APPLICATION OF A NOVEL ENDONUCLEASE SENSITIVITY ASSAY TO IDENTIFY NEW GENES THAT AFFECT DNA REPAIR AND CHROMOSOME STABILITY

THESIS

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CHAPTER I

INTRODUCTION

Lesions within the strands of chromosomal DNA can be caused by various clastogens (damaging agents that break DNA strands) including ionizing radiation such as X-rays and gamma rays, chemicals such as bleomycin and methyl methanesulfonate (MMS), or by endonuclease enzymes such as *Eco*RI. Most DNA damaging agents can cause multiple types of lesions to occur within the chromosome including damaged bases, sugar alterations, single-strand or even double-strand breaks (1). Double-strand breaks (DSBs) are one of the most damaging types of lesions to occur in DNA (1, 2). DSBs can occur as one of two types: as a direct result, as with exposure to ionizing radiation and endonucleases, or through a more indirect series of processes as seen following treatment with MMS (2). Normal cells have the ability to repair most DSBs and remain viable. However, cell defects can leave double-strand breaks unrepaired or repaired inaccurately, which can have many possible effects on cells including an increase in DNA mutations, altered metabolism, cancer and loss of cell viability (3).

To maintain genomic stability, cells have mechanisms to repair broken DNA. In eukaryotes such as *Saccharomyces cerevisiae* (budding yeast) chemically- and physically-induced DSBs are repaired almost exclusively by two main mechanisms that

are implemented according to the type of damage occurring to the DNA (2, 4). Nonhomologous end-joining (NHEJ) is a pathway that involves the direct rejoining of broken ends through the use of multiple proteins and enzymes (Figure 1A) (2, 4). Nonhomologous end-joining requires minimal energy, but can be error-prone and increase mutations (4). Although NHEJ is regarded as a secondary pathway in yeast, it has been shown to be essential for the repair of certain DSBs (2, 5). In yeast, there are at least 11 genes that are required for NHEJ: *YKU70, YKU80, DNL4, LIF1, NEJ1, SIR2, SIR3, SIR4, RAD50, MRE11,* and *XRS2* (2, 3, 4, 6). Previous research has shown that the proteins involved in NHEJ can be grouped into four different complexes: Yku70/Yku80, Dnl4/Lif1/Nej1, Sir2/Sir3/Sir4, and Rad50/Mre11/Xrs2 (Mrx) (in humans, Xrs2 is replaced by Nbs1). Each of these protein complexes (except the Sir protein complex) binds to the ends of double-stranded linear DNA at sites where DSBs occur in the cells, and are essential for the NHEJ repair pathway to occur (2, 6).

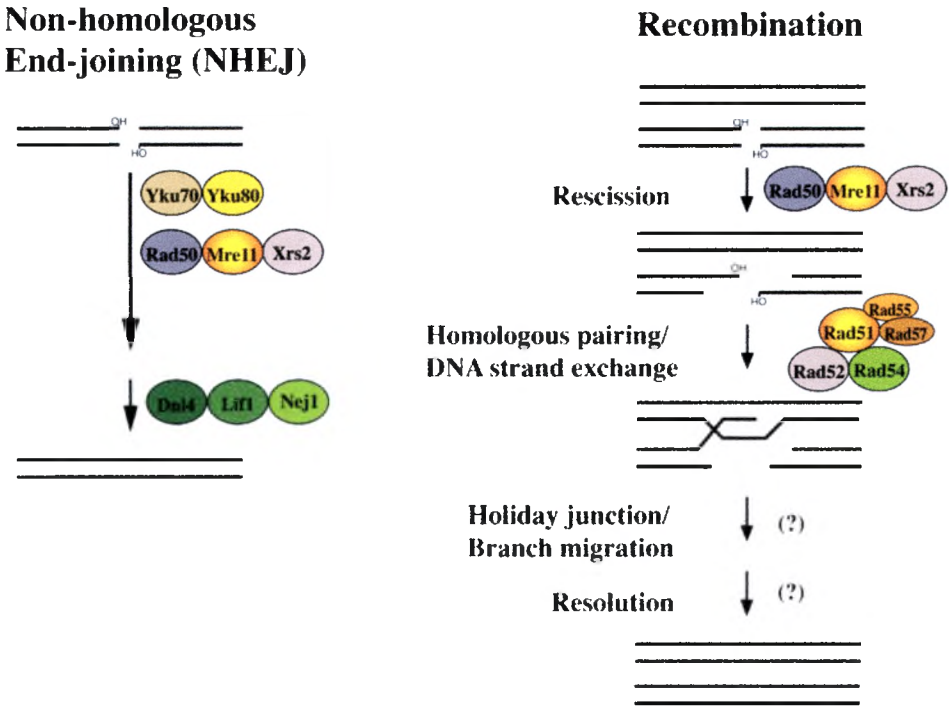

Figure 1A. Model for NHEJ in *S. cerevisiae*. B. Model for Homologous Recombination (HR).

The Yku70/Yku80 complex forms a heterodimer and initiates NHEJ by binding at both ends of the DSB (2). The Mrx complex then binds to the DNA-Yku structure and acts as a bridging factor between the two DNA ends (6). The Mrx complex has multiple functions including a 3'-5' double-stranded DNA (dsDNA) exonuclease activity in the presence of manganese as well as single-stranded DNA (ssDNA) endonuclease activity which are associated with the Mre11 component of the complex (6). The Rad50 subunit of Mrx has ATP-binding activity as well as possible adenylate kinase activity (7) and it has been suggested that the Xrs2 subunit interacts with the Dnl4/Lif1/Nej1 (DNA Ligase IV) complex to stimulate ligation of two separate molecules (6). The nuclease activity is largely dispensable for most NHEJ mutants. The precise mechanism of involvement of

the Sir2/Sir3/Sir4 protein complex is not known, but it may act only indirectly (8). Next, the Dnl4/Lif1/Nej1 complex is recruited to the break site. The Dnl4 subunit of the DNA Ligase IV complex is a DNA ligase that is ATP-dependent (2) and, as mentioned above, the Lif1 subunit is suggested to associate with the Xrs2 subunit in Mrx (6, 8). Once the cohesive ends have been processed and gaps have been filled, Dnl4 ligates the ends and the break is repaired (6). Mutations in the NHEJ pathway, specifically *rad50, mre11,* and *xrs2* mutants result in increased sensitivity to DSBs (2, 3). Of the four protein complexes involved in NHEJ, Mrx is unique because it is also required for the second repair pathway.

