the current study have strong homology to human and animal proteins (e-value <10^{-4})[a]

RAD52 Group:	Protein	Human	Mus/Rattus[b]
	Rad50	$3e^{-65}$	$3e^{-127}/4e^{-65}$
	Rad51	$2e^{-170}$	$4e^{-170}/5e^{-170}$
	Rad52	$2e^{-49}$	$6e^{-50}/2e^{-50}$
	Rad54	$3e^{-153}$	$2e^{-148}/4e^{-154}$
	Rad55	$2e^{-4}$	$4e^{-6}/5e^{-6}$
	Rad57	$8e^{-20}$	$2e^{-18}/6e^{-18}$
	Mre11	$9e^{-130}$	$2e^{-132}/2e^{-130}$
	Xrs2	-	*1.9/3.8*
This Study:	Gnd1[c]	$5e^{-158}$	$2e^{-178}/4e^{-178}$
	Top3	$4e^{-149}$	$2e^{-152}/1e^{-97}$
	Atp2	$4e^{-148}$	$3e^{-55}/2e^{-167}$
	Lip5	$1e^{-110}$	$1e^{-112}/5e^{-114}$
	Gcn5	$2e^{-83}$	$5e^{-82}/4e^{-82}$
	Rpl12b[c]	$4e^{-85}$	$1e^{-85}/3e^{-85}$
	Ccr4	$3e^{-83}$	$3e^{-80}/1e^{-80}$
	Rad5	$5e^{-83}$	$3e^{-83}/3e^{-85}$
	Cdc40	$2e^{-75}$	$5e^{-76}/3e^{-76}$
	Adk1	$5e^{-75}$	$5e^{-73}/9e^{74}$
	Spt5[c]	$2e^{-69}$	$6e^{-72}/3e^{-71}$
	Srv2	$2e^{-67}$	$1e^{-65}/6e^{-66}$
	Ado1	$3e^{-64}$	$3e^{-65}/3e^{-66}$
	Akr1	$5e^{-58}$	$9e^{-57}/6e^{-58}$
	Bck1	$1e^{-56}$	$8e^{-57}/1e^{-57}$
	Exo1	$2e^{-56}$	$9e^{-55}/1e^{-54}$
	Ubp8	$3e^{-54}$	$2e^{-54}/2e^{-53}$
	Trm9	$1e^{-44}$	$4e^{-46}/1e^{-35}$
	Not5	$5e^{-40}$	$4e^{-40}/4e^{-6}$
	Mms2	$4e^{-38}$	$1e^{-37}/7e^{-38}$
	Sco1	$4e^{-38}$	$1e^{-39}/2e^{-39}$
	Arp5	$3e^{-36}$	$3e^{-36}/1e^{-34}$
	Rpl31a	$7e^{-34}$	$4e^{-34}/2e^{-34}$
	Bud32	$2e^{-29}$	$5e^{-31}/1e^{-31}$
	Ubr1	$1e^{-27}$	$2e^{-31}/3e^{-31}$
	Vma7	$8e^{-27}$	$6e^{-26}/7e^{-27}$
	Rpb9	$3e^{-24}$	$2e^{-24}/7e^{-25}$
	Cax4	$2e^{-19}$	$3e^{-20}/1e^{-19}$
	Rpl39	$4e^{-19}$	$3e^{-19}/1e^{-19}$
	Rtf1	$5e^{-19}$	$3e^{-19}/1e^{-18}$
	Lsm7	$2e^{-18}$	$2e^{-19}/3e^{-19}$
	Ctf4	$4e^{-18}$	$6e^{-13}/2e^{-15}$
	Cgi121	$4e^{-16}$	$7e^{-17}/5e^{-17}$
	Rvs161	$2e^{-15}$	$1e^{-16}/3e^{-16}$
	Npl3[c]	$6e^{-15}$	$5e^{-15}/2e^{-15}$
	Nup84	$6e^{-13}$	$4e^{-12}/3e^{-11}$
	Sfp1	$4e^{-10}$	$6e^{-10}/3e^{-10}$
	Taf14	$2e^{-8}$	$4e^{-8}1e^{-6}$
	Dcc1	$6e^{-8}$	$2e^{-7}/3e^{-5}$
	Bik1	$7e^{-8}$	$3e^{-7}/6e^{-8}$
	Rpc53[c]	$1e^{-7}$	$2e^{-8}/5e^{-8}$
	Tsr2[c]	$1e^{-7}$	$4e^{-8}/1e^{-6}$
	Img2	$7e^{-6}$	$6e^{-8}/4e^{-7}$
	Eaf1	$6e^{-5}$	$6e^{-4}/1e^{-4}$

[a] The values shown are exponents, e.g., $3e^{-65}$ is $3x10^{-65}$.

