CHARACTERIZATION OF NEW GENES REQUIRED FOR DNA DOUBLE-STRAND BREAK REPAIR IN *SACCHAROMYCES CEREVISIAE*

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CHAPTER I

INTRODUCTION

Deoxyribonucleic acid (DNA) is a double-stranded nucleic acid. The two strands of DNA are anti-parallel with one strand running in the 5'-3' direction and the other strand running in the 3'-5' direction. Both the strands of DNA have strong affinity for each other, which occurs due to the presence of hydrogen bonds that hold adjacent base-pairs together and stacking interactions between the bases. DNA is the hereditary material that contains the genetic information used in the development and functioning of living organisms and some viruses. Based on their location, DNAs can be classified into two types: nuclear DNA (present in the cell nucleus) and mitochondrial DNA (present in mitochondria). The DNA within our cells may be exposed to DNA damaging agents such as ionizing radiation (X-rays and gamma rays), chemicals such as bleomycin and MMS (methyl methanesulfonate) and by nuclease enzymes such as EcoRI (1). Damage that is inflicted upon DNA can be either single-stranded or double-stranded DNA lesions. Of the two forms of damage, the most damaging is the DNA double-strand break (DSB) (2). If DSBs are left unrepaired, they can lead to broken chromosomes and cell death and if repaired improperly, they can lead to chromosome mutations, translocations and cancer. DSBs are a type of damage that can occur either after exposure to ionizing radiation or through treatment of cells with chemicals such as MMS (1, 3).

In response to the threat of DSBs, cells have evolved mechanisms to repair broken DNA. The yeast *Saccharomyces cerevisiae* is a single-celled lower eukaryote that has genes homologous to those in humans, which are multi-cellular higher eukaryotes. In contrast to multicellular organisms, yeast cells have the ability to grow as haploids or diploids, which facilitates creation and analysis of genetic mutants (4).

Mechanism for repair of DNA DSBs

In eukaryotes such as *S. cerevisiae,* repair of physical and chemically induced DSBs can be accomplished by at least two independent major pathways, homologous recombination (HR) or non-homologous DNA end-joining (NHEJ) (1, 5). Vastly conserved pathways have been found for both HR and NHEJ in all eukaryotes, from yeast to humans. Homologous recombination and NHEJ pathways are generally utilized in all eukaryotic organisms, however, one of the mechanisms is predominant among a given species. HR is an "error free" DNA repair mechanism that involves a multi-step process in which DSBs are repaired using multiple proteins and other homologous chromosomes within the same cell (6, 7). HR repair is the primary pathway for DSB repair in *S. cerevisiae,* whereas it is considered to be the secondary pathway in humans (5, 8). In contrast to HR, NHEJ repair is an error prone mechanism (5). NHEJ is the primary pathway of DSB repair in humans while it is the secondary pathway for repair in *S. cerevisiae* (9).

Both repair pathways have their own mechanism and proteins involved in DSB repair. The proteins exclusively involved in repair of DSBs by the homologous recombination pathway include Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, the Rpa complex as well as other uncharacterized proteins (7). The protein complexes exclusively

involved in NHEJ include Yku70/Yku80, Sir2/Sir3/Sir4 and Dnl4/Lif1/Nej1. The protein that is common in both the pathways is the Mrx complex which is composed of Mre11, Rad50 and Xrs2 (1, 3, 5, 8). DSB repair in mammalian cells requires Ku70 and Ku80. The Yku70 and Yku80 genes involved in DSB repair in yeast cells are homologs of Ku70 and Ku80 (5).

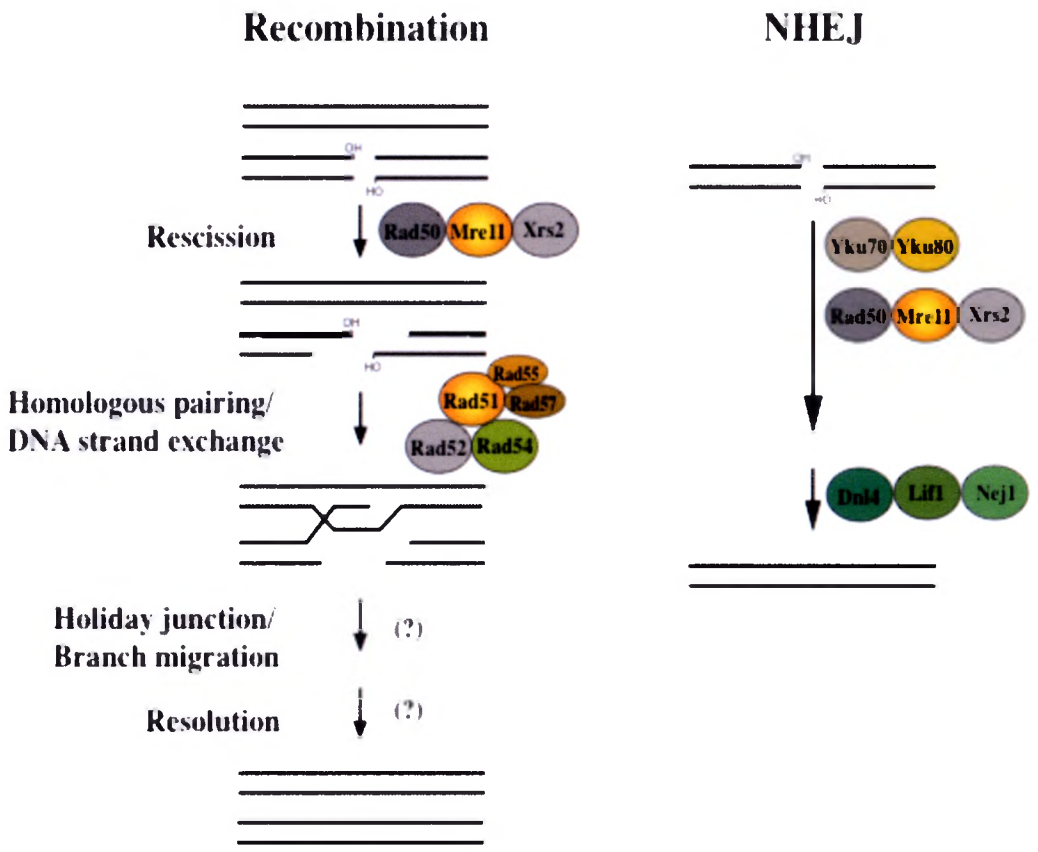

Figure 1. Illustration of the proteins involved in both known DSB repair pathways.

Structural similarity with human genes is exhibited by yeast genes involved in homologous recombination (Rad51, Rad52, Rad54, Rad55, Rad57, Rad59) and genes involved in NHEJ (Yku70, Yku80, Rad50, Mre11, Sir2, Dnl4). Also, both

human and yeast cells display many similar protein:protein interactions such as Rad51:Rad52, Rad51:Rad54, Ku70:Ku80, and Rad50:Mre11 (3).

Homologous recombination involves initial resection of DSB ends by the Mrx complex (Mre11, Rad50, Xrs2) that creates 3' overhangs on both strands of DNA at break sites. Other proteins such as Exo1, Sae2, Sgs1 and Dna2 have also been implicated in resection. These events are followed by homologous pairing, annealing, and strand exchange mediated by a complex containing Rad51, Rad52, Rad54, Rad55 and Rad57 (3, 7).

NHEJ involves binding of protein complexes (except the Sir protein complex) to the ends of linear double-stranded DNA (1, 8). The Yku70/Yku80 heterodimer binds at both ends of the DSB and initiates NHEJ. The Mrx (Mre11, Rad50, Xrs2) complex comes in and binds to the DNA-Yku structure and acts as a bridging factor between the two DNA ends. Next, the Dnl4/Lif1/Nej1 complex binds at the break site. Dnl4 is a DNA ligase that is ATP-dependent. Once the cohesive ends have been processed and gaps have been filled, Dnl4 ligates the ends and the break is repaired. The subunits of the Mrx complex perform additional functions. The Mre11 subunit of the Mrx complex has manganese-dependent 3'-to-5' dsDNA exonuclease and ssDNA endonuclease activities that are active on both linear and circular DNA structures *in vitro* and behaves as 5'-3' exonuclease *in vivo*. The Mre11 complex is required for DSB repair, checkpoint responses to DSBs as well as checkpoint activation after UV irradiation (8). The Rad50 subunit of the Mrx complex has ATP-binding activity. The function of the Xrs2 subunit of the Mrx complex is not clearly known, but it has been suggested that Xrs2 binds to Lif1, a component of the DNA ligase IV complex that is

involved in Mrx-mediated repair by NHEJ (8, 10). More recent studies have identified cell-cycle checkpoints, sister chromatid cohesion and nucleosome remodeling as important processes in DSB repair, particularly with homologous recombination.

Mutations in Rad50, Mre11 or Xrs2 (Nbs1 in humans) can have a devastating effect both in yeast and in human cells. Mutation of the Mrx complex in yeast cells results in shortened telomeres, increased sensitivity to clastogenic chemicals such as MMS and bleomycin and increased sensitivity to ionizing radiation and leads to reduced recombination in meiosis. Mutations in Mrx in humans show several similar metabolic defects as seen in yeast cells. Mutation of human hMre11 and hNbs1 (Xrs2 in yeast) results in the human disorders Nijmegen breakage syndrome and ataxia telangiectasia-like disorder (ATLD). Cells derived from individuals with these disorders exhibit high sensitivity to ionizing radiation. Inactivation of the human hMRE11 gene also results in increased incidence of cancer development (10).

DNA damage causes cells to arrest at the checkpoint in G2 phase

Checkpoint mechanisms occur during the cell cycle. Damage-induced cell cycle arrest is regulated by a large number of checkpoint genes that are involved in monitoring DNA integrity. Cell cycle checkpoints arrest the cell cycle in G_2 phase and provide DNA the time to be repaired before attempting to complete the cell cycle (11, 12). In yeast, exposure to MMS or ionizing radiation results in G_2 arrest and this arrest is more prolonged in recombination–deficient mutants such as *rad51* or *rad52* cells.

Chemical agents and endonucleases induce DSBs in cellular DNA

Damaging agents such as MMS and bleomycin induce DSBs by different

mechanisms. MMS is a DNA alkylating agent and a known carcinogen that methylates DNA at N^7 of guanine and N^3 of adenine. N^3-methyladenine inhibits DNA synthesis and needs to be repaired. Previous studies have found cells to be more sensitive to MMS while passing through S phase (5, 9). Cells exposed to MMS display a reduction in the rate of replication fork progression mainly due to physical obstruction caused by alkylated DNA and this can cause formation of DSBs. MMS–induced N^3-methyladenine lesions are corrected by three pathways: bypass repair, recombinational repair and base excision repair (14). Bleomycin is an antibiotic and is used in cancer treatment. Bleomycin kills cells primarily by induction of DNA strand breaks. Bleomycin produces several mutagenic lesions by a process involving free radicals (15).

Researchers have also expressed endonucleases in cells such as EcoRI, HO, I-SceI, etc., to induce DSBs. This approach is advantageous compared to chemicals and radiation because these nucleases produce DSBs and no other lesions. Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA. Recognition sequences vary in length between 4 and 8 nucleotides. Restriction endonucleases are categorized into three main types (Types I, II and III) based on their composition, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence. Out of these three endonucleases, Type II enzymes are most commonly available and used as restriction enzymes. Type II endonucleases are homodimers and require Mg^{2+} for their action (16). Some restriction endonucleases cut symmetrically and leave blunt ends (no overhangs) and other restriction enzymes cut asymmetrically and leave 5' overhangs or 3' overhangs. Type II endonucleases that produce 5' overhangs reach DNA from the major

groove and use an alpha helix to distinguish between their specific sites whereas Type II restriction endonucleases that produce 3' overhangs or blunt ends reach DNA from the minor groove and use beta sheets to distinguish their specific sites (17).

HO is an endonuclease enzyme that naturally exists in yeast cells that makes a site-specific DSB in the *MAT* locus on chromosome III in late G_1 HO endonuclease produces DSBs with 3' overhangs that are 4 nucleotides long (3). Rare-cutting restriction enzymes such as homing endonucleases recognize long, 14-40 bp sequences. The homing endonuclease I-SceI is an intron-encoded enzyme that is present in the mitochondria of *S. cerevisiae*. The I-SceI DNA target site is an 18 bp sequence (Figure 2). The enzyme cleaves this sequence to produce a DSB with 3' overhangs that are 4 nt long. I-SceI induces homologous recombination in cells by making a single DSB (18).

Figure 2. Recognition site of homing endonuclease I-SceI.

The restriction endonuclease enzyme EcoRI produces DSBs within DNA and has the recognition sequence GAATTC. EcoRI cleaves covalent bonds between G and A at staggered positions on both strands and the resulting fragments are joined by weak hydrogen bonds which can be easily broken to generate four base 5'-overhangs (Figure 3). The amino acids which are involved in hydrogen bonding to its target sequence are glutamic acid 144 (E144), arginine 145 (R145), and arginine 200 (R200).

EcoRI forms a dimeric globular protein complex with a molecular weight of 62 kDa that has increased specificity in the presence of Mg^{2+} (19, 20).

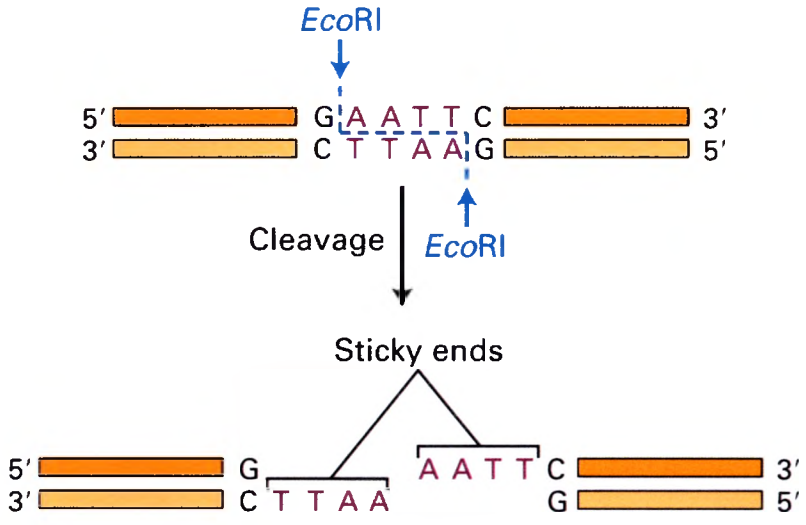

Figure 3. Schematic of EcoRI cleavage of double-stranded DNA.