The alternative pathway is homologous recombination (HR), which involves an intricate, multi-step process in which DSBs are repaired virtually error-free using multiple proteins and other homologous chromosomes within the same cell (9, 10) (shown schematically in Figure 1B). In *S. cerevisiae*, HR is considered to be the primary pathway for DSB repair, while it is the secondary pathway for DSB repair in humans (4, 6). Genes associated with homologous recombination include *RAD50, MRE11, XRS2, RAD51, RAD52, RAD54, RAD55, RAD57* and *RAD59*. The proteins associated with these genes form two complexes including the Mrx (discussed above) and the Rad51/Rad52/Rad54/Rad55/Rad57 complex. Other DNA-associated proteins such as Rpa (single-stranded DNA binding proteins) are also active in this pathway (10).

Mrx creates 3'-overhangs on both strands at the break site, which provide long cohesive ends. This resection activity is critical for HR but not NHEJ. The Rad51/Rad52/Rad54/Rad55/Rad57 complex is responsible for functions such as homologous pairing, annealing, and strand exchange that are necessary for homologous

recombination to occur (3, 10). Although many specifics of the mechanism of homologous recombination are still unknown, functions of several of the proteins are known. Mrx processes the ends to produce single-strand DNA overhangs that are subsequently bound by other proteins, as described above, but later events are unknown. The Rad52 protein has been shown to be most critical, as *rad52* mutants have resulted in more severe sensitivity to DSBs than *rad51, rad54,* or *rad59* mutants in most assays (4, 11). While mutations in *MRX* genes proved to greatly decrease repair of DSBs by the NHEJ repair pathway (mentioned above), such mutations result in a less severe decrease in recombinational repair of DSBs (3). These results are attributed to a back-up mechanism seen in homologous recombination provided by the Exo1 exonuclease protein, which functions the same, yet less efficiently, as the Mrx complex (9). This allows homologous recombination to take place, at a low level, even when Mrx is not available. However, the homologous recombination pathway is unable to be utilized in a *rad52* mutant (9).

Double-strand breaks also occur naturally during cell development and are essential for some processes such as meiotic recombination (3). Once a DSB occurs, it is usually repaired by NHEJ or HR; however, other processes occur in response to a DSB. These include binding of the DNA ends by proteins that are involved in nucleosome remodeling, cohesion of sister chromatids, and cell cycle checkpoint responses (Figure 2) (8, 10).

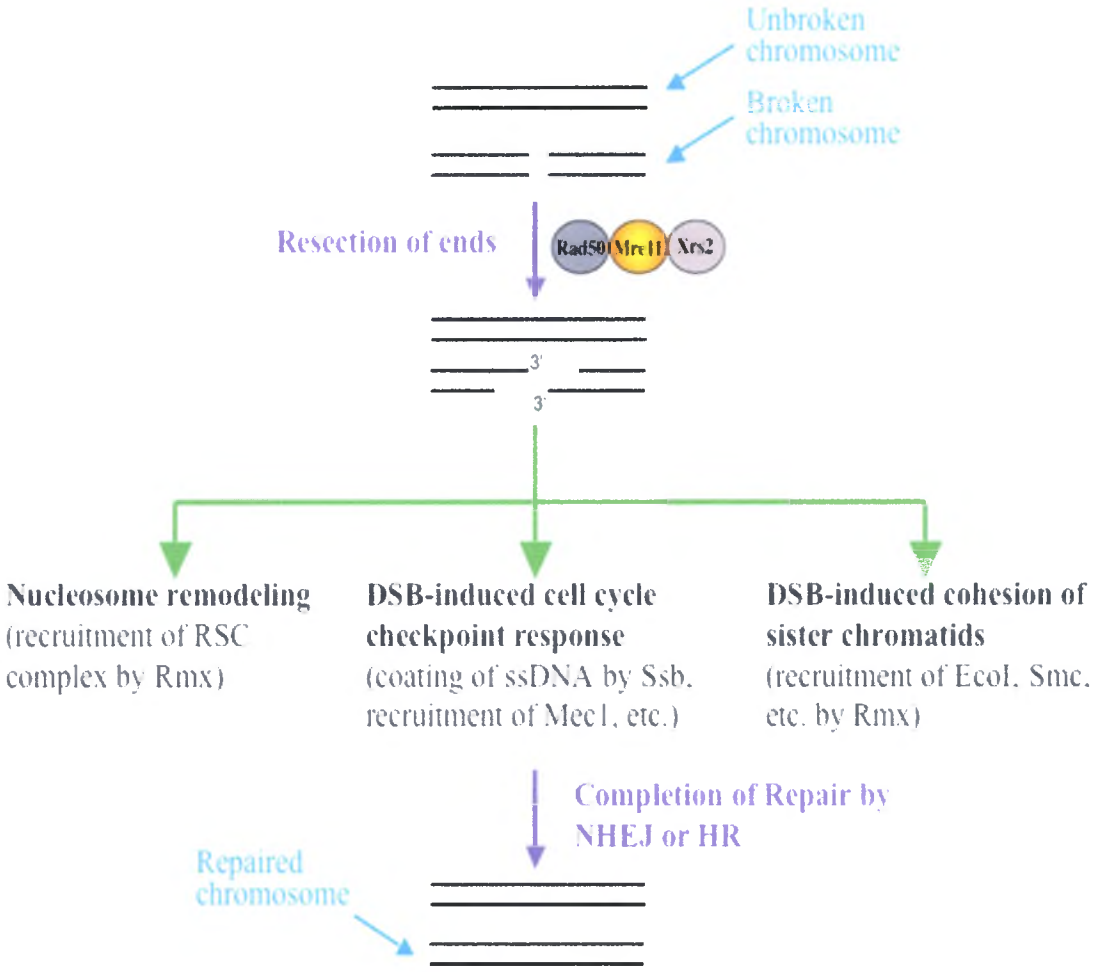

Figure 2. Schematic of processes that occur in response to a DSB.