[b] Mus, mouse; Rattus, rat.

[c] These proteins are encoded by genes whose coding regions overlap one or more other large open reading frames. In most of these cases, the overlapping ORF is unlikely to be a functional gene.

In the final part of this project, we wished to increase the efficiency of transfer of plasmid DNAs into yeast cells to improve assays for DNA repair using the 73 EcoRI^s mutants. Many different assays have been developed for measuring either homologous recombination or NHEJ repair proficiency that require transformation of plasmids into yeast cells. This process is extremely inefficient and improvement is needed.

The transformation of overnight liquid cultures of yeast cells with plasmids in stationary phase cells was first addressed. The basic protocol developed by Soni *et al.* using stationary phase cells was chosen to try and improve it (21). This protocol involved spinning down cells from overnight liquid YPDA cultures to make a pellet. Polyethylene glycol, lithium acetate, DMSO and plasmid DNA were then added. Polyethylene glycol promotes association of the plasmid DNA with the cells, whereas the lithium ions and DMSO disrupt the cell membrane and the cell wall to allow DNA to enter. The goal of the method is to disorganize the cell membrane and cell wall to allow DNA to pass inside without the cell dying.

A series of pretreatments were implemented into the protocol to test their effect on the efficiency of transformation using the yeast plasmid pRS316 (*CEN/ARS URA3*) (26). BY4742 cells were tested with three different salts, lithium acetate (LiAc), potassium chloride (KCl), and rubidium chloride (RbCl) as pretreatment washes (Figure 12). In the salt pretreatment series, 500 µL of 0.1 M of each salt was added to the cell pellets and the cells were resuspended by brief vortexing. The cells were incubated in the salt for 20 minutes at 42 ^{o}C. Four separate transformations were done to test each variable and average numbers of Ura$^+$ colonies produced and standard deviations were calculated. Cells with the LiAc pretreatment had a 2.6 fold increase in colonies

compared to cells with no pretreatment. However, standard deviations were consistently high in these experiments (Figure 12 and data not shown) and therefore the results represented a trend, but were not statistically significant. Pretreatment of the cells at lower temperatures did not improve transformation efficiencies (not shown).

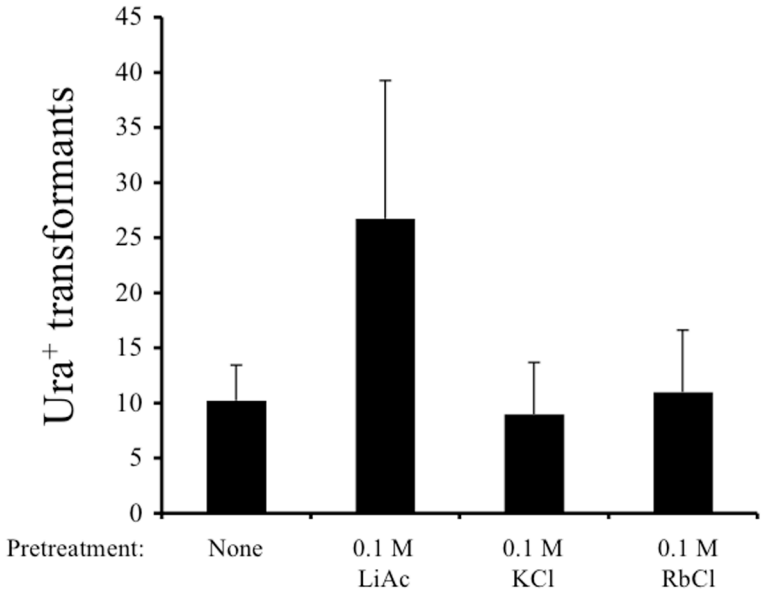

Figure 12. Pretreatment of stationary phase cells with salts prior to transformation with plasmid pRS316 using the method of Soni *et al.* Error bars indicate standard deviations.