EcoRI-induced DSBs cause inhibition of the growth of *rad52* strains and cell cycle arrest in G_2 phase because these mutants are known to be deficient in HR. *rad52* cells have increased rates of chromosome loss and are sensitive to X-rays, gamma rays and chemicals such as MMS and bleomycin (3).

Expression of heterologous genes in yeast cells

Different promoters are used for expression of proteins in yeast cells. Promoters can either be constitutive or regulated. The promoters *GAL1* and *GAL10*, which are tightly regulated by the galactose-glucose system, have been widely used for heterologous gene expression in yeast cells. The galactose-regulatable *GAL1* promoter in yeast cells has previously been used to express EcoRI (21, 22). The expression of EcoRI is increased in the presence of galactose and repressed in the presence of glucose.

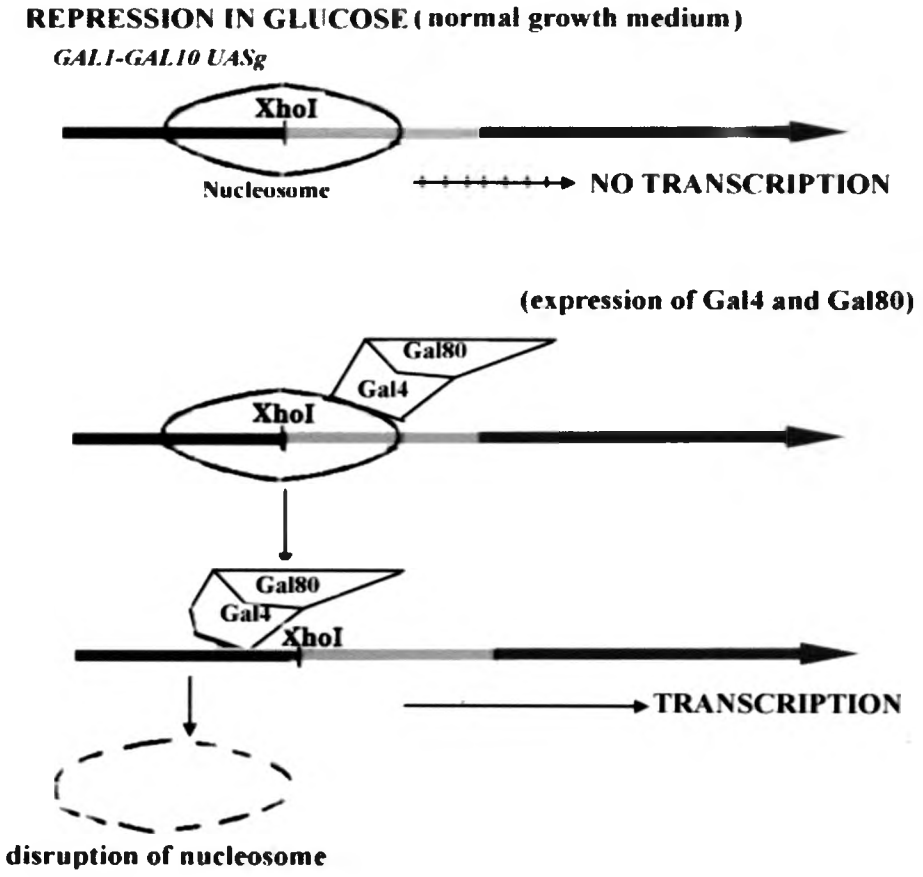

Figure 4. *GAL1* promoter expression in yeast cells.

Galactose induction occurs due to expression of chromosomal proteins Gal4 and Gal80 which makes a complex that disrupts nucleosomes and binds DNA at UASg sequences to activate transcription (Figure 4) (23).

HO endonuclease induce mating type switching in yeast cells

Haploid *Saccharomyces* cells have the potential to change mating type between a and α cells, a process that is accomplished by an intrachromosomal gene conversion between an expressor locus (*MAT)* and one of two repositories of mating type information, *HML* or *HMR.* The replacement of *MAT**a*** with the *MATα* allele is due to the presence of an additional silenced copy of both the *MATa* and *MATα* alleles. Two silent cassettes, *HMR* (Hidden MAT Right) which carries a silenced copy of the *MATa* allele and *HML* (Hidden MAT Left) that carries a silenced copy of the *MATα* allele are present at opposite ends of the chromosome. These additional loci contain unexpressed genetic information for a (*a1* and *a2* genes) or α (*α1* and *α2* genes), respectively. Mating type switching is initiated when a DSB is introduced by the enzyme HO endonuclease, which cleaves only at the *MAT* locus (24). A homology search is initiated after HO cleavage at *MATa* whereby genes at *HMLα* are recruited to the break site for repair. After repair, the *MATa* allele is replaced by that of *MATα.* This type of recombination event is called gene conversion because during restoration of the break the genetic information is transferred to the *MAT* locus from an HM site with no reciprocal exchange (24).

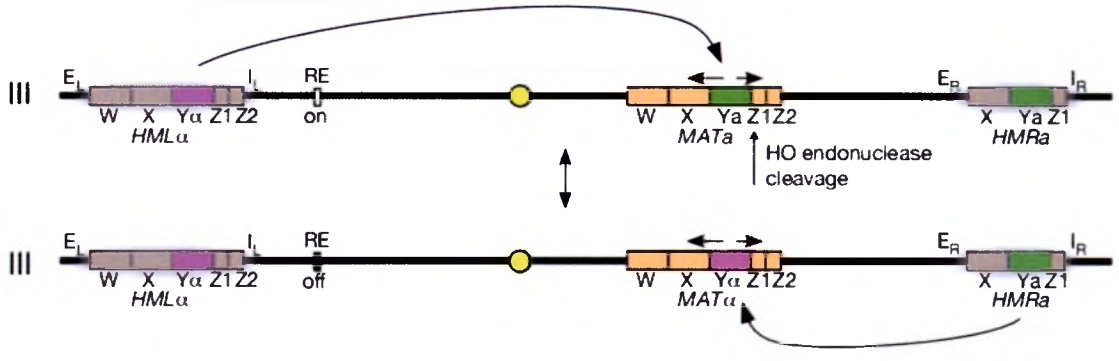

Figure 5. Schematic of *Saccharomyces cerevisiae* chromosome III. This figure illustrates the expressed *MAT* locus and silenced *HML* and *HMR* loci.

Previous studies of EcoRI and HO endonuclease-induced DSBs in yeast

Expression of the bacterial endonuclease EcoRI in yeast cells has been employed for several studies of DSB repair. In the Lewis lab, past studies tried to identify new genes involved in DSB repair. This was accomplished by screening a set of approximately 5000 different yeast mutants to identify those that were sensitive to gamma radiation. Over190 genes were found to be required for normal resistance to radiation in diploid yeast cells (25, 26). A student in the Lewis lab, Jennifer Summers, recently tested approximately half of these genes to determine which ones are essential for DSB repair. In these experiments, a *GAL1p:.EcoRI* expression plasmid was transformed separately into haploid mutants and sensitivity to expression of EcoRI was assessed. Thirty-three strains were found to be sensitive to EcoRI, implicating many genes that had not previously been associated with DSB repair pathways. In a similar fashion, graduate student Rachel Roberts screened half of the gamma-sensitive mutants as haploid cells, for sensitivity to HO endonuclease. She identified 10 genes that were essential for resistance to this nuclease.

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 and HO expression inside yeast cells. The relative sensitivities of the mutants to EcoRI, HO endonuclease, ionizing radiation and the DNA strand breaking chemicals MMS and bleomycin were used to classify the mutants into specific groups. Furthermore, the role of one of the newly identified genes, *CNM67*, in DSB repair was characterized in more detail.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Ethidium bromide (EtBr) was obtained from Shelton Scientific, Incorporated (Shelton, CT). Dimethyl sulfoxide (DMSO), ampicillin, lysozyme, RNase, and potassium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Sonicated salmon sperm carrier DNA was purchased from Stratagene (La Jolla, CA). Sodium dodecyl sulfate (SDS), sodium chloride and polyethylene glycol (PEG) 4000 were purchased from Mallinckrodt (Paris, Kentucky). Ethylenediaminetetraacetic acid (EDTA), agarose and bleomycin were obtained from EMD Chemicals, Inc. (Darmstadt, Germany). Tris base was purchased from VWR International (West Chester, PA). Lithium acetate, magnesium chloride, calcium chloride, Hoechst 33258, glutamic acid (monopotassium salt) were obtained from Sigma Chemical Company (St. Louis, MO). Klenow DNA polymerase and 1 Kb standard DNA ladder were purchased from New England Biolabs (Beverly, MA). Deoxynucleotide triphosphate (dNTP) mix was purchased from Takara (Madison, WI).

Bacteriological and Yeast Media

Raffinose, D-(+)-galactose, D-(+)-glucose, plate agar, and all amino acids were purchased from Sigma Chemical Co. (St. Louis, MO). 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).

Yeast Strains and Plasmids

BY4742 was used as a wild type strain and has a genotype of *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (27). BY4742 cells with a deletion of the *CNM67* gene (*cnm67Δ::HygB*^{r}) were designated as YLKL1089. YLKL197 (m53) and YLKL199 (m163) were used as wild type parental strains for chromosome:chromosome recombination assays (5, 28). The yeast deletion strain library was obtained from Open Biosystems (Huntsville, Al).

Cell culture solutions and media

For general 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). To assess mitochondrial function, 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 minus agar. For plasmid selection, 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. Plates with bleomycin were prepared using synthetic media or YPDA supplemented with aliquots of a stock solution of 0.5 mg/ml bleomycin to achieve varying final concentrations. *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). *E. coli* cells containing newly constructed plasmids were stored at -80 °C in 15% (v/v) glycerol.

II. METHODS

DNA Purification

Plasmid DNA was purified using either a rapid boiling lysis protocol (29) or a Qiagen Spin Column Miniprep Kit (Maryland, VA). Yeast chromosomal DNA was purified using either a kit from Epicentre or a chemical-based method developed in this lab (30).

Yeast Transformations

Yeast transformations were performed using either a rapid DMSO-based transformation protocol of Soni *et al.* (31) or the lithium acetate based high efficiency protocol of Gietz *et al.* (32).

E. coli Transformations

Plasmid DNA was transformed into DH5α *E. coli* cells using the protocol of Miller and Chung (33).

Gel Electrophoresis

Gel electrophoresis was performed using 0.6%, 0.7% and 0.8% 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 at a voltage of 110 -130V and were stained with EtBr. A Kodak IS440 CF Imager and Kodak 1D imaging software were used to capture gel images.

Gamma Irradiation of Yeast Cells

Arrays of yeast cells were irradiated using ^{137}Cs source at the national institution of Environmental Health Sciences (NIEHS) as part of a collaboration with Michael Resnick and James Westmoreland.

Replica-plating

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

Deletion of the CNM67 Gene in Haploid Yeast Strains

The *CNM67* gene was deleted in the haploid *MAT* alpha strain BY4742. PCR-based methods were applied to construct the gene deletion. DNA of the plasmid pFA6MX4::G418 was used in initial experiments and served as template for preparative PCR reactions. Genomic DNA was subjected to 32 cycles of PCR amplification with primers gCNM67a2 (GGATTCTCCTGTATCAGAGAATGGGGAAATTAAAGACGGAGAGCCAATCCC ACATGTGACTGTCGCCCGTACATT) and gCNM67b2 (CATCTTCTCCAGGATATGATCATACAGATGGTCCAGACACCTCTCCAAATGG ACAAGTTCTTGAAAACAAGAATC) at 94 °C for 2 min followed by cycling using 94 °C for 30 sec, 49 °C for 40 sec and 72 °C for 1.5 min. PCR fragments were resolved on 0.8% agarose gels to confirm size and yield. The oligonucleotide primers were utilized to

introduce short flanking regions of homology to the 5' and 3' ends of the *CNM67* gene. The PCR product was then integrated into the *CNM67* gene in BY4742 cells by homologous recombination as described below.

Transformation- The PCR product was transformed into BY4742 cells using a lithium acetate based high efficiency transformation protocol. After the heat treatment step, cells were spun down in a centrifuge and resuspended in YPDA broth. Cells were then spread on YPDA plates supplemented with ampicillin to create lawns of cells on the surface (for transformants that had integrated pFA6MX4 into *CNM67*).

Replica plating- Lawns of cells from each YPDA plate were replica-plated onto a YPDA plate that had been supplemented with G418, permitting only G418-resistant ($G418^r$) colonies to grow. $G418^r$ colonies were streaked onto fresh YPDA+G418 media and chromosomal DNA was then purified. Purified DNA was subjected to PCR, which used the same conditions as above but with different primers. The test primers 5'CNM67 (CTTGTGAAAATTCGCGTTGCGCGAC) and 3'CNM67 (CTTGGCTGGTCAGACTTTTACTATGTA) were used to confirm the deletion of the *CNM67* gene. The PCR product was resolved on a 0.8% agarose gel that used BY4742 DNA as a control. The gel showed one band for BY4742 which was used as a control, but two bands with the putative *cnm67* mutants with one band the same as wild type and the other band at the expected size of the *cnm67* mutant gene. Repetition of this experiment did not resolve the problem so a new selectable marker was used.

Deletion of the *CNM67* gene was accomplished in later experiments by employing Hygromycin B. pAG32 was used, which had a Hygromycin B resistance gene

instead of G418. Primers that were used for the PCR reaction were gCNM-c (CGGAGAGCCAATCCCACAAAACTGGCTAAATGAAAATCATGTCGGAAAATC CATCTATGTGACTGTCGCCCGTACATT) and gCNM-d (GGTGATCTATGGAATCCATCCTGCGCGTTGACATAAAATCATAGTATGCTTG ATCCGACAAGTTCTTGAAAACAAGAATC). Test primers that were used to confirm deletion of the *CNM67* gene were 5'cnm-2 (GAGTTTTAGGTACAACTTCTCGTTACATA) and 3'cnm-2 (CTCAAGATCAGAAGCATGCGTTTCGACCC). Insertion of the HygB gene into *CNM67* was successful and resulted in creation of *cnm67Δ::HygB* strains that were confirmed by PCR.