During nucleosome remodeling, the Mrx complex, specifically Mre11, recruits a large multisubunit Rsc protein complex for ATP-dependent mobilization of nucleosomes presumably to aid in the repair of DSBs (12). The tethering of sister chromatids by the cohesin complex also occurs in response to DSBs. This process involves the recruitment of proteins such as EcoI and SmcI by Mrx. It has been suggested that this tightened

association of chromatids occurs to aid in homologous recombination repair since sister chromatids are the preferred partners in diploid cells (13,14, 15, 16).

During the cell cycle, a checkpoint mechanism is in place in which the cycle is temporarily paused when chromosomal DNA is damaged (3, 4). Cells arrest during the G_2 phase of the cell cycle, which allows the DNA to be repaired before completing the cell cycle. This arrest occurs in response to DSBs and involves a series of events including the coating of single-stranded DNA by the Ssb protein as well as recruitment of Mec1 by the Mrx (17, 18). In yeast, exposure to ionizing radiation or MMS results in G_2 arrest, a phenomenon also observed in human cells (4, 5). It has also been shown that DSBs induced by *in vivo* expression of the restriction endonuclease *Eco*RI result in G_2 phase cell arrest (4). An increased amount of DSBs needing repair results in more cells in arrest and reduced cell viability.

Human and yeast cells with defective DSB repair genes exhibit many similar phenotypes. Cells that lack genes involved in both pathways, such as *RAD50, MRE11,* or *XRS2* (or *NBS1* in humans), can have devastating effects. In humans, a defect in the *MRN* genes can lead to certain diseases that can ultimately result in cancer (19). In humans and mice, cells having a mutation that leads to a defect in NHEJ, such as *YKU70* or *YKU80*, have been shown to have an increase in cancer incidence as well as Severe Combined Immunodeficiency (SCID), a disease caused by an inability to produce the normal assortment of antibodies (20). Yeast cells that are deficient in DSB repair due to lack of one of the necessary repair proteins exhibit specific phenotypes. For example, *rad51* and *rad52* mutants are known to be deficient in homologous recombination and have increased rates of chromosome loss. These mutants also exhibit sensitivity to X-

rays, gamma rays, and chemicals such as MMS and bleomycin (3, 4, 19). Both of these mutants also show sensitivity to *in vivo* expression of site-specific DNA endonucleases such as *Eco*RI (3, 4).

DNA damaging agents such as MMS and bleomycin induce DSBs by different mechanisms. Bleomycin is an antibiotic that is used in cancer treatment due to its ability to directly attack and damage DNA with some preference for growing cancer cells versus non-dividing normal cells. Several mutagenic lesions are produced by this drug through a process involving free radicals. The activated Bleomycin complex produces sugar-carbon radicals, which can lead to abasic sites, single-stranded breaks, mutations called base adducts, and DSBs (21). In contrast, MMS causes a specific type of damage to DNA leading to a DSB that requires repair. MMS is a known carcinogen that methylates DNA, primarily at adenine and guanine bases, which are processed by repair enzymes creating potentially lethal lesions (22).

The restriction endonuclease enzyme *Eco*RI produces DSBs within DNA and has a recognition sequence of G˅AATTC, leaving a four base 5'-overhang after cleavage (Figure 3) (23, 24). *Eco*RI forms a dimeric, globular protein complex with a molecular weight of 62 kDa that has increased specificity in the presence of Mg^{2+} (23, 25).

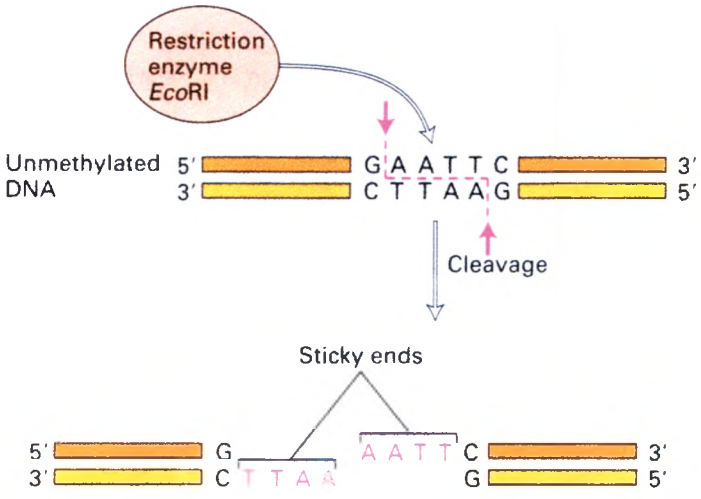

Figure 3. Diagram of *Eco*RI cleavage of double-stranded DNA.

*Eco*RI expression has been performed, *in vivo*, to monitor the outcome of precise cohesive-ended DSBs and their repair, and has been used as an inducer of DSBs to determine the essential genes needed for both repair pathways (23, 24, 26). Use of *Eco*RI expression *in vivo* is advantageous over use of radiation or chemicals because *Eco*RI *only* produces consistent DSBs with precisely defined end structures and the other agents generate many types of DNA damage (23, 24).

Although *Eco*RI exists naturally and originates from *E. coli* bacterial cells, the enzyme can be expressed in a controlled fashion in non-*E. coli* cells. *Eco*RI has previously been expressed from a galactose-regulatable *GAL1* promoter in yeast cells (Figure 4) (4, 27).

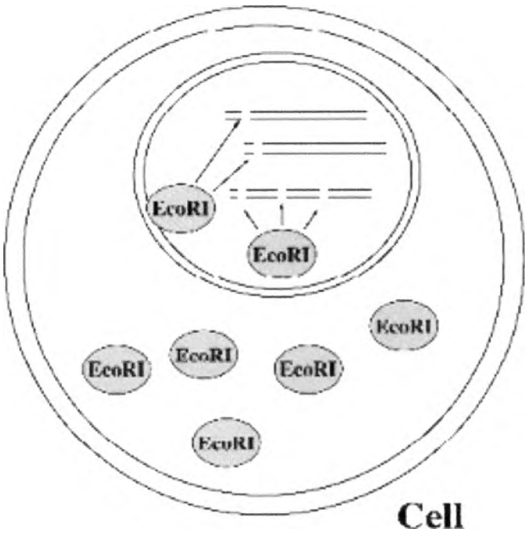

Figure 4. *Eco*RI expression. *Eco*RI expression from a plasmid induces multiple DSBs within each chromosome.