BY4742 cells were also tested by pretreating them with dithiothreitol (DTT) solution. DTT and other reducing agents have been used in transformation protocols in the past, but they are not in common usage among yeast researchers (35-38). Once the cells were spun down, 500 µL of 0.1 M DTT was added. The cells were then incubated at 42 ^{o}C for 20 minutes, followed by spreading to selective plates. The temperature of 42 ^{o}C was chosen because preliminary results by an undergraduate in the lab, Jennifer Lilley, demonstrated that 42 ^{o}C gave better results than lower incubation temperatures.

The pretreatment of cells with DTT dramatically increased the efficiency of transformations by about 4.5 fold. This result gave rise to further experiments utilizing DTT.

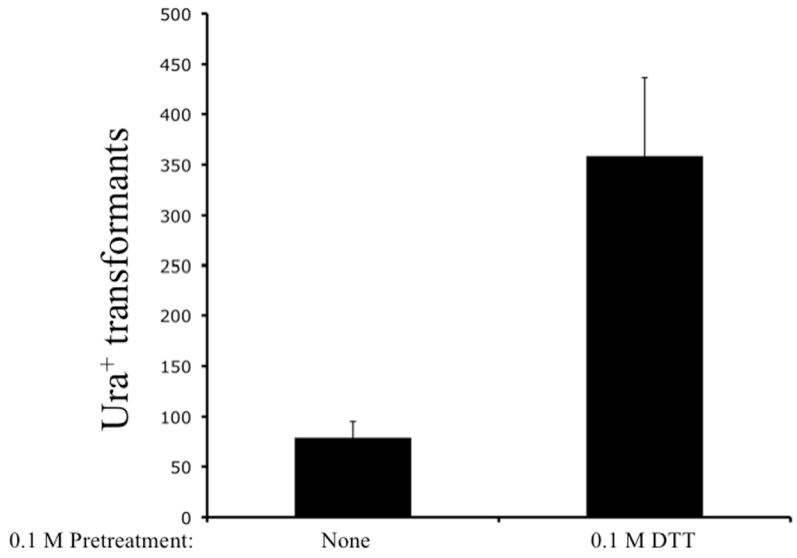

Figure 13. Pretreatment of stationary phase cells with DTT.

A number of different yeast background strains were tested to see if the addition of DTT also worked on other popular laboratory strains. The different strains were BY4741, BWGI-7a, S1, SK-1, T334, and YPH102 (24, 26-30). These six different strains were tested with pretreatment of 0.1 M DTT at 42 ^{o}C (Figure 14). Each strain's efficiency for transformation increased with the DTT pretreatment by at least 7 fold. The highest increase was with the strain S1, which increased by 42 fold.