Double imprinting

To test bleomycin-sensitivity, *cnm67* gene mutants and WT cells were streaked onto YPDA plates, grown for 2 days at 30 °C and replica-plated onto YPDA plates supplemented with bleomycin. The first YPDA plate was immediately used as a new master plate to replica- plate cells to YPDA+bleomycin plates. The double imprints were grown at 30 °C for 2-3 days. WT cells were resistant to killing by bleomycin, but *cnm67* mutants showed strong sensitivity when exposed to bleomycin.

Recombination assays

For this study two recombination assays were done: Plasmid:chromosome recombination assays and chromosome:chromosome recombination assays.

Plasmid:chromosome recombination assay

Preparation of plasmid DNA to be transformed. DNA minipreps were prepared from E.coli DH5 alpha cells containing pLKL37Y. pLKL37Y DNA was then digested with BseRI under the following conditions: 79.36 µl pLKL37Y DNA, 88.64 µl ddH_2O, 20 µl 10X NEB 4 buffer, 12 µl *Bse*RI (40 units) in a final volume 200 µl. The digested DNA was precipitated and complete digestion was verified by 0.6% agarose gel electrophoresis.

Transformation. The high efficiency transformation method was used to co-transform the *LEU2* vector pRS315 (100 ng) and BseRI-cut pLKL37Y (400 ng) into WT cells and into EcoRI/gamma-sensitive mutants. Prior to transformation, a mix was made for each experiment. For a typical transformation of 10 samples, a mix for a total of 11 transformations was made with 2640 µl 50% PEG-4000, 396 µl 1 M lithium acetate, 55 µl sonicated salmon sperm carrier DNA, 5.6 µl pRS315, 13.9 µl pLKL37Y and 763 µl H_2O. A 351 µl aliquot of the mix was then used per transformation. Tubes were kept in a shaker at 30 °C for 15 min and cells were then given heat shock treatment at 42 °C for 15 min. Cells were spun down for 0.5 min in a centrifuge, resuspended in 300 ul of H_2O and 1/10 dilution was done and cells were spread on to Glu-Leu and Glu-His-Ura plates. Transformation efficiencies were calculated as the number of recombinants per µg of pLKL37Y DNA normalized to the efficiency of transformation using the unbroken vector pRS315.

Chromosome:chromosome recombination assay

The *CNM67* gene was deleted in YLKL197, which has a genotype of *MATa ura3-1 leu1-c trp5-c lys2-2 tyr1-2 his7-1 ade2-1 ade6 met13c cyh2*^{r} and YLKL199, which has a genotype of *MATα ura3-1 leu1-12 trp5-2 lys2-1 his1 ade2-1 ade5 can*^{r}. The method employed to delete the *CNM67* gene in these strains was the same as done for BY4742. YLKL197 cells containing the *cnm67::HygB*^{r} insertion were designated YLKL1090. Similarly, *cnm67::HygB*^{r} YLKL199 cells were named YLKL1091.

Double-imprinting assays were done to test the sensitivity of *cnm67* mutants to bleomycin as described above. The cells from the strains that were sensitive to bleomycin were repatched onto YPDA + HygB media and kept in an incubator for 2-3 days at 30 °C.

CNM67 diploids were made by crossing YLKL197 and YLKL199. *cnm67* mutant diploids were made by crossing *cnm67*Δ-YLKL197 and *cnm67*Δ-YLKL199. To make diploid WT cells (*CNM67*), YLKL197 and YLKL199 were streaked into each other on a YPDA plate. The same technique was used to make *cnm67* diploids by streaking cnm67Δ-YLKL197 and cnm67Δ-YLKL199 into each other on a YPDA plate. Cells were grown for 2 days at 30 °C. Cells were then replica-plated onto Glu - His plates to select for diploids.

For chromosome:chromosome recombination assays, WT and *cnm67* diploid strains were streaked onto YPDA plates. After 2-3 days of growth at 30 °C, colonies from each diploid strain were harvested, diluted and spread to Glu - Leu and Glu - Trp plates to detect *LEU1* and *TRP5* recombinants and to YPDA plates to determine total cells.

HO endonuclease survival assay for screening of strains from the yeast deletion strain library collection

Transformation of mutants with pGALHO. A total of 95 mutants from the yeast gene deletion library (Open Biosystems; Huntsville, AL) were transformed with pGALHO (*GAL10p::HO URA3*) (47) by using the DMSO-based quick transformation method and spread onto Glu - Ura plates. Three colonies from each transformation were then patched onto Glu - Ura plates.

Screening method used to identify repair-deficient strains. All mutant cells containing pGALHO were patched to selective media containing 1% raffinose (Raff) to alleviate glucose repression of the *GAL10* promoter. These patches were then replica-plated, using velvets, to a 1% raffinose minus uracil (Raff – Ura) plate. The Raff - Ura plate was then immediately used as a new master plate to replica-plate cells to 1% raffinose + 3% galactose minus uracil and 2% galactose minus uracil plates and grown at 30 °C for 2-3 days. Mutants were then classified as having WT sensitivity (mutants that grew on all three media), moderate sensitivity (mutants that grew on Raff - Ura and Raff + Gal - Ura but not on Gal - Ura) and high sensitivity (mutants that were able to grow only on Raff - Ura plates). Transformation with the vector pRS316 was then done to serve as controls to confirm the sensitivity of moderately and highly sensitive mutants using dilution pronging.

Dilution pronging survival assays. All mutants that were found to be sensitive to HO endonuclease using replica-plating were then further quantitatively tested using survival pronging assays. Both mutants containing pGALHO and mutants containing

pRS316 were initially cultivated on 1% Raff – Ura plates by allowing growth at 30 °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 concentration of $2 – 3 \times 10^7$ cells was added to H_2O in a sterile 96-well microtiter dish for 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 rows 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 as well as 2% Gal-Ura. After 3-4 days of growth at 30 °C, the plates were evaluated for sensitivity to HO-induced DSBs.

Cell Cycle Analysis of EcoRI Sensitive Mutants

WT cells, mutants that showed sensitivity to *Eco*RI, and 14 control DSB repair-deficient mutants were harvested from either synthetic or YPDA plates into sterile deionized H_2O, diluted 1/40, briefly sonicated for 10-12 sec at 3 watts using a Sonics Vibracell Ultrasonic Processor (Newtown, CT), and quantitated using a hemocytometer on a phase contrast microscope. Cells were then diluted into three YPDA cultures at $3x10^6$ cells/ml for each mutant and cultures (500 µl) were shaken vigorously for 4 hrs at 30 °C. Cells from each culture were then sonicated and a hemacytometer was used to analyze cells that were unbudded, small-budded, or large-budded. Large-budded cells were defined as cells in which the size of the bud was ≥50% of the size of the mother cell (3, 5).

CHAPTER III

RESULTS AND DISCUSSION

The major goal of this project was to expand upon earlier studies in the Lewis lab that identified new genes involved in repair of broken chromosome in yeast (34, 35). One of those studies (35) involved testing a large number of mutants that were previously found to be sensitive to gamma radiation as diploids for sensitivity to killing by *in vivo* expression of the endonuclease EcoRI. The gamma radiation screens were published by Bennett *et al.* (25, 26) and resulted in the identification of 195 genes that cause diploid yeast cells to become gamma^s when they are inactivated. Two other known RAD52 group genes, *RAD54* and *MRE11,* were not tested by Bennett *et al.* but are known to be gamma^s, bringing the total number of genes to 197. Between the previous study (35) and the current tests, all 197 mutants were transformed with EcoRI expression plasmids as haploid strains to test sensitivity to EcoRI expression.

In the other study (34) in the Lewis lab, the same mutants were tested for sensitivity to expression of HO endonuclease, which makes a single DSB on chromosome III. This mechanism of action is different from EcoRI, which creates many DSBs when expressed in yeast cells (3). In these earlier EcoRI and HO sensitivity studies, only one-half of the original gamma-sensitive mutants (25, 26) were tested for EcoRI or HO sensitivity. The current project has expanded upon both previous projects and tested all genes required for resistance to gamma rays in diploids to determine if they are also required for repair of HO and EcoRI-induced DSBs. In addition, the role of one of the new genes, *CNM67,* in DSB repair was

explored in more detail.

Yeast deletion strain library

The yeast deletion strain library is a collection of approximately 5000 different haploid mutants in which one non-essential gene is knocked out in each mutant (25). The strain background of the library is BY4742, and the mutant strains are *MAT*α (mating type). The library is stored at -80 °C in multiple 96-well microtiter dishes. Each well of the microtiter dish contains a different mutant with columns and rows prepared and labelled as shown in Figure 6.

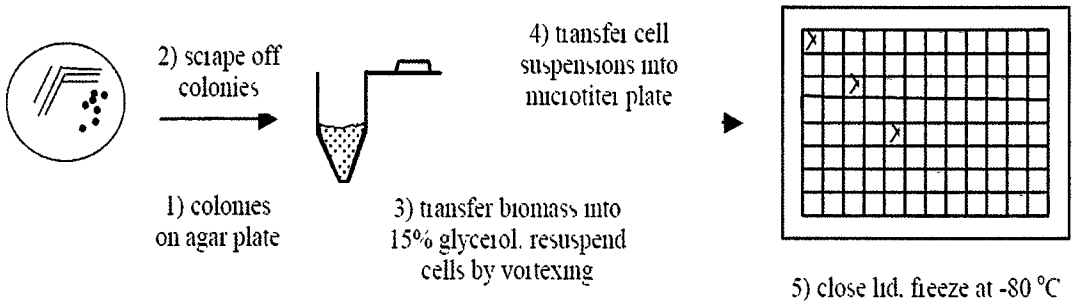

Figure 6. Representation of a microtiter dish from the yeast deletion strain library. Source: http://www.kuhner.com/_upl/files/b020_250_1_.pdf

Application of dilution pronging cell survival assays

The EcoRI survival assay developed by Jennifer Summers in the Lewis lab was used to screen mutants from the yeast deletion library for sensitivity to EcoRI. For these experiments, mutant cells were transformed with the *GAL1p::EcoRI* expression plasmid YcpGal::R1b (24) and grown on glucose minus uracil (Glu – Ura) plates to keep expression of EcoRI repressed. Initially double-imprint replica plating was used to characterize mutants for EcoRI sensitivity, where plates with different media were used.

Raffinose was used because *GAL1p* is not repressed nor is it induced and resulted in faster induction upon transfer to galactose than going directly from glucose to galactose. EcoRI expression is fully induced in galactose plate media. In this approach, a plate containing patches of cells was replica-plated to a 1% raffinose-uracil plate, followed immediately by using this second plate as a new master for a second round of replica-plating, transferring cells to 1% Raff - Ura, 1% Raff + 3% Gal – Ura and 2% Gal - Ura. Fewer cells are transferred to each plate with this method, which allowed easier detection of EcoRI-induced killing.

Dilution pronging assays are a more quantitative method than conventional replica-plating or double-imprint replica-plating and was used for this project also. These experiments involved harvesting cells and placing them in the first well on the left side of a 96 well microtiter dish at a specific concentration. Usually 2 x 10^7 cells in 220 µl ddH_2O were serially diluted 5-fold horizontally for six wells along the microtiter dish. A metal pronger was placed in the microtiter dish wells and then placed onto a plate, transferring the cells onto the surface. This method allowed quantification of cell growth rates and survival. BY4742 was used as a control for these experiments.

In the recent EcoRI sensitivity study, Jennifer Summers identified 33 new mutants as EcoRI-sensitive. In the new experiments for the current study, 96 additional mutants were screened and 28 of these were found to be EcoRI-sensitive. Out of all mutants tested, including 7 known RAD52 group DSB repair-deficient strains treated as controls (*mre11, rad50, rad51, rad52, rad54, rad55* and *rad57),* a total of 107 mutants did not show sensitivity to EcoRI expression. Twenty mutants could not be tested because of their poor growth on plates. All mutants from both studies (combined) that were not sensitive are listed in Table 1a and mutants that could not be tested are listed in Table 1b.

One mutant, *cdc40*, was Ura^+ and therefore could not be transformed with the

URA3 plasmid YCpGal::RIb. Another mutant, *ade12*, did not yield colonies despite repeated transformation attempts. Two other mutants (*rus167* and *xrs2)*, were not present in the MATα haploid strain library. Finally, the *cwh36* locus was not tested because it does not encode a functional gene. A few mutants were tested for EcoRI sensitivity that were not identified in the original radiation study as sensitive to gamma rays as diploids. These mutants, which are marked with an asterisk in the tables, were tested because they had been linked to DSB repair in previous studies. For example, *dnl4, nej1, sir2, sir3* and *sir4* have previously been linked to the NHEJ pathway of DSB repair.

Mutants were classified as R (Resistant to EcoRI), S (moderately sensitive), or SS (Strongly sensitive). Moderately sensitive mutants exhibited < 25-fold killing relative to vector controls on plates with galactose only. Strongly sensitive mutants exhibited ≥ 25-fold killing on plates with galactose only.

Table 1a. Mutants categorized as not sensitive to *in vivo* expression of EcoRI[a]

apn1	*her2*	*pfd1*	*slx8*
ard1	*hmo1*	*pfk2*	*smi1*
arp8	*hof1*	*pho2*	*srb5*
asf1	*hpr1*	*plc1*	*srs2*
atp4	*iwr1*	*pol32*	*sse1*
bdf1	*jem1*	*pso2*	*ssz1*
bem1	*ldb7*	*rad1*	*tho2*
bfr1	*lge1*	*rad10*	*tif4631*
bmh1	*lhs1*	*rad17*	*tps1*
bre1	*lif1**	*rad18*	*tps2*
*cbc2**	*loc1*	*rad24*	*tup1*
chl1	*map1*	*rad27*	*ubc13*
ckb1	*mbp1*	*rad59*	*vid31*
deg1	*mdj1*	*rad6*	*vps33*
dhh1	*mec3*	*rad61*	*vps65*
dia4	*mnn11*	*rad9*	*yaf9*
*dnl4**	*mrp10*	*rai1*	*ybr100w*[b]
doc1	*mrpl31*	*rdh54*	*ydj1*
dot1	*mrt4*	*ref2*	*ydl041w*
*dun1**	*mus81*	*rim1*	*ydr532c*
eap1	*nat1*	*rmd6*	*yjl193w*
efg1	*nat3*	*rpl34b*	*yku70**
eos1	*nej1**	*rsa1*	*yku80**
est1	*not4*	*rsc1*	*ylr358c*
fab1	*npl6**	*rsc2*	*yml009c-a*
fil1	*nup120*	*scp160*	*ypl066w*
fun12	*nup133*	*sgs1*	*ypl071c*
glo3	*nup170*	*sir2**	*zuo1*
gos1	*nup188*	*sir3**	
grr1	*pat1*	*sir4**	

* Mutants not listed in Bennett papers that described gamma-sensitive diploid mutants

[a] Genes listed in the table are taken from the 1st part of this project (35) and the current work combined

[b] *ybr100w* contains a deletion of part of the *MMS4* gene and does not encode a discrete protein

Table 1b. Mutants not tested[a]

Mutant	Reason Not Tested
ade12	Could not get Ura^+ colonies after transformation with pGAL1p::EcoRI plasmid.
ada2, arp5, asm4, bik1, bud30, bud32, ccr4, clc1, gon7, hfi1, mdm10, mdm20, pre9, rsm7, rtf1, she1, sac6, sco1, ye1033w, ygl218w	Grows poorly
cdc40	Ura^+
*rvs167, xrs2**	Absent from library
cwh36	Not a gene. Partial deletion of *VMA9*

*Mutants not listed in Bennett papers

[a] Genes listed in the table are taken from the 1st part of this project (35) and the current work combined.