This system is useful due to the ability to regulate *Eco*RI expression by altering the sugar source for the cell. The expression of *Eco*RI is induced by galactose and repressed by glucose; however, expression can also be regulated using a mix of two sugars such as raffinose (no repression) and galactose. This allows a low level of expression to be compared with full induction as seen with galactose alone (28, 29, 30). Galactose induction occurs through a series of positively and negatively regulated mechanisms in which the Gal4 activator protein binds to the *GAL* promoter and stimulates transcription and, therefore, expression of *Eco*RI. The Gal80 protein binds and inhibits Gal4 when cells are grown in glucose, keeping *Eco*RI expression off. In galactose, the Gal3 protein inhibits Gal80, allowing expression of *Eco*RI to be activated by Gal4 (Figure 5). Glucose repression occurs through inhibition of this mechanism (31, 29). This expression system has been useful for studying and identifying the proteins

involved in the DSB repair pathways. It has previously been shown that *Eco*RI expression in yeast produced DSBs that were repaired efficiently in wildtype (WT) cells, while HR or NHEJ mutants exhibited DNA damage-induced growth inhibition and/or cell death (3).

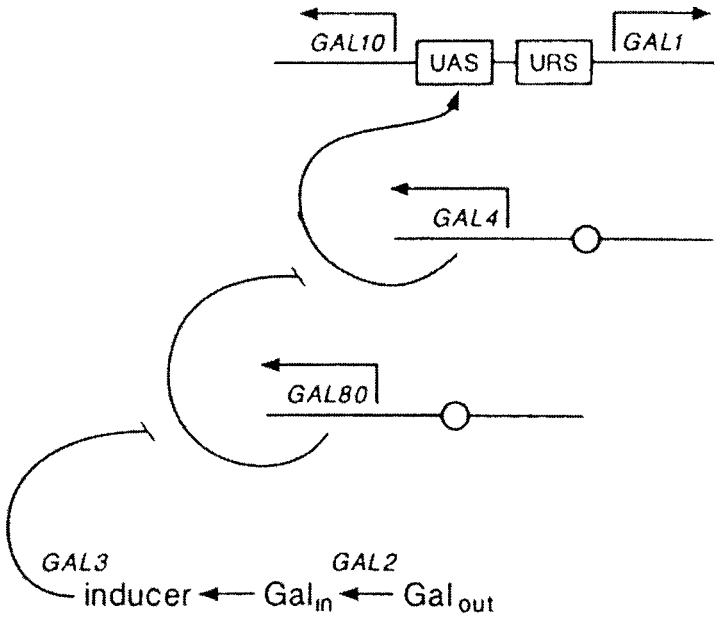

Figure 5. Simplified mechanism of galactose induction at *GAL* promoters. In the presence of galactose, the Gal3 protein inhibits *GAL80* allowing Gal4 to bind to the *GAL* promoter for transcription and expression of *Eco*RI (31).

In an effort to discover other genes required for resistance to radiation, a genome-wide screen was previously performed of a collection of 5000 yeast mutants that each had a different gene inactivated to identify mutants that were hypersensitive to gamma radiation (1, 22, 32, 33, 34, 35). This type of radiation produces DSBs as well as several

other types of oxidative damage to DNA. Out of the 5000 mutants, 169 were found to be radiation-sensitive. Seventeen of the 169 genes were previously associated with recombinational repair of DSBs. Subsequent experiments demonstrated that these 17 mutants were sensitive to radiation *and* to the DNA damaging chemicals MMS and bleomycin (22, 32, 33). Interestingly, 78 of the remaining 152 mutants were subsequently found to be sensitive to radiation plus the chemicals MMS and bleomycin, also suggesting that they might be involved in DSB repair (1).

The primary goals of this project were to develop a novel *in vivo Eco*RI endonuclease survival assay using a *GAL1* promoter expression system that demonstrates the killing of mutants known to be defective in homologous recombination or NHEJ but does not kill wildtype cells. The assay was then applied to the collection of 95 mutants to identify new genes that are specifically required for repair of DSBs and are therefore critical for chromosome stability and prevention of mutations.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Lithium acetate and glycerol were purchased from Invitrogen Life Technologies (Carlsbad, CA). Ethidum bromide (EtBr) was purchased from Shelton Scientific, Inc. (Shelton, CT). Dimethyl sulfoxide (DMSO), ampicillin, methyl methanesulfonate (MMS), lysozyme, RNase, potassium chloride, calcium chloride, and magnesium chloride were purchased at Sigma Chemical Co. (St. Louis, MO). Shrimp alkaline phosphatase (SAP) was purchased from Stratagene (La Jolla, CA). Sodium dodecyl sulfate (SDS), sodium chloride, ammonium sulfate, magnesium sulfate, sucrose, boric acid, and polyethylene glycol (PEG) 4000 were purchase from Mallinckrodt (Paris, Kentucky). Ethylenediaminetetraacetic acid (EDTA) and bleomycin were obtained from EMD Chemicals, Inc. (Darmstadt, Germany). Tris base was purchased from VWR International (Westchester, PA). Triton X-100 was purchased from J.T. Baker (Phillipsburg, NJ). Klenow DNA polymerase, T4 DNA Ligase, and 1Kb DNA standard ladder were purchased from New England Biolabs (Beverly, MA), and a deoxynucleotide triphosphate (dNTP) mix was purchased from Takara (Madison, WI).

Bacteriological and Yeast Media

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

Yeast Strains and Plasmids

The yeast strain BY4742 has a genotype of *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (30). YLKL834 has a genotype of *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1::hisG-URA3-hisG mre11Δ. G418*. Plasmids used for this study include pRS316 (*CEN/ARS URA3*) and pRS426 (*2μ URA3*) (38), pLKL31Y (*CEN/ARS TRP1 GAL1p.:EcoRI*), and YCpGal:RIb (*CEN/ARS URA3 GAL1p::EcoRI*) (27). The yeast deletion strain library was obtained from Open Biosystems (Huntsville, Al) (32). The library is a MAT alpha strain collection kept frozen in 96-well microtiter dishes with BY4742 as the strain background. In each well, the cells are suspended in YPD Broth + G418 (200μg/ml) + 15% glycerol and the microtiter dishes are stored at -80°C.