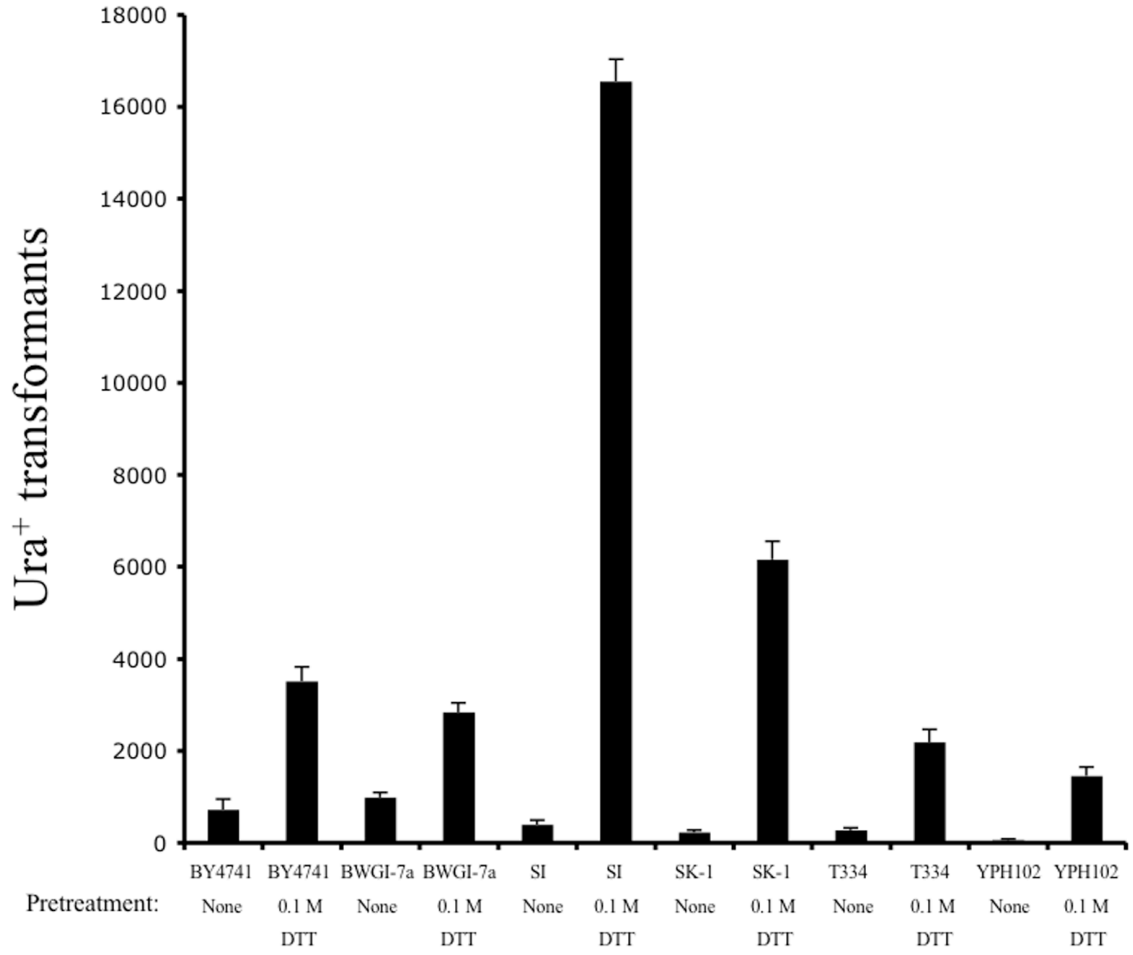

Figure 14. Effect of pretreatment with DTT on transformation efficiencies in various yeast strain backgrounds.

DTT was also tested to see if it could increase the efficiency of transformations by putting it in the PEG/Li mix that is added to the cells along with pRS316 plasmid DNA. The logic behind this was to increase the efficiency with DTT, but to eliminate the 20-minute wait time that is required when cells are pretreated. Figure 15 shows the data for the transformations with the DTT added into the PEG/Li mix. DTT had a similar effect on the cells as the DTT pretreatment. DTT increased the efficiency of transformations by about 4.6 fold.

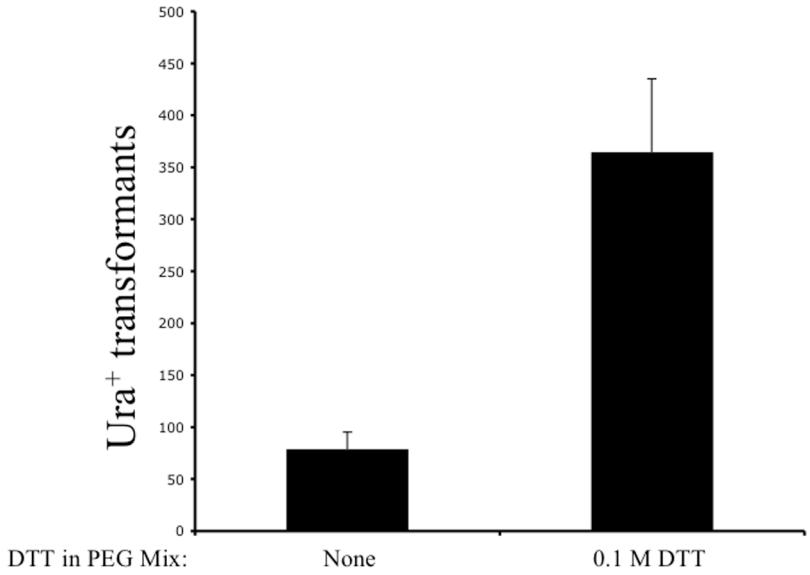

Figure 15. Effect of including DTT in the PEG/Li mix instead of pretreating with DTT.