Thirty-one mutants were classified as strongly sensitive to EcoRI and these genes are shown in Table 2a. The seven genes at the top of Table 2a (*rad50* - *rad57* plus *mre11*) are known to be sensitive to EcoRI (3, 5) and were used as controls. Thirty mutants were classified as moderately $EcoRI^S$ (Table 2b). Two of these 30 (*exo1* and *rad5*) were not found in the original gamma-sensitivity screen, so only 28 new mutants from the gamma screen were moderately $EcoRI^s$. Thus, the total number of new EcoRI-sensitive mutants was 61 with 59 of them having been identified in the gamma screen.

Table 2a Mutants characterized as most EcoRI-sensitive[a]

*mre11**	*rad52*
rad50	*rad54**
rad51	*rad55*
	rad57
akr1	
(anc1)taf14	*htl1*
apq13	*img2*
atp2	*lrp1*
bck1	*mms2*
bud19	*mms22*
cax4	*mms4*
cgi121	*not5*
cis3	*sam37*
cnm67	*sfp1*
ctf4	*ume6*
ctf8	*vma7*
dcc1	*vph2*
gcn5	*ybr099c*
gnd1	*ydr433-w*
hsp150	*yml009w-b*

*Mutants not listed in Bennett papers

[a] Genes listed in table 2a and 2b are taken from the 1st part of this project (35) and the current work combined

Table 2b Mutants characterized as moderately sensitive

adk1	*rvs161*
ado1	*sae2*
bur2	*slm4*
ddc1	*spt10*
*exo1**	*spt20*
ids2	*srv2*
lip5	*trm9*
lsm7	*tsr2*
mct1	*ubr1*
mrps35	*vid21*
nup84	*ydr417c*
och1	*ylr235c*
*rad5**	*yml012c-a*
rem50	*ynr068c*
rpb9	
rpl31a	

*Mutants not listed in Bennett papers

Assessment of resistance to MMS and bleomycin in EcoRI-sensitive mutants

Bleomycin is known to cause the introduction of DSBs into DNA molecules. Previous studies done on the control mutants used for this project (e.g., *rad50, rad51, rad52, rad54, rad57* and *rad59*) had shown sensitivity to physical and chemical DNA damaging agents (14). Here we tried to compare the effects of clastogens such as MMS and bleomycin to those of EcoRI, where the sensitivity of each new mutant to MMS and bleomycin was determined. Pronging survival assays were carried out on solid medium as described before in the presence of varying concentrations of bleomycin and MMS.

To test for bleomycin sensitivity, the 61 EcoRI-sensitive mutants plus seven control DSB repair mutants were pronged to synthetic Glu plates and to Glu plates containing 2 μg/ml or 4 μg/ml bleomycin. Wild type BY4742 cells were used as a control. The parameters that were used to categorize mutants were as follows: ≥ 125- fold killing (SS), < 125-fold killing (S), and survival equal to that of WT (R).

Another agent known to induce strand breaks in DNA is MMS. To test for MMS sensitivity, the same set of 61 EcoRI-sensitive strains and seven controls were pronged to Glu, Glu + 1 mM MMS, and Glu + 2 mM MMS plates. The parameters that were used for characterization were the same as described above. Results that were obtained from MMS and bleomycin assays are shown in Table 3 for the most sensitive EcoRI mutants and Table 4 with the moderately sensitive EcoRI mutants.

Table 3. Resistance of strongly EcoRI^S mutants to MMS and bleomycin[a]

Mutant	MMS		Bleomycin		Mutant	MMS		Bleomycin	
	1 mM	2 mM	2 µg/ml	4 µg/ml		1 mM	2 mM	2 µg/ml	4 µg/ml
Controls									
WT	R	R	R	R	*rad52*	SS	SS	S	SS
mre11	SS	SS	S	SS	*rad54*	SS	SS	S	SS
rad50	SS	SS	S	SS	*rad55*	SS	SS	R	SS
rad51	SS	SS	S	SS	*rad57*	SS	SS	R	SS
Most *EcoRI*^{S}									
akr1	R	R	R	R	*hsp150*	R	R	R	R
anc1-taf14	S	SS	R	S	*img2*	R	R	R	S
atp2	S	SS	S	S	*lrp1*	R	R	R	S
apq13	S	SS	S	SS	*mms2*	S	SS	R	R
bck1	S	S	R	R	*mms4*	S	SS	R	R
bud19	S	S	SS	SS	*mms22*	SS	SS	R	S
cax4	R	R	SS	SS	*not5*	R	R	R	R
cgi121	R	R	S	S	*sam37*	S	SS	S	SS
cis3	S	SS	R	S	*sfp1*	R	R	R	S
cnm67	R	S	R	SS	*vma7*	S	S	SS	SS
ctf4	SS	SS	R	S	*ume6*	S	S	R	R
ctf8	R	SS	R	R	*vph2*	R	R	SS	SS
dcc1	S	SS	R	R	*ybr099c*	S	SS	R	R
gnc5	R	SS	R	S	*ydr433-w*	R	SS	SS	SS
gnd1	S	S	R	SS	*yml009w-b*	S	SS	R	S
htl1	R	S	SS	SS					

[a]The results shown are a summary of previous work (35) and the current work.

Table 4. Resistance of moderately EcoRI^S mutants to MMS and bleomycin

Mutant	MMS		Bleomycin		Mutant	MMS		Bleomycin	
	1 mM	2 mM	2 µg/ml	4 µg/ml		1 mM	2 mM	2 µg/ml	4 µg/ml
Moderate EcoRI^s									
adk1	R	R	SS	SS	*rpl31a*	R	R	R	R
ado1	S	S	SS	SS	*rvs161*	R	R	R	SS
bur2	R	S	R	S	*sae2*	R	S	R	R
ddc1	R	S	R	R	*slm4*	S	SS	S	SS
exo1	R	R	R	R	*spt10*	R	S	SS	SS
ids2	R	R	R	R	*spt20*	R	R	S	SS
lip5	R	R	R	S	*srv2*	R	R	R	R
lsm7	S	S	R	S	*trm9*	S	S	S	S
mct1	S	SS	R	S	*tsr2*	R	R	R	S
mrps35	R	S	R	S	*ubr1*	S	S	R	S
nup84	R	S	S	SS	*vid21*	R	S	R	S
och1	R	S	S	SS	*ydr417c*	S	SS	S	SS
rad5	SS	SS	S	SS	*ylr235c*	SS	SS	R	S
rem50	R	SS	R	S	*yml012c-a*	S	SS	R	S
rpb9	R	SS	S	SS	*ynr068c*	SS	SS	S	SS

By looking at the results obtained from survival assays it was observed that all control mutants showed strong sensitivity towards MMS and bleomycin and were characterized as SS at the highest concentration of each drug. Twenty-one of the 31 most EcoRI^s mutants were sensitive to 2 mM MMS and 20 of the 31 mutants were sensitive to 4 µg/ml bleomycin. Twenty of the 30 moderately EcoRI^s mutants were sensitive to 2 mM MMS, whereas 24 of the 30 mutants showed sensitivity to 4 µg/ml bleomycin. Overall, 43 of the 61 mutants were MMS-sensitive and 45 were bleomycin-sensitive. Surprisingly, 7 of the 61 mutants were not sensitive to either MMS or bleomycin.

Assessment of resistance to gamma radiation in EcoRI^s mutants

Ionizing radiation such as gamma rays causes damage to DNA by generating free

radicals within the cell. In this project, all 61 mutants that were sensitive to EcoRI were tested for their sensitivity to gamma radiation at different doses. Dilution pronging assays were done as described above. The assays were performed first on several mutants that are known to be defective in HR, which included *mre11, rad50, rad51, rad52, rad54* and *rad57*. A total of 2×10^7 cells were placed in the wells on the left sides of each microtiter dish and were then diluted 5-fold serially from left to right. For each experiment the first row on each plate was WT cells. The subsequent rows consisted of mutants that had been identified to be sensitive to EcoRI. Each set of strains was pronged to YPDA plates and were then exposed to a ^{137}Cs source at doses of 30 and 60 krad. WT cells showed no sensitivity to gamma radiation whereas mutants that were used as controls exhibited strong sensitivity (Figure 7A). In Figure 7B, the first row represents WT cells whereas seven subsequent rows consisted of mutants that showed sensitivity to EcoRI. At the high dose, WT cells and mutants *adk1*, *bur2*, *ddc1* and *srv2* showed no effect, but killing was seen for *exo1*, *mct1*, and *spt10* cells.

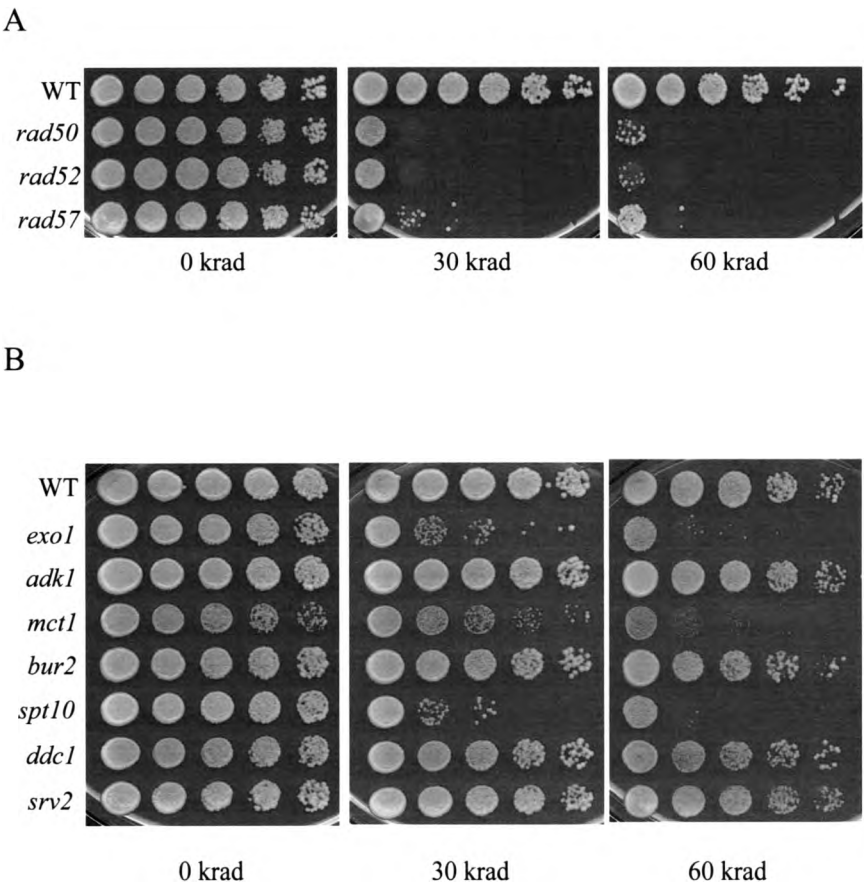

Figure 7. Dilution pronging assays testing radiation resistance of control and new mutants. The control for this assay was WT. (A) Homologous recombination mutants. (B) Mutants that exhibited EcoRI sensitivity.

The criteria that were established to categorize mutants were: SS (strongly sensitive), significant growth in only 1-2 columns; S (sensitive) growth in only 3-4 columns; and R (Resistant), growth similar to WT cells. Out of all 61 EcoRI-sensitive mutants, only 7 mutants showed sensitivity at 30 krad and only 12 mutants showed sensitivity at 60 krad. These mutants and their sensitivities are listed in Table 5.

Table 5. Characterization of gamma sensitivities of EcoRI^S mutants

Mutant	30K	60K	Mutant	30K	60K
mre11	SS	SS	*rad52*	SS	SS
rad50	SS	SS	*rad54*	SS	SS
rad51	SS	SS	*rad57*	SS	SS
adk1	R	R	*nup84*	R	R
ado1	R	S	*och1*	R	R
akr1	R	R	*rad5*	R	R
apq13	R	R	*rem50*	R	R
atp2	R	R	*rpb9*	S	SS
bck1	R	S	*rpl31a*	R	R
bud19	R	R	*rvs161*	R	R
bur2	R	R	*sae2*	R	R
cax4	S	S	*sam37*	R	R
cgi121	SS	SS	*sfp1*	R	R
cis3	R	R	*slm4*	R	R
cnm67	R	S	*spt10*	S	SS
ctf4	R	R	*spt20*	R	R
ctf8	R	R	*srv2*	R	R
dcc1	R	R	*taf14*	R	R
ddc1	R	R	*trm9*	R	R
exo1	S	SS	*tsr2*	R	R
gnc5	R	R	*ubr1*	SS	SS
gnd1	R	R	*ume6*	R	R
hsp150	R	R	*vid21*	R	R
htl1	S	SS	*vma7*	R	S
img2	R	R	*vph2*	R	R
ids2	R	R	*ybr099c*	R	R
lip5	R	S	*ydr417c*	R	R
lrp1	R	R	*ydr433-w*	R	R
lsm7	R	R	*ylr235c*	R	R
mct1	R	SS	*yml012c-a*	R	R
mms2	R	R	*yml009w-b*	R	R
mms22	R	R	*ynr068c*	R	R
mms4	R	R			
mrps35	R	R			
not5	R	R			

All 61 mutants that exhibited sensitivity to EcoRI expression *in vivo* were grouped into profiles based on their sensitivity to all agents. All mutants that were found

to be sensitive to EcoRI and their cross sensitivities to other DNA damaging agents are summarized in Table 6. Among the 61 mutants noted above, more than half were sensitive to MMS and bleomycin. Specifically, 43 of 61 EcoRI^S mutants were sensitive to MMS and 45 of the 61 mutants were sensitive to bleomycin. It was interesting to see that mutant *bck1* exhibited sensitivity to all DNA damaging agents except bleomycin, whereas mutants *cax4*, *cgi121* and *lip5* were sensitive to bleomycin, gamma radiation and EcoRI but were resistant to MMS. Only 13 of the mutants were found to be sensitive to gamma irradiation. *rad52* mutants (shown in bold) are sensitive to all four DNA damaging agents. Only 8 of the 61 new mutants were similarly sensitive to all agents.