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). For the *Eco*RI assay, yeast cells were grown on synthetic media with drop-out mix (0.17% yeast nitrogen base without amino acids), sugar (2% glucose or 2% galactose or 1% raffinose + 0.2-3% galactose), 2% bacto agar, and all essential amino acids minus the amino acids used for selection. Plates supplemented with magnesium were prepared using synthetic media and varying concentrations of magnesium sulfate. Plates with methyl methanesulfonate (MMS) were prepared using synthetic media or YPDA supplemented with aliquots of a stock solution of 11.2 M MMS to achieve varying final concentrations. Bleomycin plates were made using synthetic media plus aliquots of a stock solution of 0.5 mg/ml Bleomycin to obtain various 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

Plasmid DNA Purification

Plasmid DNA was purified using either a rapid boiling lysis protocol (38) or a Qiagen Spin Column Miniprep Kit (Maryland, VA).

Yeast Transformations

Yeast transformations were performed using a rapid lithium acetate and DMSO-based transformation protocol (39).

E. coli Transformations

E. coli transformations were completed using frozen competent DH5α cells prepared by the protocol of Miller and Chung (40).

Gel Electrophoresis

Gel electrophoresis was performed using 0.6% agarose gels in 1X TBE (90 mM tris-borate, 2 mM EDTA) running buffer in a Life Technologies Horizon 11-14 gel rig at a voltage of 110 V. Gels were stained with ethidium bromide (EtBr) and a Kodak IS440 CF Imager and Kodak 1D imaging software were used to capture gel images.

Plasmid Construction

pLKL89Y *(pLKL31Y with the unique EcoRI site filled in).* pLKL31Y (*GAL1p:EcoRI TRP*) (5 μl of a concentrated miniprep) was digested with 30 U *Eco*RI in

1X KGB buffer (100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.6), 10 mM magnesium acetate), filled in with 5 U Klenow DNA polymerase and 50 μM dNTPs, self-ligated with 1X T4 Ligase Buffer and 400 U T4 DNA Ligase at 9°C overnight. The plasmid was then re-digested with 30 U *Eco*RI to ensure the *Eco*RI cut site was eliminated from the plasmid and transformed into competent DH5α *E. coli* cells for propagation at 37°C on LB + Amp plates. The plasmid DNA was purified as described above and visualized using gel electrophoresis on a 0.6% agarose gel. Plasmid DNA was then digested with 30 U *Eco*RI and run on a 0.6% agarose gel to verify that the *Eco*RI cut site was removed.

EcoRI Replica-plating and Double Imprinting Assays

To develop an assay that demonstrated *Eco*RI killing of known DSB repair mutants, patches of mutant and WT cells were grown on 2% raffinose synthetic plate media for 2-3 days, replica-plated to a 2% raffinose plate. The raffinose plate was then immediately used as a new master plate to replica-plate cells to 1% raffinose + 0.2-3% galactose (partial *Eco*RI induction), and 2% galactose (full *Eco*RI induction) plates and grown at 30°C for 2-3 days. Partial induction of *Eco*RI was determined to be optimum using 1% raffinose + 3% galactose plate media. Assay optimization included increasing the temperature at which the double imprints were grown to 37°C for 2-3 days, as well as increasing the concentration of $MgSO_4$ in plate media to 10 times the normal, neither of which increased the efficiency of *Eco*RI in the killing of known DSB repair mutants. The *Eco*RI double imprinting assay was then applied to the collected of mutants used in this study. Mutants were classified as R (resistant to *Eco*RI killing), SS (sensitive to *Eco*RI

killing) or SSS (most sensitive to *Eco*RI killing) according to their growth on double imprints in comparison to *Eco*RI killing of WT and known DSB repair mutant cells. Images of the cell growth inhibition on plates were captured using a Canon Powershot G3 digital camera.

Dilution Pronging Cell Survival Assays

Yeast cells were harvested from either synthetic or YPDA plates into sterile deionized H_2O, diluted 1/40, sonicated for 10 seconds at 3 watts using a Sonics Vibracell Ultrasonic Processor (Newtown, CT), and quantitated using a Hausser Scientific (Horsham, PA) Bright-Line hemocytometer on a Comcon (Russia) LOMO phase contrast microscope. Yeast cells were then added to a Becton Dickinson Labware (Franklin Lakes, NJ) 96-well mictrotiter dish to a cell concentration of $1x10^7$ or $2x10^7$ cells per 220 µl. A series of six 5-fold serial dilutions of the cells were then made using a multi-pipettor. The cells were then pronged onto control plates as well as onto plates containing either varying concentrations of MMS or Bleomycin, 2% raffinose, 1% raffinose + 3% galactose (to partially induce *Eco*RI killing), or 2% galactose (to fully induce *Eco*RI killing). Cells were grown at 30°C for 3-4 days. Images of the cell growth on plates were captured using a Canon Powershot G3 digital camera.

Cell Cycle Analysis of EcoRI Sensitive Mutants

All of the mutants exhibiting the most sensitivity to *Eco*RI including BY4742 (WT) and 14 HR or NHEJ control mutant cells were harvested, sonicated, and counted with a hemocytometer as described above, then diluted into three YPDA cultures at $3x10^6$

cells/ml for each mutant. The cultures (500 µl) were shaken vigorously at 30°C for 4 hours. Each culture was sonicated and the fraction of cells which were unbudded, small-budded, or large-budded was analyzed with a hemacytometer. A total of 100 cells were counted for each culture and the results of the three samples were averaged for each mutant. Large-budded cells were defined as cells in which the size of the bud was >50% of the size of the mother cell (4).

Cell Cycle Analysis of EcoRI Sensitive Mutants after Exposure to Bleomycin

All of the mutants exhibiting the most sensitivity to *Eco*RI including WT, six HR or NHEJ and two checkpoint control mutant cells were harvested and diluted into six YPDA cultures (1 ml) at $1x10^6$ for each mutant as described above. The cultures were shaken vigorously at 30°C for two hours, then bleomycin was added at 0.06 µg/ml to only three of the cultures for each mutant (the three cultures were used as untreated controls for each mutant). The cultures were shaken at 30°C for 5 hours, sonicated and cell types were counted and averaged as described above. Averages of cultures treated with bleomycin were compared to those untreated.