In another experiment, three published stationary phase transformation protocols were tested against each other to reveal which would have the highest transformation efficiency. The simple PEG/Li protocol from Schiestl and Gietz *et al.* (22), the PEG/Li + DTT protocol from Chen *et al.* (23), and the PEG/Li + TE + DMSO method from Soni *et al.* (21) were all compared to each other. In addition, the Soni PEG/Li + TE + DMSO protocol was also tested with the addition of DTT in the PEG/Li mix added to the cells. These four separate protocols were tested against each other (Figure 16). The new protocol involving addition of DTT had the highest efficiency. It increased the efficiency by about 11.9 fold relative to the PEG/Li + TE + DMSO protocol. It produced > 700 colonies per plate while the other three protocols only produced < 100 colonies per plate. Interestingly, the Chen *et al.* protocol also included DTT, but did not surpass the

efficiency of the original Soni *et al.* protocol. The simple Chen protocol did not include treatment of cells with DMSO, which may be important to get the highest efficiencies.

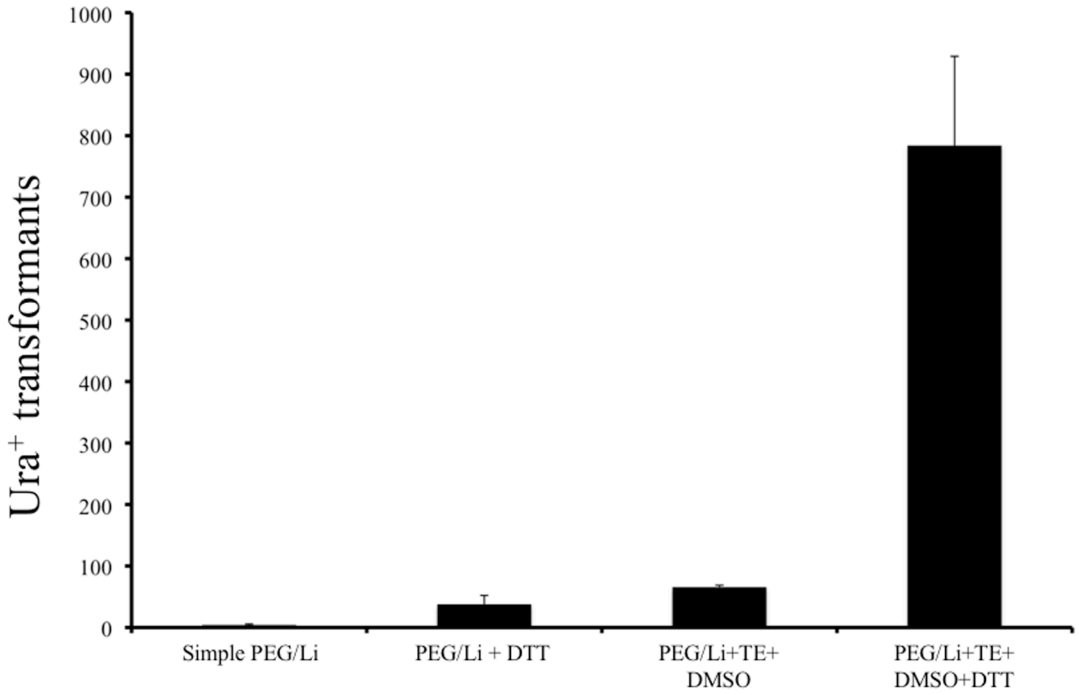

Figure 16. Comparison of four separate protocols for transformation of stationary phase yeast cells.