Table 6. Resistance of EcoRI^S mutants to DNA damaging agents[a]

EcoRI^S	MMS^S	Bleo^S	Gamma^S	***rad52***, *cnm67, htl1, vma7, ado1, mct1, rpb9, spt10, ubr1*
			Gamma^R	*(anc1)taf14, atp2, apq13, bud19, cis3, ctf4, gnc5, gnd1,lsm7, sam37, bur2, mms22, mrps35, nup84, och1, rad5, rem50, slm4, trm9, vid21, ydr417c, ylr235c, yml012c-a, ynr068c, yml009w-b, ydr433-w*
		Bleo^R	Gamma^S	*bck1*
			Gamma^R	*dcc1, mms2, mms4, ume6, ybr099c, ddc1, sae2, ctf8*
	MMS^R	Bleo^S	Gamma^S	*cax4, cgi121, lip5*
			Gamma^R	*img2, lrp1, sfp1, vph2, adk1, rvs161, spt20, tsr2*
		Bleo^R	Gamma^S	*exo1*
			Gamma^R	*akr1, hsp150, not5, ids2, rpl31a, srv2*

[a] Genes listed in the table are taken from the 1st part of this project (35) and the current work combined

Cell cycle analysis of cultures of known DSB repair mutants and EcoRI-sensitive mutants

The cell cycle consists of 4 phases, G_1/S/G_2/M. Entry into each phase of the cell cycle is carefully regulated by receptor collectives, termed cell-cycle checkpoints (36). Checkpoint control genes are important for cell cycle delay, DNA repair and apoptosis after DNA is damaged. An increased level of DNA damage is present in cells that are defective in DNA repair. In yeast cells, this unrepaired damage often results in an increased fraction of cells that are in G_2 phase during normal, log phase growth. The damage signals the cells to pause growth in G_2 to allow time to repair the damage before chromosomes are attached to the spindle and pulled apart during M phase (36, 37).

In the previous study done by graduate student Jennifer Summers in the Lewis lab, she screened 33 EcoRI^s mutants (along with thirteen known HR, NHEJ, and checkpoint mutants as controls) to measure spontaneous cell cycle arrest in G_2 phase. Log phase cultures of all mutants known to be involved in homologous recombination exhibited higher number of G_2/M cells compared to wild type cells, indicating that they had high levels of unrepaired DNA damage. In contrast, most mutants involved in NHEJ did not show high levels of G_2/M cells, which reinforces the idea that it is not the primary pathway for DNA repair in yeast. In this current project all new EcoRI^s mutants were tested to determine if they have constitutively activated DNA damage checkpoints and high levels of G_2/M cells. For this experiment cells were harvested from plates and inoculated into YPDA cultures at a starting concentration of 1 x 10^6 cells/ml. Cells were then grown for 5 hours in a shaker at 30 °C to reach mid-log growth phase. Three cultures of each strain were counted using a light microscope and the percentage of G_2/M phase cells was then calculated out of 100 cells for each culture. G_1 cells are unbudded (single cell with no bud), S phase cells have a bud < 50% of the size of the parent cell, and G_2/M cells have a bud $\geq$ 50% of the size of parent cell (Figure 8).

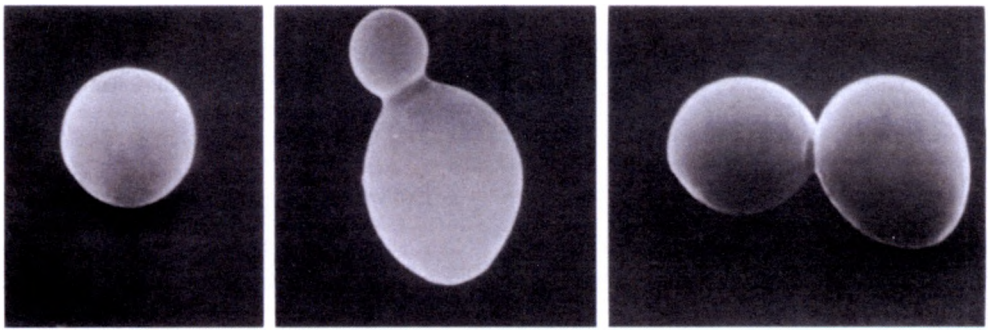

Figure 8. Representation of cells and bud size for each phase of the cell cycle (38).

The percentage of G_2/M phase cells in each set of three cultures were averaged from two separate experiments (6 cultures for each mutant) and were then graphed with standard deviations. Only 13 of the 61 EcoRI^s mutants exhibited high levels of G_2/M cells during normal growth. In Figure 9 the average percentages are shown for WT cells and the mutant cells that showed high levels of G_2/M cells. This phenotype, which was also observed in *rad50, rad51, rad52* and *rad54* cells, suggest that these 13 mutants have high levels of unrepaired damage in their DNA which causes them to pause frequently in G_2/M.

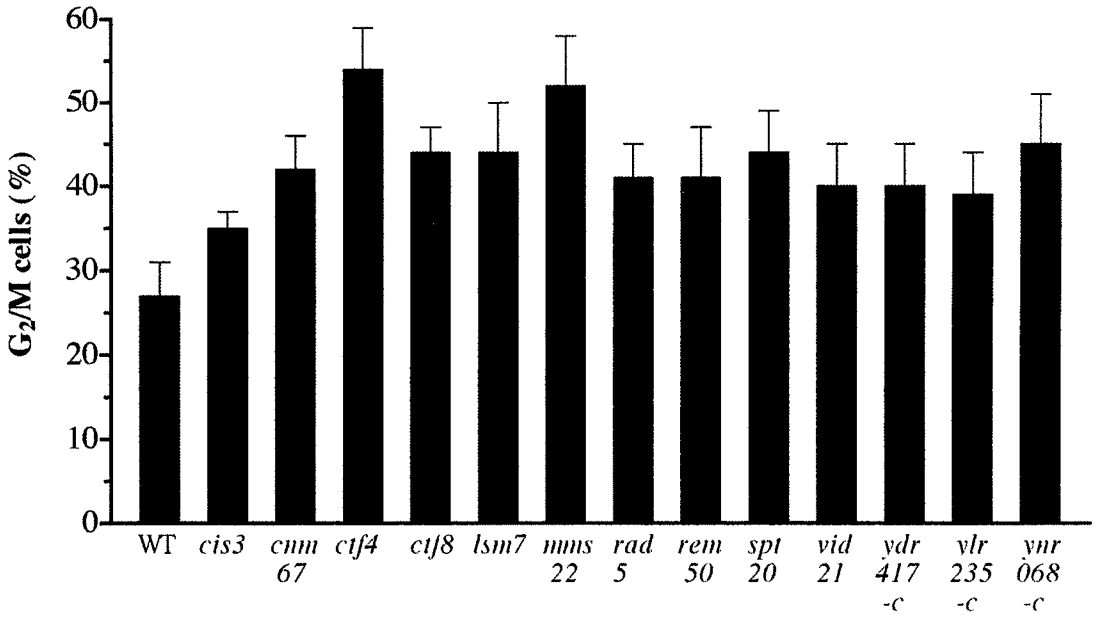

Figure 9. Characterization of spontaneous levels of G_2/M cells in mutants that were sensitive to EcoRI. Mutants that exhibited percentages of G_2/M cells greater than that of WT. Genes listed in the figure are taken from the 1st part of this project (35) and the current work combined.

The names of the 61 mutants that were found to be most sensitive and moderately sensitive to expression of EcoRI *in vivo* are summarized in Table 7 and Table 8 along with brief gene descriptions.

Table 7. Strongly EcoRI^S mutants-gene descriptions

Gene Name	Name Description	Description
*mre11**	Meiotic Recombination	Subunit of Mrx that functions in repair of DNA DSBs and in telomere stability, nuclease activity required for Mrx function; widely conserved
*rad50**	Radiation Sensitive	Subunit of Mrx complex, initiation of meiotic DSBs, telomere maintenance, and NHEJ
*rad51**	Radiation Sensitive	Strand exchange protein, forms a helical filament with DNA that searches for homology; HR repair of DSBs in DNA; homolog of Dmc1p and bacterial RecA protein
*rad52**	Radiation Sensitive	Protein stimulates strand exchange by facilitating Rad51p binding to ssDNA; anneals complementary single-stranded DNA; repair of DSBs in DNA
*rad55**	Radiation Sensitive	Protein that stimulates strand exchange by stabilizing the binding of Rad51p to ssDNA; HR of DSBs in DNA; forms heterodimer with Rad57p
akr1	Ankyrin Repeat-containing Protein	Palmitoyl transferase involved in protein palmitoylation; negative regulator of pheromone response pathway; required for endocytosis of pheromone receptors; involved in cell shape control; contains ankyrin repeats
atp2	ATP Synthase	Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase, which is a large, evolutionarily conserved enzyme complex required for ATP synthesis; phosphorylated
apq13	N/A	Dubious open reading frame, unlikely to encode a protein; not conserved in closely related Saccharomyces species; 85% of ORF overlaps the verified gene *NET1* which is involved in nucleolar silencing and RNA pol I transcription
bck1	Bypass of C Kinase	Mitogen-activated protein (MAP) kinase acting in the protein kinase C signaling pathway, which controls cell integrity; upon activation by Pkc1p phosphorylates downstream kinases Mkk1p and Mkk2p
bud19	Bud Site Selection	Dubious open reading frame, unlikely to encode a protein; not conserved in closely related Saccharomyces species; 88% of ORF overlaps the verified gene *RPL39* which encodes a component of the 60S ribosomal subunit
cax4	calmodulin-dependent in CMD 1-26	Dolichyl pyrophosphate (Dol-P-P) phosphatase, cleaves the anhydride linkage in Dol-P-P, required for Dol-P-P-linked oligosaccharide intermediate synthesis and protein N-glycosylation
cgi121	N/A	Protein involved in telomere uncapping and elongation as component of the KEOPS protein complex with Bud23, Kae1p, Pcc1p, and Gon7p; also shown to be a component of the EKC protein complex; homolog of human GCI-121

cis3	CLK1 Suppressing	Mannose-containing glycoprotein constituent of the cell wall; member of the PIR (proteins with internal repeats) family
cnm67	Chaotic Nuclear Migration	Component of the spindle pole body outer plaque; required for spindle orientation and mitotic nuclear migration
ctf4	Chromosome Transmission Fidelity	Chromatin-associated protein, required for sister chromatid cohesion; interacts with DNA polymerase alpha (Pol1p)
ctf8	Chromosome Transmission Fidelity	Subunit of a complex with Ctf18p,shares subunits with Replication Factor C and is required for sister chromatid cohesion
dcc1	DNA Damage Checkpoint	Subunit of a complex with Ctf8p and Ctf18p, shares components with Replication Factor C, required for sister chromatid cohesion and telomere length maintenance
gcn5	General Control Nonderepressible	Histone acetyltransferase, acetylates N-terminal lysines on histones H2B and H3; catalytic subunit of the ADA and SAGA histone acetyltransferase complexes; founding member of the Gcn5p-related N-acetyltransferase superfamily
gnd1	N/A	6-phosphogluconate dehydrogenase (decarboxylating), catalyzes an NADPH regenerating reaction in the pentose phosphate pathway; required for growth on D-glucono-delta-lactone and adaptation to oxidative stress
htl1	High-Temperature Lethal	Component of the RSC chromatin remodeling complex; RSC functions in transcriptional regulation and elongation, chromosome stability, and establishing sister chromatid cohesion; involved in telomere maintenance
hsp150	Heat Shock Protein	O-mannosylated heat shock protein that is secreted and covalently attached to the cell wall via beta-1,3-glucan and disulfide bridges; required for cell wall stability; induced by heat shock, oxidative stress, and nitrogen limitation
img2	Integrity of Mitochondrial Genome	Mitochondrial ribosomal protein of the small subunit
lrp1	Like an rRNA Processing Protein	Substrate-specific nuclear cofactor for exosome activity in the processing of stable RNAs; required for telomere length maintenance; homolog of mammalian nuclear matrix protein C1D involved in regulation of DNA repair and recombination.
mms2	Methyl Methanesulfonate	Protein involved in error-free postreplication DNA repair; forms a heteromeric complex with Ubc13p that has a

	Sensitivity	ubiquitin-conjugating activity; cooperates with chromatin-associated RING finger proteins, Rad18p and Rad5p
mms4	Methyl Methanesulfonate Sensitivity	Subunit of the structure-specific Mms4p-Mus81p endonuclease that cleaves branched DNA; involved in recombination and DNA repair
mms22	Methyl Methanesulfonate	Protein acts with Mms1p in a repair pathway that may be involved in resolving replication intermediates or preventing the damage caused by blocked replication forks; required for accurate meiotic chromosome segregation
not5	N/A	Subunit of the CCR4-NOT complex, which is a global transcriptional regulator with roles in transcription initiation and elongation and in mRNA degradation
sam37	Sorting and Assembly Machinery	Component of the mitochondrial outer membrane sorting and assembly machinery (SAM) complex; required for the sorting of some proteins to the outer membrane after import by the TOM complex
sfp1	Split Finger Protein	Transcription factor that controls expression of many ribosome biogenesis genes in response to nutrients and stress, regulates G2/M transitions during mitotic cell cycle and DNA-damage response, involved in cell size modulation
taf14	TATA Binding Protein-associated Factor	Subunit of TFIID, TFIIF, INO80, SWI/SNF, and NuA3 complexes, involved in RNA polymerase II transcription initiation and in chromatin modification;
ume6	Unscheduled Meiotic Gene Expression	Key transcriptional regulator of early meiotic genes, binds URS1 upstream regulatory sequence, couples metabolic responses to nutritional cues with initiation and progression of meiosis, forms complex with Ime1p, and also with Sin3p-Rpd3p
vma7	N/A	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
vph2	Vacuolar pH	Integral membrane protein required for vacuolar H+-ATPase (V-ATPase) function, although not an actual component of the V-ATPase complex; functions in the assembly of the V-ATPase; localized to the endoplasmic reticulum
ybr099c	N/A	Dubious open reading frame unlikely to encode a protein, completely overlaps the verified gene *MMS4*
ydr433w	N/A	Dubious open reading frame unlikely to encode a functional protein. Overlaps *NPL3*, involved in mRNA processing and transport

yml009w-b	N/A	Dubious ORF unlikely to encode a functional protein, overlaps *SPT5* gene involved in RNA pol I and pol II transcription, deletion mutation confers an increase in Ty1 transposistion

*Well-characterized recombination genes used as controls.
[a] Genes listed in the Table 7 and Table 8 are taken from the 1st part of this project (35) and the current work combined. Descriptions are taken from the Saccharomyces Genome Database.