CHAPTER III

RESULTS AND DISCUSSION

This research project focused on the identification and characterization of new genes that are involved in one or both of the DSB repair pathways. First, an assay based on sensitivity to *in vivo* expression of the endonuclease *EcoRI* was developed and demonstrated to be lethal in known DSB repair mutants but not in WT cells. The assay was then used to screen a collection of 95 mutants from a yeast strain deletion library which had been previously shown to exhibit similar characteristics to known DSB repair mutants, including sensitivity to gamma radiation, MMS, and other DNA damaging agents that can cause DSBs. Next, a subset of the collection of sensitive mutants with the most sensitivity to *Eco*RI was tested for MMS and bleomycin-resistance to compare results with these clastogens to that of *Eco*RI. Finally, these mutants were also screened for alterations in their cell cycle checkpoint responses to DNA damage to further clarify their role in DNA repair.

Development of an assay that permits screening of a large collection of yeast mutants for sensitivity to expression of EcoRI endonuclease in vivo

Several parameters were tested during the development of the *Eco*RI survival assay (see Methods). The key to this assay is that the expression of *Eco*RI endonuclease inside haploid yeast cells is lethal in mutants unable to repair DSBs but not in WT cells, which are proficient in both HR and NHEJ. My approach involved comparing growth of WT cells containing the plasmid YCpGal:RIb (*GAL1p::EcoRI URA3*) to growth of repair-deficient *mre11* mutants containing this plasmid. *mre11* mutant cells were selected for these tests because they are known to be highly sensitive to *Eco*RI killing (3). Several approaches were taken to optimize the assay using plates containing raffinose minus uracil (Raff - Ura), raffinose plus galactose (Raff + Gal - Ura), and galactose (Gal - Ura) media. The amount of galactose to use for varying levels of induction of *Eco*RI was the most important variable to optimize. Using raffinose as the main sugar source avoided the problem of glucose repression of the *GAL1* promoter resulting in faster induction upon transfer to galactose (31). By using raffinose plate media, the *GAL1* promoter is off and *Eco*RI expression is not induced. Full induction of *Eco*RI expression was achieved by using galactose only plate media. For partial induction of *Eco*RI a mix of raffinose and galactose plate media was used. The optimum concentrations of raffinose and galactose were found to be 1% raffinose + 3% galactose. This concentration resulted in strong growth inhibition of *mre11* cells by *Eco*RI, but not WT cells (data not shown).

Once the sugar concentrations were optimized, other variables were tested to improve the assay. Initially, the assay involved using a replica-plating technique (Figure

6). In replica-plating, colonies on a plate are transferred to a velvet cloth and duplicated by pressing subsequent plates onto the cloth. It was determined that replica-plating a single time did not show as strong of a growth inhibition effect as replica-plating twice.

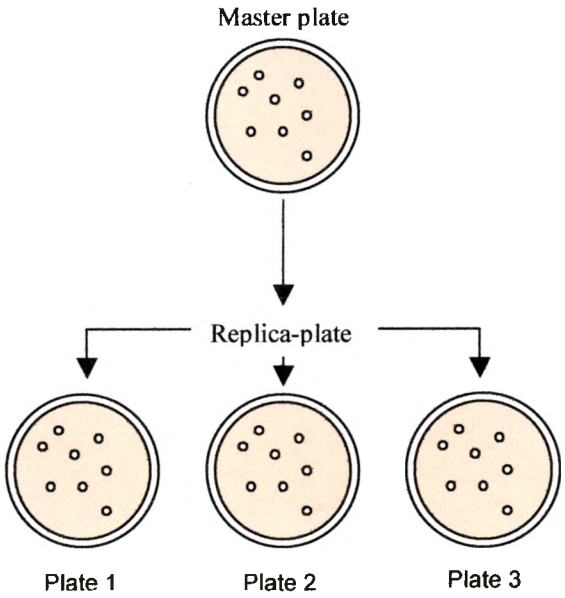

Figure 6. Diagram of replica-plating technique. During the replica-plating process, the pattern of colonies or patches on a master plate is duplicated onto subsequent plates.

This method of double-imprinting was found to better demonstrate the effective killing of *mre11* mutants by *Eco*RI. In this approach, a plate containing patches of cells is replica-plated to another, followed immediately by using this second plate as a new master for a second round of replica-plating (see diagram in Figure 7). With this method, fewer cells are transferred to each plate, which allows easier detection of *Eco*RI-induced killing.