Pretreatment with DTT was also assessed using log phase cells and a "high efficiency" protocol developed by Schiestl and Gietz (22). Logarithmically growing cells exhibit higher transformation efficiencies than stationary phase cells. Cells from overnight cultures were diluted, grown to log phase over 2 – 3 hours and then spun down. Five hundred μL of 0.1 M DTT was added to the cells and they were incubated for 20 minutes at 42 ^{o}C. The pretreated cells were then pelleted by centrifugation for 30 seconds and the standard Schiestl and Gietz protocol involving simple treatment of log phase cells with PEG and LiAc followed. The efficiency increased about 3 fold when DTT was present (Figure 17).

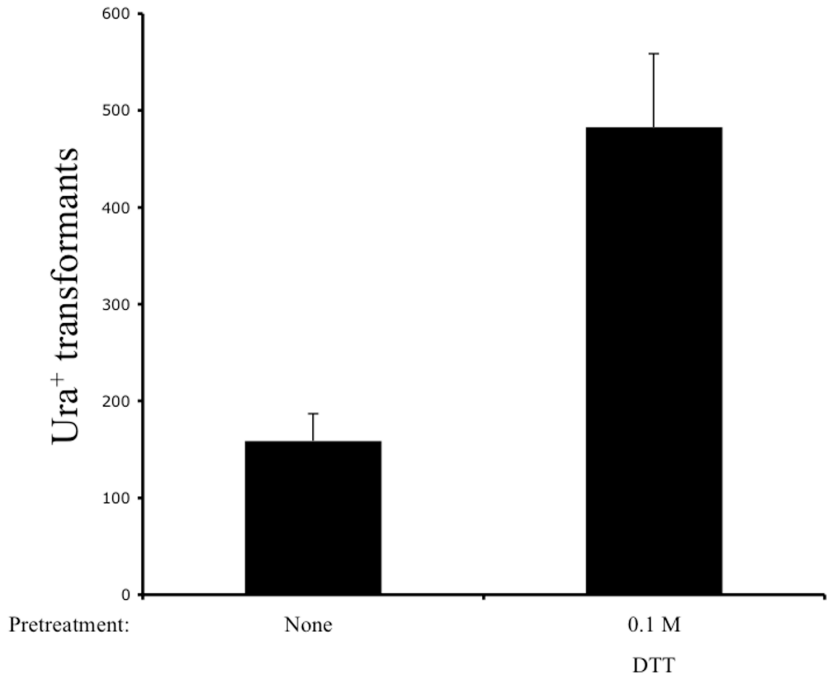

Figure 17. Pretreatment of log phase cells with DTT increases transformation efficiency.

DTT was also tested by putting it into the PEG/Li mix when transforming log phase cells, eliminating the 20 minute preincubation. The molarity of the DTT once put into the PEG/Li mix was 0.1 M. The resulting increase in efficiency, displayed in Figure 18, was about 3.5 fold.

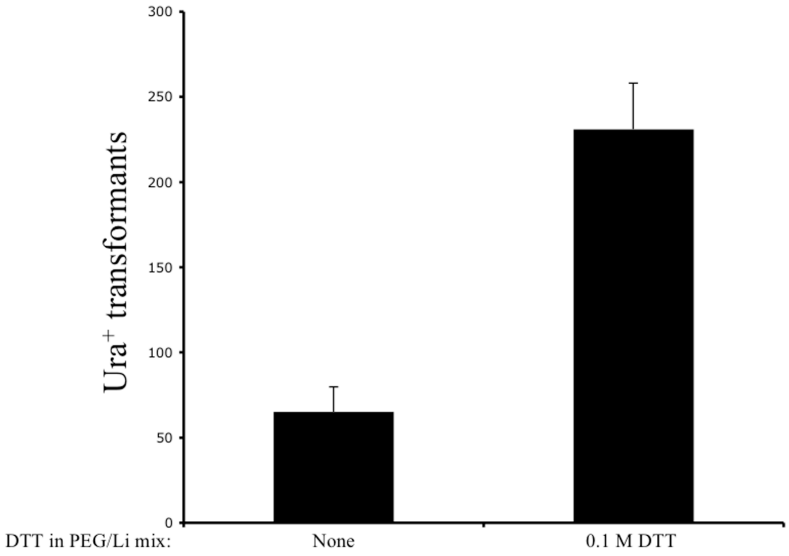

Figure 18. Effect of including DTT in the PEG/Li mix during transformation of log phase cells.