Table 8. Moderately EcoRI[S] mutants-gene descriptions

Gene Name	Name Description	Description
*rad54**	Radiation Sensitive	DNA-dependent ATPase, stimulates strand exchange by modifying the topology of double-stranded DNA, involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis; member of the SWI/SNF family
*rad57**	Radiation Sensitive	Protein that stimulates strand exchange by stabilizing the binding of Rad51p to single-stranded DNA; involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis; forms heterodimer with Rad55p
adk1	Adenylate Kinase	Adenylate kinase, required for purine metabolism; localized to the cytoplasm and the mitochondria; lacks cleavable signal sequence
ado1	ADenOsine Kinase	Adenosine kinase, required for the utilization of S-adenosylmethionine (AdoMet); may be involved in recycling adenosine produced through the methyl cycle
bur2	Bypass UAS Requirement	Cyclin for the Sgv1p (Bur1p) protein kinase; Sgv1p and Bur2p comprise a CDK-cyclin complex involved in transcriptional regulation through its phosphorylation of the carboxy-terminal domain of the largest subunit of RNA polymerase II
ddc1	Defective in sister Chromatid Cohesion	DNA damage checkpoint protein, part of a PCNA-like complex required for DNA damage response, required for pachytene checkpoint to inhibit cell cycle in response to unrepaired recombination intermediates; potential Cdc28p substrate
eaf1	Esa1p-Associated Factor	Component of the NuA4 histone acetyltransferase complex; required for initiation of pre-meiotic DNA replication, probably due to its requirement for significant expression of *IME1*

exo1	Exonuclease	5'-3' exonuclease and flap-endonuclease involved in recombination, double-strand break repair and DNA mismatch repair; member of the Rad2p nuclease family, with conserved N and I nuclease domains
ids2	IME2-Dependent Signaling	Protein involved in modulation of Ime2p activity during meiosis, appears to act indirectly to promote Ime2p-mediated late meiotic functions; found in growing cells and degraded during sporulation
lip5	Lipoic acid	Protein involved in biosynthesis of the coenzyme lipoic acid, has similarity to *E coli* lipoic acid synthase
lsm7	Like SM	Lsm (Like Sm) protein, part of heteroheptameric complexes (Lsm2p-7p and either Lsm1p or 8p): cytoplasmic Lsm1p complex involved in mRNA decay; nuclear Lsm8p complex part of U6 snRNP and possibly involved in processing tRNA, snoRNA, and rRNA
mct1	Malonyl-CoA:ACP Transferase	Predicted malonyl-CoA:ACP transferase, putative component of a type-II mitochondrial fatty acid synthase that produces intermediates for phospholipid remodeling
mrps35	Mitochondrial Ribosomal Protein, Small subunit	Mitochondrial ribosomal protein of the small subunit
nup84	Nuclear Pore	Subunit of the nuclear pore complex (NPC), forms a subcomplex with Nup85p, Nup120p, Nup145p-C, Sec13p, and Seh1p that plays a role in nuclear mRNA export and NPC biogenesis
och1	Outer Chain elongation	Mannosyltransferase of the cis-Golgi apparatus, initiates the polymannose outer chain elongation of N-linked oligosaccharides of glycoproteins
rad5	Radiation Sensitive	DNA helicase proposed to promote replication fork regression during postreplication repair by template switching, contains RING finger domain
rtt109	Regulator of Ty1 Transposition	Histone acetyltransferase critical for cell survival in the presence of DNA damage during S phase, acetylates H3-K56, plays a role in regulation of Ty1 transposition
rpb9	RNA Polymerase B	RNA polymerase II subunit B12.6; contacts DNA, mutations affect transcription start site; involved in telomere maintenance
rpl31a	Ribosomal Protein of the Large subunit	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl31Bp and has similarity to rat L31 ribosomal protein; associates with the karyopherin Sxm1p
rvs161	Reduced Viability on Starvation	Amphiphysin-like lipid raft protein; subunit of a complex (Rvs161p-Rvs167p) that regulates polarization of the actin cytoskeleton, endocytosis, cell polarity, cell fusion and viability following starvation or osmotic stress

sae2	Sporulation in the Absence of spo Eleven	Endonuclease that processes hairpin DNA structure with the Mrx complex; involved in meiotic and mitotic DSB repair
slm4	Synthetic Lethal with Mss4	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p, gene exhibits synthetic genetic interaction with *MSS4*
spt10	Supressor of Ty	Putative histone acetylase, sequence-specific activator of histone genes, binds specifically and highly cooperatively to pairs of UAS elements in core histone promoters, functions at or near the TATA box
spt20	Suppressor of Ty	Subunit of the SAGA transcriptional regulatory complex, involved in maintaining the integrity of the complex
srv2	Suppressor of RasVal19	CAP (cyclase-associated protein) subunit of adenylyl cyclase complex; N-terminus binds adenylyl cyclase and facilitates activation by RAS; C-terminus binds ADP-actin monomers, facilitating regulation of actin dynamics and cell morphogenesis
trm9	tRNA Methyltransferase	tRNA methyltransferase, catalyzes esterification of modified uridine nucleotides in tRNA(Arg3) and tRNA(Glu), likely as part of a complex with Trm112; deletion confers resistance to zymocin
tsr2	Twenty S rRNA accumulation	Protein with a potential role in pre-rRNA processing
ubr1	N/A	Ubiquitin-protein ligase (E3) that interacts with Rad6p/Ubc2p to ubiquitinase substrates of the N-end rule pathway; binds to the Rpn2p, Rpt1p, and Rpt6p proteins of the 19S particle of the 26S proteasome
ydr417c	N/A	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified ORF *RPL12B*, encoding a component of the 60S ribosomal subunit
ylr235c	N/A	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene *TOP3*, which encodes DNA topoisomerase III
yml012c-a	N/A	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene UBX2 (ubiquitin-associated protein degradation)

(ynr068c)	N/A	Game *et al* (39) have demonstrated that this strain has a secondary mutation in *RAD52*

* Well-characterized recombination genes used as controls.

The EcoRI^S mutants could be grouped according to their function (Table 9). Many of the genes are known to be involved in DNA metabolism. However, there are some mutants that are not involved in DSB repair according to their function but are still sensitive to EcoRI and other clastogens that are known to be involved in DNA damage. Four of 61 EcoRI^S mutants are known to have mitochondrial function, but how they are related to DSB repair is not known. Similarly, six mutants are known to have cell membrane functions whose possible roles in determining sensitivity to EcoRI, MMS, bleomycin and radiation are unknown.

Table 9. EcoRI-sensitive mutants and their functions

Function or process	Genes[a]
Sister chromatid cohesion	*CTF4, CTF8, DCC1, HTL1*
Histone acetylation/deacetylation	*EAF1, GCN5, RTT109, SPT10*
Nuclease processing of DNA	*EXO1, MMS4/YBR099C, SAE2, YLR235C/TOP3*
Chromatin-associated proteins	*MMS2, RAD5*
Chromosome stability/segregation	*CGI121, CNM67, DDC1, MMS22*
Transcription regulation	*ANC1, APQ13/NET1, BUR2, NOT5, RPB9, SFP1, SPT20, UME6, YML009W-B/SPT5*
RNA processing/modification	*LRP1, LSM7, TRM9, TSR2, YDR433W/NPL3*
Cell membrane/cell wall	*CIS3, HSP150, RVS161, SAM37, VMA7, VPH2*
Protein glycosylation/palmitoylation	*AKR1, CAX4, OCH1*
Mitochondrial proteins	*ATP2, IMG2, MCT1, MRPS35*

[a] Gene names separated by a forward slash, e.g., *MMS4/YBR099C*, indicate that two overlapping open reading frames (ORFs) are present In each case, one ORF corresponds to a verified gene and the other is speculative

Use of an novel HO endonuclease survival assay to identify new DSB repair mutants

The primary goal of the 2nd part of this project was to complete the screen for mutants that are sensitive to expression of HO endonuclease-induced DSBs. HO endonuclease of *S. cerevisiae* is a homing endonuclease that makes a site-specific DSB at a 45-bp target sequence on chromosome III as shown in Figure 10.

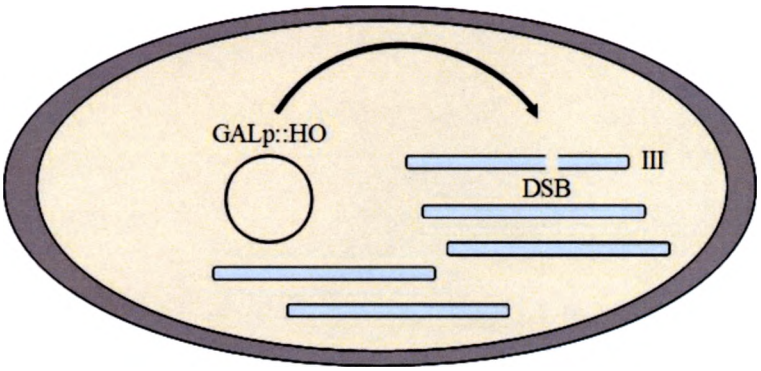

Figure 10. Expression of HO endonuclease from a *GAL* promoter-containing plasmid induces a single DSB on chromosome III.

Previous work by Rachel Roberts in the Lewis lab screened a total of 101 mutants for sensitivity to HO endonuclease, including several mutants that are known to be involved in homologous recombination (e.g., *mre11, rad50, rad51, rad52, rad54, rad55* and *rad57*). Out of 101 mutants, only 10 mutants were identified that exhibited sensitivity to HO endonuclease. In this new project, 95 additional yeast mutants were screened with HO endonuclease. The HO sensitivity assay developed by graduate student Rachel Roberts in the Lewis lab was used to complete the screen.

Strains were transformed with the plasmid pGALHO, which has the HO gene under the control of a galactose-induced promoter for expression of the endonuclease. The ability to repair HO-induced DSBs was monitored by growing cells in media

containing Raff – Ura, Raff + Gal - Ura and Gal - Ura because expression of HO is turned off in the presence of glucose or raffinose, partially turned on in Raff + Gal and fully expressed in the presence of galactose alone.

Double replica imprints to identify mutants sensitive to HO endonuclease

A double replica-plating technique was employed to test all mutants for HO endonuclease sensitivity. This technique consisted of transforming mutant cells with pGALHO. The cells were patched onto 1% Raff - Ura plates. The cells from 1% Raff - Ura plates were replica-plated onto 1% Raff + 3% Gal - Ura and 2% Gal - Ura plates. Double imprints were done using the first Raff - Ura replica plate as a new master plate and cells were replica-plated onto fresh plates with selective media. The growth of mutant cells transformed with pGALHO was compared to that of WT cells and classified as either non-inhibited (cells that grew well on all plates), moderately inhibited (cells that grew well on Raff + Gal - Ura plates but no growth on Gal - Ura plates) and severely inhibited (grew only on Raff plates but no growth on Raff + Gal and Gal plates). Of all new mutants tested by double imprinting only 24 mutants exhibited provisional sensitivity to HO endonuclease.

Dilution pronging survival assays

Pronging survival testing is a more quantitative assay and was used to further investigate the mutants that were found to be potentially sensitive to HO endonuclease through double-imprint replica-plating. The pronging method employed for this project involved harvesting cells and placing them in the first well of a microtiter dish at a specific concentration. Five-fold serial dilution was done for each mutant containing either pGALHO or the control vector pRS316. Diluted cells were then pronged onto plates with different sugar sources such as 1% Raff - Ura, 1% Raff + 3% Gal – Ura and

2% Gal – Ura. The parameters that were used to categorize mutants were as follows: non-inhibited (grew like their vector control), moderately inhibited ($\leq$ 5 fold killing and slow growth as compared to their vector control on Gal plates), strongly inhibited (> 5 fold killing and inhibition of growth on Gal plates).

Of all mutants tested with the dilution pronging assays none were found to be consistently sensitive to HO endonuclease. However, thirteen mutants grew extremely poorly on raffinose plates but could grow reasonably well on glucose and galactose plate (without pGALHO in the cells). These 13 mutants that grew poorly on raffinose and raffinose + galactose were then tested separately by pronging assays as described above except that the cells were pronged directly from Glu – Ura to Gal – Ura plates. It is normally preferable to induce *GAL* promoter expression by switching growth from raffinose to galactose because induction is fast. However, it is possible to transfer cells directly from Glu to Gal and assess the consequences, though subtle effects may be missed.

Two of the mutants were determined to be killed due to HO endonuclease activity as seen in Figure 11. The first 2 rows on each plate are WT cells with vector and with pGALHO. The next six rows consist of three sets of mutant strains containing vector and pGALHO (*grf10*, *pfk2,* and *ydl041w*) From this assay, it was determined that *grf10* grew like WT and was not sensitive to HO, which indicates that this gene is not involved in mating type switching. In the other two mutants, *pfk2* and *ydl041w,* expression of HO induced strong killing, which indicates that these genes play a significant role in mating-type switching.