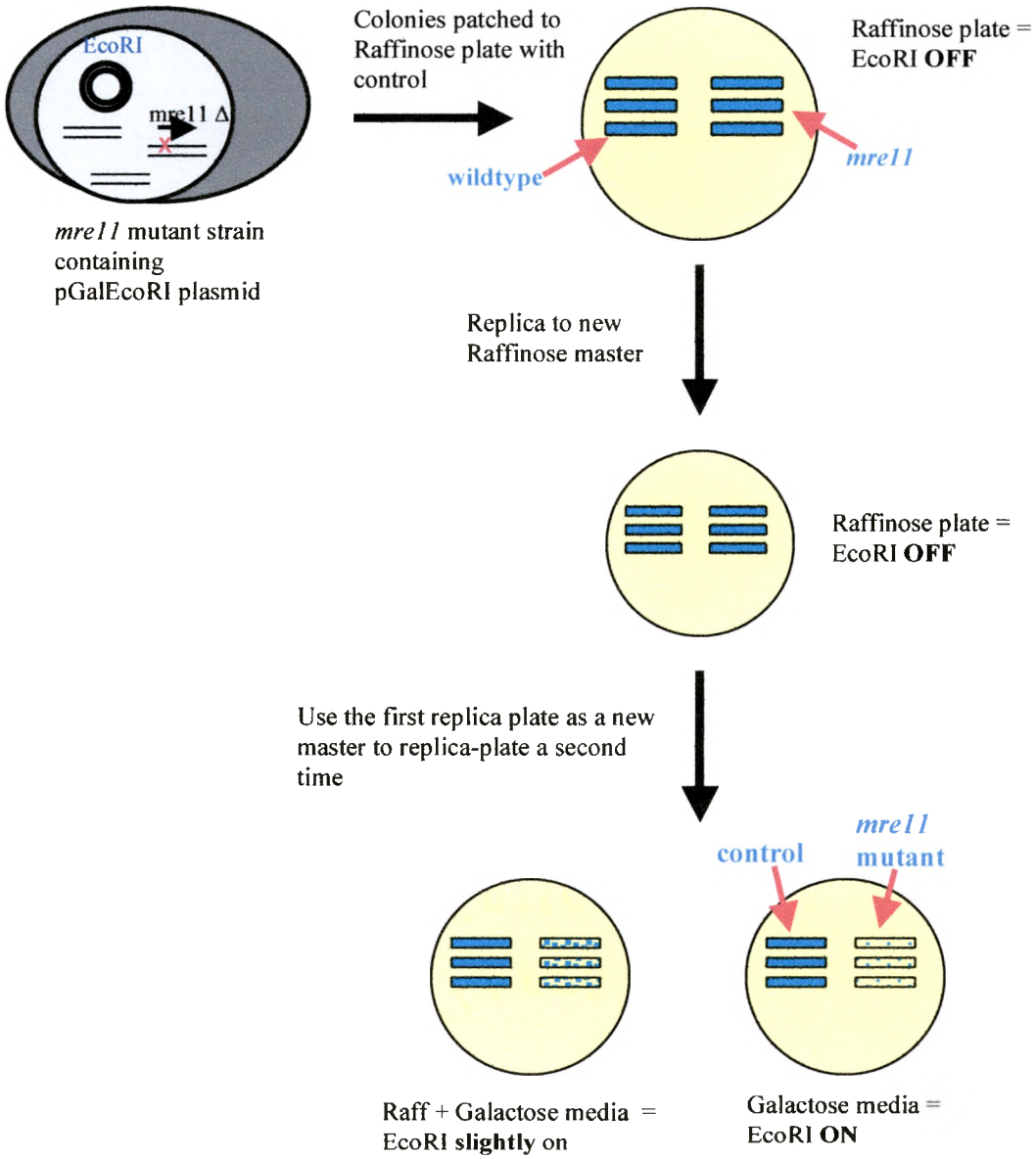

Figure 7. Diagram illustrating the double-imprinting method used for *Eco*RI survival assays. Wildtype and mutant cells growing on raffinose media are replica-plated to a new raffinose plate, which is then replica-plated to Raff + Gal and Gal plates.

Initially, the plasmid used for *Eco*RI induction was YCpGal:RIb, which contains *URA3* as a selectable marker (4, 27). A *TRP1* gene-containing plasmid called pLKL89Y with the *GAL1* promoter fused to *Eco*RI (Figure 8) was constructed using the vector pRS314 (see Methods) to determine if *Eco*RI killing could be improved by using the new plasmid. Both of these plasmids have a centromere and are single-copy plasmids. Plates containing streaks of wildtype and *mre11* cells containing either pLKL89Y or YCpGal:RIb were replica-plated to galactose plates and growth at 30°C assessed after 3 days. It was determined that pLKL89Y did not improve the assay (data not shown); therefore YCpGal:RIb, which has been used for several previous studies (3, 4, 27) was used for this project.

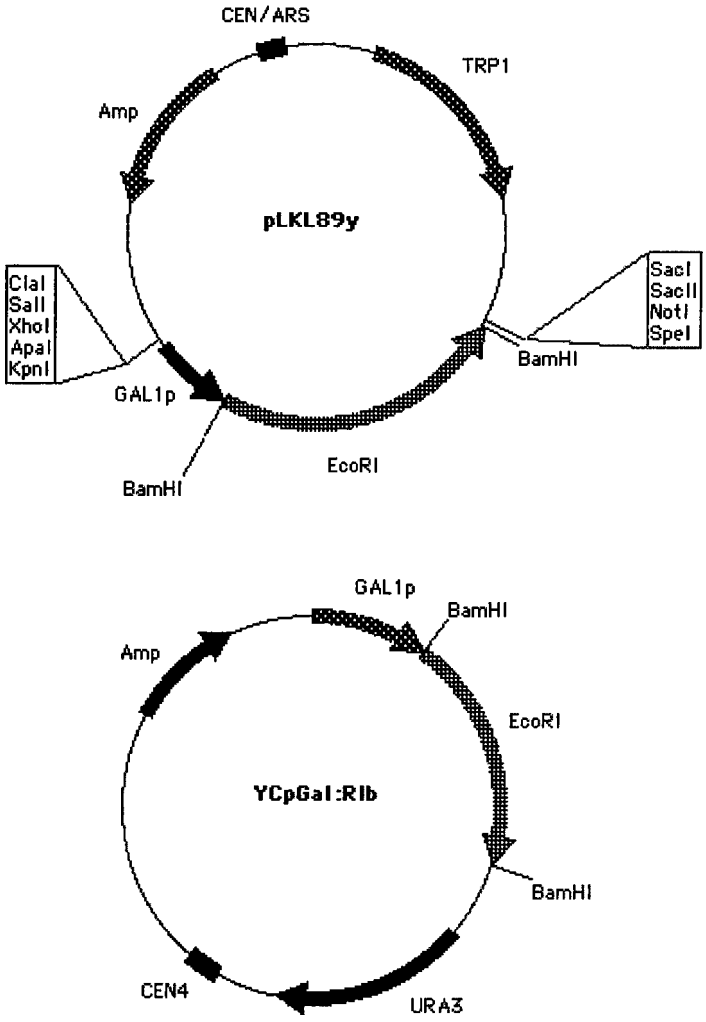

Figure 8. Plasmid map of pLKL89Y and YCpGal:RIb. Graphical representation of plasmid pLKL89Y made in this study and YCpGal:RIb (27).

Yeast cells normally grow at 30°C, but since *Eco*RI originates from *E. coli* cells, which have an optimum growth temperature of 37°C, the temperature at which the plates from the assay were grown was increased to 37°C. However, the results of the assay were not improved with the temperature increase, therefore all subsequent assays were performed at 30°C, which is the optimum growth temperature for yeast cells.

*Eco*RI requires magnesium for cleavage of DNA. To determine if increasing magnesium concentration in the growth media might increase the activity of *Eco*RI, WT and *mre11* cells containing YCpGal:RIb were replica-plated to standard galactose plates with and without magnesium (ten times the normal concentration of 4 mM). However, an increase in magnesium concentration did not improve the results of the assay (i.e., there was not more inhibition of growth on the galactose plates).