DTT is a reducing agent and is used to reduce disulfide bonds in proteins, breaking the covalent bonds and creating free sulfhydryl groups. The exterior surface of yeast cells has many proteins with disulfide linkages between cysteine residues (41, 42). It is possible that the reduction of disulfide bonds breaks down the cell wall partially and makes it an easier task for the plasmid DNA to penetrate the cell wall, allowing for more cells to take up the DNA. This increase of plasmid DNA uptake would then account for the increased number of transformants seen in the experiments. Figure 19 is a yeast cell wall representation depicting disulfide bridges between cell surface proteins.

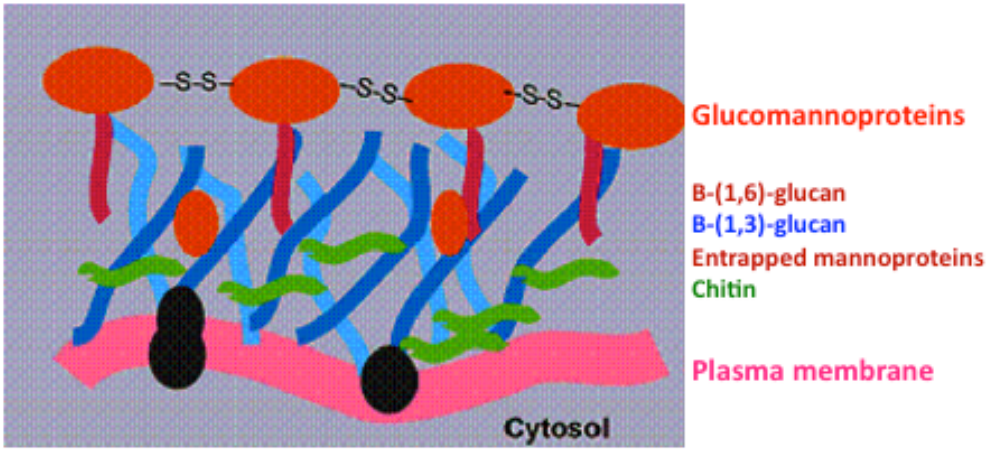

Figure 19. Yeast cell wall depicting disulfide bridges between cell surface proteins (45).

Summary and Conclusions

DNA DSBs are one of the most damaging types of lesions that DNA repair pathways must resolve. DSBs can occur from a number of sources such as ionizing radiation, chemical clastogens, and endonuclease enzymes. In eukaryotes like *Saccharomyces cerevisiae*, two repair pathways are utilized to repair DSBs. If DSBs are left unrepaired or they are repaired inaccurately an increase in DNA mutations affecting metabolism, resulting in cancer, or loss of cell viability can occur. Identifying genes that are essential for DSB repair is crucial to further our understanding of cellular factors that maintain chromosome stability and DNA sequence integrity.

Two previous groups screened 4,746 diploid mutants for gamma sensitivity and 210 genes were determined to be required for resistance to gamma radiation (15, 17). Nine of the genes were members of the RAD52 group, which are required for resistance to ionizing radiation (15). The combined work of the current study and those of Summers and Sethi in this lab demonstrated that seventy-three of the 201 remaining genes were required for resistance to EcoRI in haploids. The 73 mutants were each tested and compared in both haploid *MATα* and *MATa* cells in this work.

Of the 73 EcoRI sensitive mutants, 52 mutants showed sensitivity to EcoRI in both haploid versions (Table 6). In most of the remaining 20 mutants, one of the isolates (either *MATα* or *MATa*) had growth problems and could not be tested. Most of the new mutants showed sensitivity to MMS (45/73) and bleomycin (51/73) (Table 8). The 73 haploid mutants were also tested for gamma radiation resistance. Surprisingly, only 18 of the 73 mutants exhibited gamma sensitivity. This is surprising since all 73 genes were found to produce radiation sensitivity when inactivated in diploids. The cause of these

differences is not yet known, but there are precedents for the phenotype. Both *SRS2* and *RDH54* have been found to produce radiation sensitivity when inactivated in diploids but not in haploid cells (43, 44). In total, 10 mutants were found to be sensitive to EcoRI, MMS, bleomycin, and gamma radiation, not including the RAD52 group. These mutants show promising characteristics and are likely to be most important for DNA DSB repair.