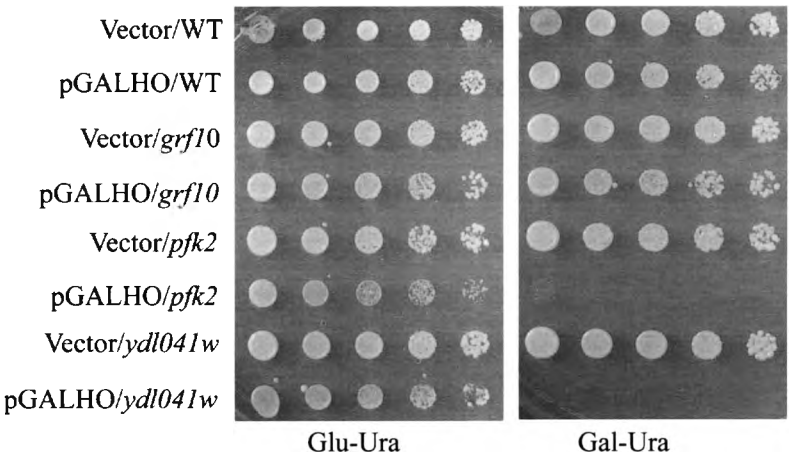

Figure 11. Dilution pronging survival assays of mutants that grew poorly on raffinose. Vector or pGALHO-containing cells grown on Glu-Ura plates were harvested and pronged to Glu – Ura and Gal – Ura plates.

All other mutants showed no effect from HO expression, indicating that these genes do not play critical roles in mating type switching. All mutants from previous and current work (excluding DSB repair defective RAD52 group mutants) that were found to be sensitive to HO endonuclease have been summarized with brief descriptions in Table 10. Interestingly, only 5 of the 12 genes have previously been associated with DNA metabolism or stability. Two of the genes (*DCC1* and *HTL1*) are involved in sister chromatid cohesion and 2 others (*DUN1* and *RAD24*) are involved in DNA damage-induced checkpoint responses. Another locus, *YML041*, overlaps the *SIR2* gene, which encodes a protein known to affect mating type switching (24, 40). Only 3 of the mutants were also found to be sensitive to EcoRI.

Table 10. *S. cerevisiae* mutants sensitive to HO endonuclease expression[a]

Gene / ORF	Description
dcc1[b]	Sister chromatid cohesion and telomere length maintenance
dun1[b]	DNA damage checkpoint, postreplicative DNA repair
grr1	Carbon catabolite repression, divalent cation transport, high affinity glucose transport, morphogenesis, sulfite detoxification
htl1	Chromatin remodeling, transcriptional regulation/elongation, chromosome stability, establishment of cohesion, telomere Maintenance
mms2	Postreplication DNA repair, contributes to ubiquitin-protein ligase Activity
mot2	Multiple transcription roles, degradation of mRNA
nat3[b]	Acetylation of N-terminal residues of proteins
pat1	mRNA decapping factor associated with Topoisomerase II
slx8	Substrate-specific ubiquitin ligase
rad24[b]	DNA damage checkpoint, loads Rad17-Mec3-Ddc1 onto DNA
pfk2	Beta subunit of heterooctameric phosphofructokinase involved in glycolysis, indispensable for anaerobic growth, activated by fructose-2,6-bisphosphate and AMP, mutation inhibits glucose induction of cell cycle-related genes.
yml041	Dubious open reading frame, overlaps the verified gene *SIR2/YDL042C*

[a] Genes listed in the table summarize results from the earlier study (34) and the current work.
[b] *dcc1*, *dun1*, *nat3* and *rad24* mutants exhibited only modest effects on growth/survival; all other mutants displayed 5-fold or more killing when HO was expressed. Descriptions are from the *Saccharomyces* Genome Database (www.yeastgenome.org)

Use of PCR-based gene targeting to delete the CNM67 gene

In *S. cerevisiae,* Cnm67, a novel yeast protein, is encoded on chromosome XIV and localizes to the microtubule organizing center, the spindle pole body (SPB). The role of *CNM67* in DSB repair was investigated further for this project because it was the only

EcoRI-sensitive mutant that had all phenotypes similar to *rad51* and *rad52* mutants (Table 11), which are known to be defective in homologous recombination.

Table 11. Comparison of phenotypes of *cnm67* and *rad51/rad52* mutants

Phenotype	*cnm67*	*rad51*	*rad52*
$EcoRI^S$	S	S	S
$Gamma^S$ (60 krad)	S	SS	SS
$Bleomycin^S$ (4 μg/ml)	S	SS	SS
MMS^S (2 mM)	SS	SS	SS
G_2/M cells	High G_2/M	High G_2/M	High G_2/M

PCR-based gene targeting was used to delete the *CNM67* gene using chimeric primers. *cnm67* deletion strains were constructed using a chimeric primer PCR technique in which the Hygromycin B resistance ($HygB^r$) selectable marker present in plasmid pAG32 was PCR amplified and attached to 50-55 bp of *CNM67* homology from each side of the targeted coding region. The 50-55 bp of homology on the 5' side of the ORF was directly adjacent to and included the start codon. Likewise, the 3' 50-55 bp of homology was adjacent to, and included the stop codon, thus generating precise disruption of the ORF. A high efficiency transformation method was used to transform PCR amplified product into WT haploid cells (BY4742), where homologous recombination between the PCR product and the *CNM67* gene on chromosome X1V resulted in deletion of the gene as shown in Figure 12.

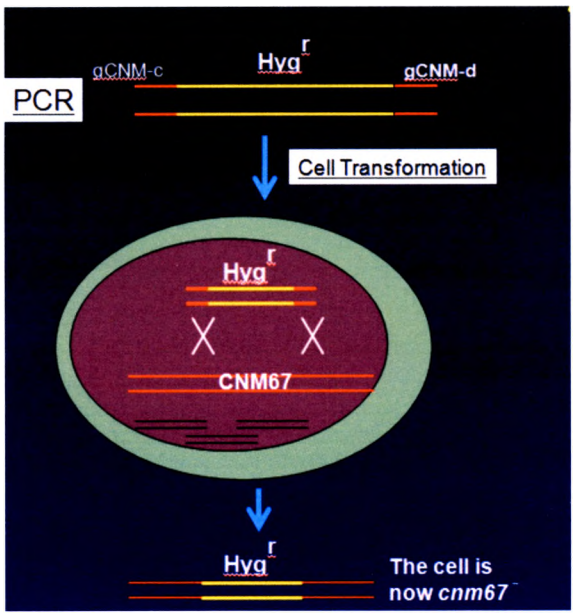

Figure 12. Deletion of the *CNM67* gene in yeast *S. cerevisiae.*

To verify correct homologous recombination of the deletion module with the yeast genome, genomic DNA was isolated from the Hygromycin B-resistant transformant colonies and used as template in PCR reactions using test primers. PCR was also done with normal *CNM67* cell DNA, which was used as a control for verification. Both the PCR reactions, one with the normal *CNM67* gene and one with the deleted *cnm67* gene were used to distinguish the correct product when analysed by agarose gel electrophoresis. The product size of the HygB^r fragment with a deleted *cnm67* gene was different from the PCR product observed with the WT *CNM67* gene as seen in Figure 13.

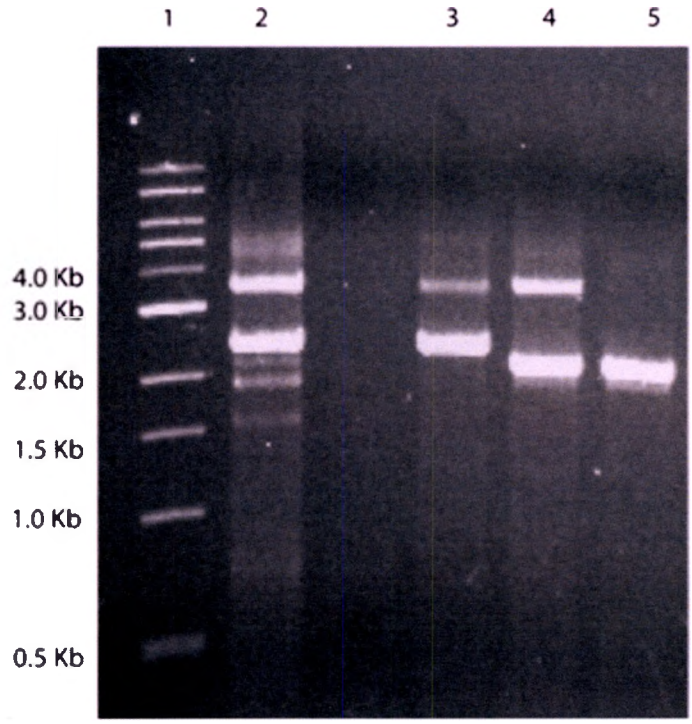

Figure 13. Confirmation of disruption-deletion of *CNM67*. The PCR fragments were electrophoresed on a 0.6% agarose gel. Lane 1, 1 Kb ladder; Lane 2, PCR analysis of the WT *CNM67* locus; Lanes 3, 4 and 5, putative *cnm67:: HygB*^{r} deletion strains.

The expected size for the WT *CNM67* fragment was 2.2 kb and the expected size for the *cnm67Δ* fragment was ~1.9 kb. WT cell DNA migrated to the expected size as shown in lane 2. The upper band at ~ 3.2 kb is frequently seen in *CNM67* PCRs and represents an unknown product made from chromosomal DNA in both *CNM67* and *cnm67* strains and it was therefore ignored. Among 3 independent HygB^r colonies tested in lanes 3, 4 and 5, isolate 1 produced a band at the size of WT DNA, which indicates that gene disruption was unsuccessful. DNA from two others migrated to the expected size, which is less than WT as shown in lanes 4 and 5, indicating that gene disruption of *CNM67* by insertion of HygB^r was successful.

Previous work in this lab on *cnm67* mutants has shown their sensitivity to bleomycin. Double-imprint replica-plating was done to confirm the deletion of the *CNM67* gene, where WT cells and *cnm67* mutant cells were streaked onto YPDA plates and later replica-plated to YPDA plates with or without 2 μg/ml bleomycin. The first YPDA plate was immediately used as a new master plate to replica-plate cells to YPDA + bleomycin plates. WT cells were resistant to killing by bleomycin, but *cnm67* mutants showed strong sensitivity when exposed to bleomycin (not shown).

Analysis of cnm67 mutants by plasmid-chromosome recombination assays

cnm67 mutants were found to be sensitive to EcoRI, MMS, bleomycin and gamma radiation, which are all agents that cause DSBs. These results suggest that this gene may play an important role in DSB repair pathways. The plasmid-chromosome recombination assay developed by Rachel Roberts (34) was used to test *cnm67* strains from the yeast deletion library and mutants made as described above. In addition, several other gamma sensitive mutants were tested. BseRI-cut pLKL37Y plasmid DNA was chosen for this assay. As shown in Figure 14, the plasmid DNA was digested with the restriction enzyme BseRI. The broken ends of the plasmid, which contains a functional *HIS3* gene, can undergo homologous recombination with the mutant *his3* allele on the chromosome, thereby yielding a functional integrated *HIS3* gene and also an integrated *URA3* gene. All of the plasmid becomes stably integrated into chromosome XV through strand exchange and the cells then go from His^- Ura^- (requiring histidine and uracil to grow) to His^+ Ura^+.

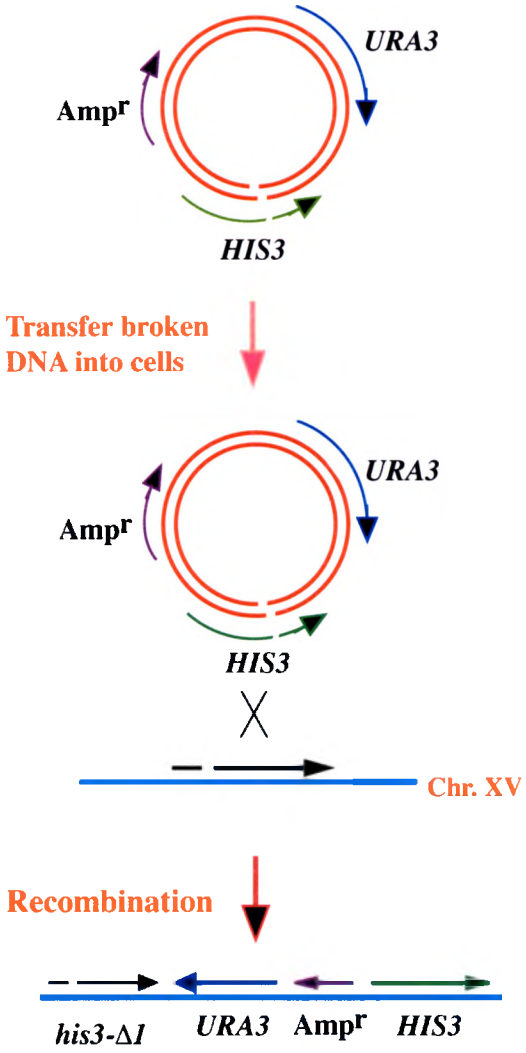

Figure 14. Schematic depicting induction of homologous recombination repair by transforming yeast cells with BseRI-cut pLKL37Y.

Recombination assays were performed by transforming 400 ng of BseRI-cut pLKL37Y DNA into WT cells and mutant cells. In these experiments, cells were also transformed with 100 ng of the unbroken *LEU2* vector pRS315. 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) to control for variation in transformation efficiencies. As seen in Figure

15, recombination efficiencies of the library *cnm67* mutant and new *cnm67::HygB* strains were ≤ 2 fold different from WT. A difference of 2 fold or less is not considered significant in light of the large standard deviations in these assays. In contrast to *cnm67* cells, the recombination frequency of *rad51* mutants was 110 fold lower compared to WT cells (Figure 15).

These results indicate, although *cnm67* cells are qualitatively similar to *rad51/rad52* mutants in several phenotypes, according to the results showing ≤ 2 fold difference in repair efficiency *CNM67* is not critical for DSB repair by homologous recombination in plasmids.

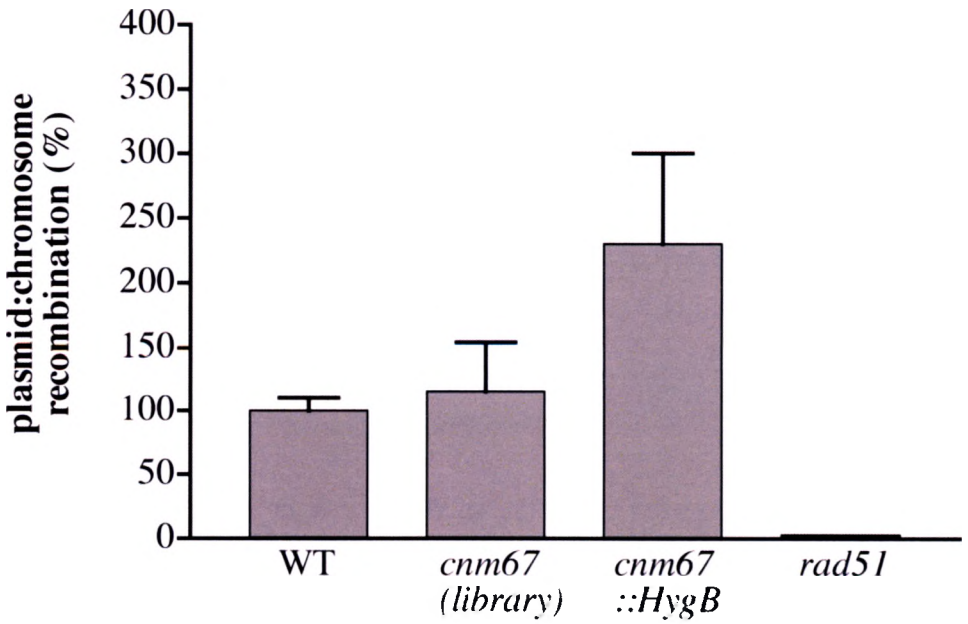

Figure 15. Analysis of DNA repair in *cnm67* and *rad51* mutants using plasmid:chromosome recombination assays.

Most of the 61 EcoRI^S mutants were also sensitive to MMS and bleomycin, but

only 12 mutants were also sensitive to gamma radiation. Interestingly, 4 of these gamma-sensitive mutants did not show any effect on homologous recombination (Figure 14). Although a 50% reduction was observed in *htl1* cells, this was not significantly different from levels seen in WT cells. Other mutants such as *sae2, cgi121* and *exo1* cells exhibited only 1.5-3 fold increases in repair efficiency from WT cells. Other gamma-sensitive mutants were also tested but their transformation efficiencies were unusually low producing less than 10 colonies per plate, which is not enough to produce valid statistical data and these experiments will have to be repeated under conditions that provide high colony counts.

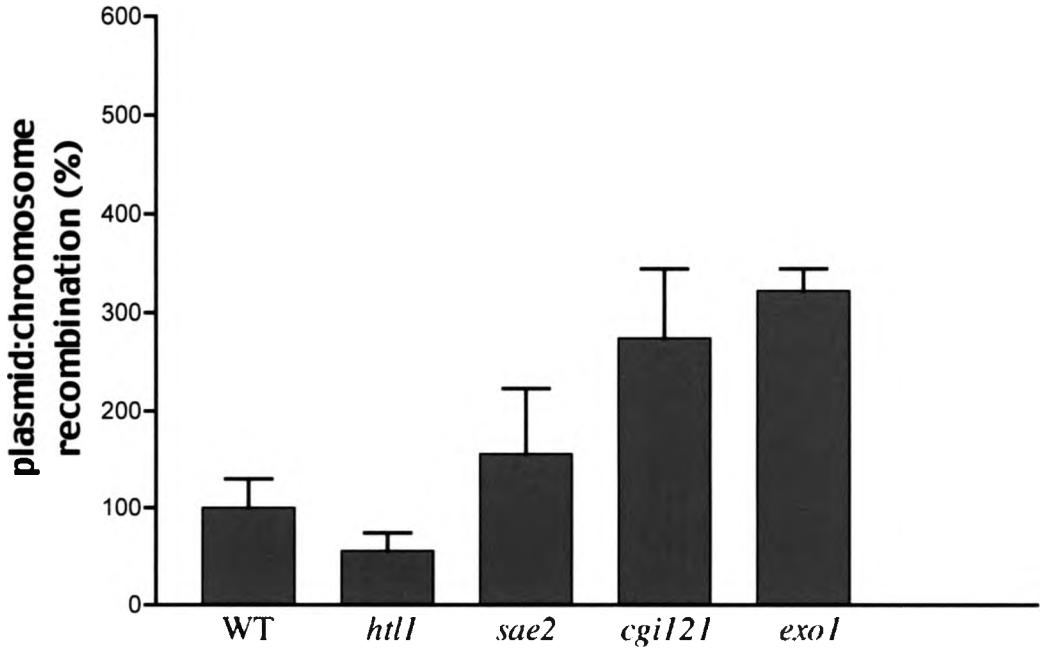

Figure 16. Analysis of plasmid::chromosome recombination in four EcoRI-sensitive mutants that were also gamma-sensitive.

Use of chromosome:chromosome recombination assays to analyze cnm67 mutants

Chromosome:chromosome recombination assays were done to further analyze

cnm67 mutants and investigate this gene's role in homologous recombination. For chromosome:chromosome recombination assays, the *CNM67* gene was deleted in the strains YLKL1090 and YLKL1091 (28). Diploid strains were created by crossing YLKL1090 and YLKL1091. In the diploids, one chromosome is *leu1-c* and its homologous chromosome contains *leu1-12* (Figure 17). Reciprocal recombination between the two chromosomes results in restoration of the *LEU1* gene (cells can now grow on plates lacking leucine). Because the small mutations in these alleles are widely separated, spontaneous recombination events anywhere in the middle of the genes can join, for example, the good upstream end of *leu-12* to the good downstream end of *leu1-c* (Figure 17), producing a functional *LEU1* gene. These same diploid cells also have 2 different alleles of *TRP5*, *trp5-c* and *trp5-2,* which can also recombine to produce *TRP5* cells.

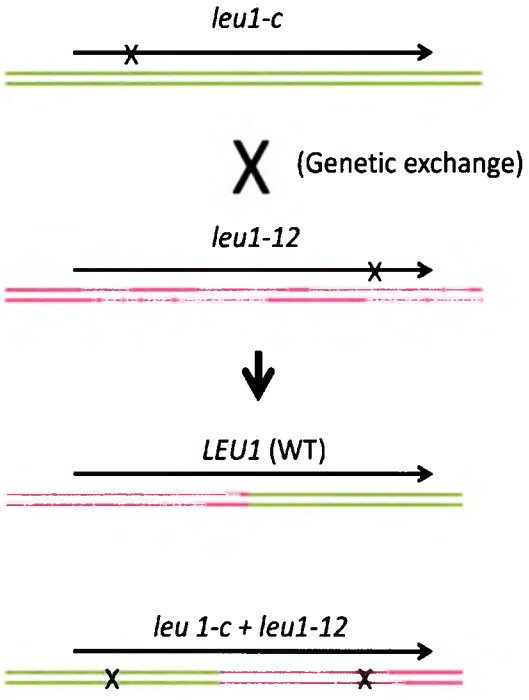

Figure 17. Schematic of chromosome:chromosome recombination assay.

In order to investigate what fraction of the recombinants from the reciprocal recombination assay were restored by homologous recombination, cells from 8 separate YPDA liquid cultures were spread onto non-selective YPDA plates. Cells were also spread onto selective plates lacking leucine and selective plates lacking tryptophan. The method of the median (5, 28) was used to calculate recombination rates in WT and *cnm67* $^{-/-}$ cells (Table 14). A less than 2-fold change in recombination rate was observed in *cnm67* mutant cells at both *leu1* and *trp5,* which was not considered significant.

Thus, these chromosome:chromosome recombination assays done to analyze diploid *cnm67* mutants at two different loci have shown that there is no significant change in recombination rates from WT, suggesting that *CNM67* is not critical for spontaneous homologous recombination between chromosomes.

Table 12. Recombination rates in WT and *cnm67* mutant cells

Alleles	Recombination rate[a] in WT cells	Recombination rate[a] in *cnm67* $^{-/-}$ cells
leu1-12/leu1-c	4.2 X 10^{-6}	7.0 X 10^{-6}
trp5-2/trp5-c	3.4 X 10^{-6}	4.3 X 10^{-6}

[a] Number of events per cell per generation

Summary and Conclusions

Yeast cells have been frequently used for fundamental studies on gene function as they can be employed either as haploids or diploids. In this project, we have tested a large set of mutants and identified their role in DSB repair after exposure to gamma radiation and different DNA-damaging agents such as bleomycin, MMS, EcoRI and HO. A part of this project also involved characterizing the *CNM67* gene in more detail since it exhibits phenotypes similar to *rad51/rad52* mutants known to be defective in recombination.

Prior experiments done by Jennifer Summers in the Lewis lab screened a total of 95 mutants previously shown to be gamma-sensitive as diploids (25, 26, 35). Another study done by Game *et al.* (39) screened a set of gamma-sensitive mutants and identified 33 mutants that were sensitive to gamma radiation. Interestingly, 9 of these 33 mutants were not found by Bennett and were not investigated in the current study.

In this project another 101 mutants were tested for EcoRI sensitivity, completing the analysis of all mutants identified in the screens by Bennett *et al.* Replica-plating assays were used initially to screen the mutants. The mutants identified as potentially sensitive through replica-plating assays were then further analyzed by dilution pronging survival assays. Sixty-one mutants (33 in the initial work by Jennifer Summers and 28 in the current work) were found to have either strong or moderate sensitivity to EcoRI expression which indicates that these mutants have reduced ability to repair DNA damage caused by endonuclease-induced DSBs. Thirty-one of the 61 mutants were found to have strong sensitivity whereas thirty mutants were moderately sensitive to EcoRI. In addition, 107 mutants did not exhibit sensitivity to EcoRI expression and 20 mutants could not be tested because of their growth problems on the plate media used for the assays.

All 61 mutants that were sensitive to EcoRI were then further tested for their sensitivity to bleomycin and MMS at different doses. Twenty-one of the 31 most EcoRI^s mutants were sensitive to 2 mM MMS and 20 of them were sensitive to 4 μg/ml bleomycin. Twenty of the 30 moderately EcoRI^s mutants exhibited killing by 2 mM MMS whereas 24 of them showed sensitivity to 4 μg/ml bleomycin. Interestingly, seven mutants that were sensitive to EcoRI were found to be resistant to both bleomycin and MMS, which suggests that these mutants may not have a defect in DNA repair. Thirty-four of the 61 mutants were sensitive to both MMS and bleomycin and are more likely to play an important role in DNA repair. Six of the 61 EcoRI^s mutants were resistant to bleomycin, MMS and gamma. It is possible that these mutations cause an increase in EcoRI protein level, enzyme activity, nuclear transport, or accessible sites on DNA for EcoRI.

All 61 EcoRI-sensitive strains were also tested for gamma sensitivity and only 13 mutants were found to be sensitive. Of those 12 gamma-sensitive mutants, 8 strains (*cnm67, htl1, vma7, ado1, mct1, rpb9, spt10* and *ubr1)* were sensitive to all other DNA damaging agents such as EcoRI, bleomycin and MMS. Although all 61 mutants were originally identified as gamma-sensitive as diploids by Bennett et al., only 13 of them were sensitive as haploid mutants in the current work. Likely explanations for this difference are (1) some genes may produce gamma-sensitivity when inactivated in diploids but not in haploids (e.g., SGS1and RDH54 (6, 7)), and (2) the earlier studies scored growth inhibition as sensitivity, but cell killing were the only criteria used in the current work.

Interestingly, several of the 61 EcoRI^S genes had previously been associated with

sister chromatid cohesion, nuclease processing of DNA, histone acetylation/deacetylation and chromosome stability. It is logical to assume that such genes might affect DNA repair. In contrast, the possible roles of several of the other genes is unclear, e.g., mitochondrial and cell membrane proteins.

EcoRI-sensitive mutants were tested for spontaneous and induced cell cycle arrest in G_2 phase. The prior study by Jennifer Summers showed that HR deficient control mutants had high spontaneous G_2 cell arrest during log phase growth. Thirteen of the 61 *Eco*RI-sensitive mutants also exhibited high levels of G_2/M cells during normal growth, suggesting that they have elevated levels of spontaneous DNA damage that is causing them to spend more time in G_2 phase.

The mutants that were tested for EcoRI sensitivity were also tested for resistance to another endonuclease enzyme known as HO endonuclease. Interestingly, only 12 mutants exhibited sensitivity to HO endonuclease as compared to 61 mutants that were sensitive to EcoRI. The difference in the sensitivities between the two endonuclease enzymes might be attributed to the fact that DSBs induced by HO are repaired by a conserved recombination mechanism that leads to mating type switching whereas EcoRI-induced DSBs are repaired by either HR or NHEJ pathways. HO DSB repair is an intrachromosomal event where exchange occurs between the middle of chromosome III and one end of chromosome III. This exchange event does not need strong sister chromatid cohesion or another homologous chromosome and therefore is not dependent on proteins that affect interchromosomal exchange. In contrast, HR repair of EcoRI DSBs almost always involves exchange with a sister chromatid or another homologous chromosome. The mechanisms necessary for interchromosomal exchange must always be functional for efficient repair of EcoRI DSBs and therefore many more proteins are likely to be required.

CNM67, encoding a spindle pole body-associated protein, displayed

characteristics similar to that of well-known HR-deficient mutants (such as *rad51/rad52*) for sensitivity to EcoRI, radiation, MMS and bleomycin, as well as cell cycle checkpoint analysis, suggesting possible involvement in homologous recombination. Plasmid:chromosome and chromosome:chromosome recombination assays indicated that, although they had similar characteristics of mutants that are known to be defective in recombination, *cnm67* mutants did not have any effect on the homologous recombination pathway. It is possible that the DNA damage sensitivity of these strains is not due to a repair defect, but rather might be related to its function in chromosome segregation. Cnm67 was not identified as NHEJ-deficient in 2 separate library searches for NHEJ genes (41, 42) so defects in this pathway are also not the cause of the damage sensitivity. As a spindle-associated protein, Cnm67 potentially affects several processes occurring in mitosis, including alignment and separation of chromosomes in metaphase and anaphase. Absence of Cnm67 and normal spindle pole body function may make chromosome more susceptible to damage-induced chromosome loss and malsegregation, resulting in increased killing of cells after exposure to EcoRI, radiation, MMS, etc.

Testing recombination in other gamma-sensitive mutants that showed sensitivity to all DNA damaging agents such as EcoRI, MMS and bleomycin might be a good place to start future studies. Although an attempt to test the role of these mutants in DSB repair was done in this project, the transformation efficiencies for the mutants were so low that we could not get good statistical data and this could be improved in future experiments.

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