In a previous study Game *et al.* tested *ydr014w* mutants for gamma radiation and ultraviolet light resistance (33). *ydr014w* cells lacked resistance to UV light but were sensitive to gamma radiation. These characteristics are consistent with those of RAD52 group mutants and Game renamed *YDR014W* to *RAD61*. When *rad61* cells were tested in this study for EcoRI sensitivity, they displayed resistance like that of wildtype cells, and therefore are inconsistent with RAD52 group characteristics.

Of the genes found to be required for resistance to EcoRI, 41 have previously been associated with processes affecting DNA or RNA metabolism in the nucleus. The functions include sister chromatid cohesion, histone modification and nuclease processing of DNA (Table 9). At present it is unclear how mutations in genes affecting other processes such as mitochondrial metabolism and cell membrane functions cause sensitivity to EcoRI and other DNA damaging agents.

Surprisingly, a few of the new genes were found to lie directly adjacent to each other. When two or more genes are adjacent to each other, factors such as polar effects on transcription can affect resistance to exogenous and endogenous DNA damaging agents. In one case, *ubp8,* this gene was found to be only 445 bp from the RAD52 group gene *MRE11* (Figure 11a). It is possible that deletion of *UBP8* caused downstream effects on transcript stability of *MRE11*, and therefore might explain the EcoRI

sensitivity of this mutant. Forty-one of the proteins have been shown to physically interact with one or more of the other proteins. These interactions support the theory that groups of essential DNA repair proteins interact with one another in complexes.

Homologies of the protein products of the 73 EcoRI sensitive loci were compared to proteins from three primary eukaryotic genomes: human, mouse, and rat. The RAD52 group displayed strong homology to proteins in these three genomes, resulting in e-values as high as e^{-170}. Thirty-six of the new proteins also showed strong homology (e^{-4} or better).

Improving the efficiency of plasmid DNA transformations in both stationary and log phase cells was also a major part of this project. In stationary cells, a series of pretreatments were compared. The pretreatments ranged from incubation in salts such as LiAc, KCl, and RbCl, to reducing agents like DTT. Of the salt pretreatments, LiAc increased the efficiency of transformation by 2.6 fold. DTT pretreatment increased the efficiency by double the amount of the salt increase, 4.5 fold. The DTT pretreatment was also tested in 6 common yeast strain backgrounds. An increase in efficiency of transformation was observed for all of the strains, with the highest being 42 fold in the yeast strain S1. DTT was also added to the PEG/LiAc mix to eliminate the 20-minute incubation time required when pretreating cells and an increase of 4.6 fold was observed. Four common transformation protocols for stationary cells were then compared against each other, one being the PEG/Li + TE + DMSO + DTT method which was developed in this study. Of the four protocols, the new PEG/Li + TE + DMSO + DTT protocol produced the best results (Figure 16). The precise function of DTT in these assays is not

known, but it is likely that the chemical breaks disulfide bridges in proteins on the surfaces of cells, allowing easier passage of plasmid DNA into the cells.

Future studies will include testing the new EcoRI sensitive mutants in gene targeting assays. In these experiments, plasmids with a DSB are transformed into cells, where they undergo homologous recombination with a chromosome that causes the whole plasmid to integrate into the chromosome. This assay can measure large decreases in recombination like those of *rad51* or *rad52* mutants, and it is also sensitive enough to detect small defects in DSB repair. These transformation-based gene targeting assays will be improved by incorporation of findings from this thesis project indicating that addition of DTT enhances transformation efficiencies.

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VITA

Jennifer Ann DeMars was born in San Antonio, Texas on November 19, 1986, the daughter of Joseph DeMars and Barbara DeMars. After completing her work at Southwest High School, San Antonio, Texas, in 2005, she entered St. Mary's University, San Antonio Texas where she studied chemistry. She received the degree of Bachelor of Science from St. Mary's University in May 2009. During the spring of 2010, she entered the Graduate College of Texas State University-San Marcos where she studied biochemistry.

Permanent Address: 10340 Kearney Rd.

Atascosa, Texas 78002

This thesis was typed by Jennifer Ann DeMars