Yeast deletion strain library

The yeast deletion strain library is a collection of 5000 different haploid mutants in which one non-essential gene is knocked out in each mutant (32). The strain background of the library is BY4742, and the mutant strains are MAT α (mating type alpha). The library is stored at -80°C in multiple 96-well microtiter dishes (Figure 9). Each well of the microtiter dish contains a different mutant with columns and rows labeled as shown in the figure.

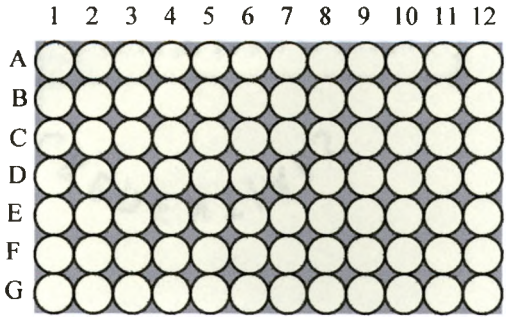

Figure 9. Representation of a microtiter dish from yeast deletion library. Each well contains a different yeast mutant strain stored in YPDA broth plus 15% glycerol.

Optimized EcoRI survival assay applied to control mutants

As demonstrated in Figure 10, eleven control mutant strains (known to be involved in HR, or in both the HR and NHEJ pathways) including *mre11, rad50, rad51, rad52, rad54, rad55,* and *rad57* cells were transformed with YCpGal:RIb and vector pRS316 separately, patched to master 2% Raff plates and grown at 30°C for 3 days. These patch plates were replica-plated to new master 2% Raff plates, and then replica-plated a second time (double-imprinted) to 1% Raff + 3% Gal and 2% Gal only plates. The double-imprint plates were incubated for 3 days. Each of the mutants was characterized as having moderate sensitivity (SS) or severe sensitivity (SSS) (Table 1). Mutants classified as SSS showed strong growth inhibition on both the Raff + Gal and Gal plates, while SS mutants exhibited strong growth inhibition only on Gal plates.

Table 1. *Eco*RI sensitivity of control mutants

Mutant	*Eco*RI Sensitivity
mre11	SSS
rad50	SSS
rad51	SSS
rad52	SSS
rad54	SS
rad55	SSS
rad57	SS

The *mre11* mutant containing YCpGal:RIb, which showed severe sensitivity to *Eco*RI (Figure 10) was chosen to use as a control for subsequent experiments involving

the screening of unknown mutants. The *mre11* cells containing the pRS316 vector grew well on galactose plates. As shown in the figure, two patches (single streaks) for each mutant were included on each plate.

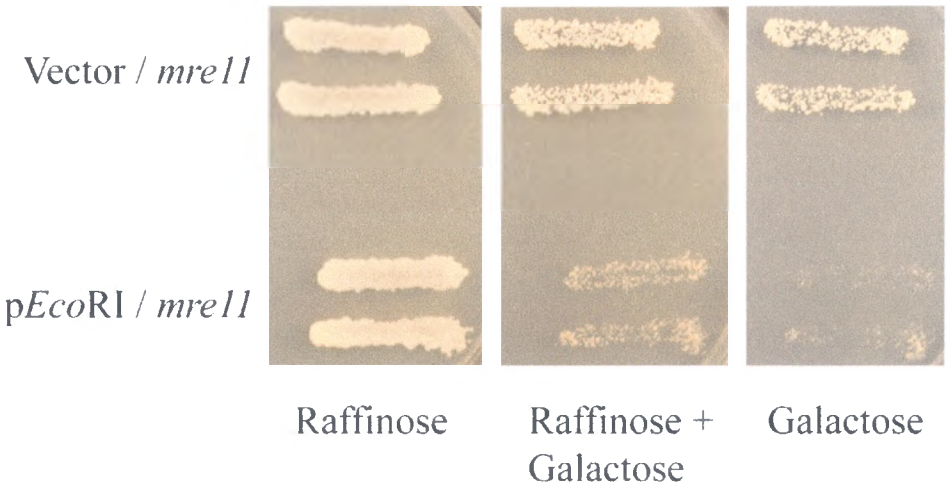

Figure 10. Double imprint of *mre11* cells showing severe sensitivity to *Eco*RI. Patches of *mre11* cells containing the vector pRS316 or YCpGal:RIb (p*Eco*RI) were replica-plated two times (double-imprinted) to plates containing different sugars. These *mre11* cells show severe sensitivity to *Eco*RI on Raff + Gal (low *Eco*RI expression) as well as Gal (full *Eco*RI expression) plate media.

EcoRI survival assay applied to mutants obtained from the yeast deletion strain library

A total of 95 mutants from the yeast deletion strain library were tested for *Eco*RI sensitivity. This included 71 mutants known to be sensitive to gamma radiation, bleomycin, and MMS (32, 33). In addition, 9 mutants identified in a previous screen for MMS-sensitive mutants by Chang *et al.* (22) that were sensitive to all mutagens they used

(MMS, gamma, HU, and UV) were tested (*dun1, mec3, mms2, mms4/ybr099c, npl6, rad5, rad17,* and *rad24*). Also, 5 mutants linked to HR in previous studies of DSB-induced recombination were included (*rad1, rad9, rad10, rdh54* and *exo1*) (41, 42, 43, 44), plus an additional 9 mutants previously linked to the NHEJ double-strand break repair pathway (*dnl4, lif1, nej1, rad27, sir2, sir3, sir4, yku70,* and *yku80*) (2). Finally, a mutant lacking Ddc1 was tested because it is part of a trimeric protein complex formed with two other proteins in the list, Rad17 and Mec3 (45).

This set of 95 mutants was transformed with YCpGal:RIb and *Eco*RI assays were used to screen the set of mutants for sensitivity to *Eco*RI. *mre11* cells are included as a control (Figure 11). For example, in the figure *Eco*RI-sensitive *mre11* cells were replica-plated in the top of the plate along with four unknown mutants (*rdh54, hpr1, ctf8,* and *rad5*) to Raff-Ura, Raff + Gal – Ura, and Gal-Ura plates and grown at 30°C. As shown in the figure, *mre11* cells were strongly sensitive to *Eco*RI, growing poorly on Raff + Gal and Gal plates. *hpr1* and *ctf8* strains exhibited strong sensitivity essentially identical to *mre11* cells and were categorized as SSS (highly sensitive). In contrast, *rdh54* and *rad5* mutants were only strongly inhibited on Gal plates and were therefore classified as SS (moderately sensitive) mutants.

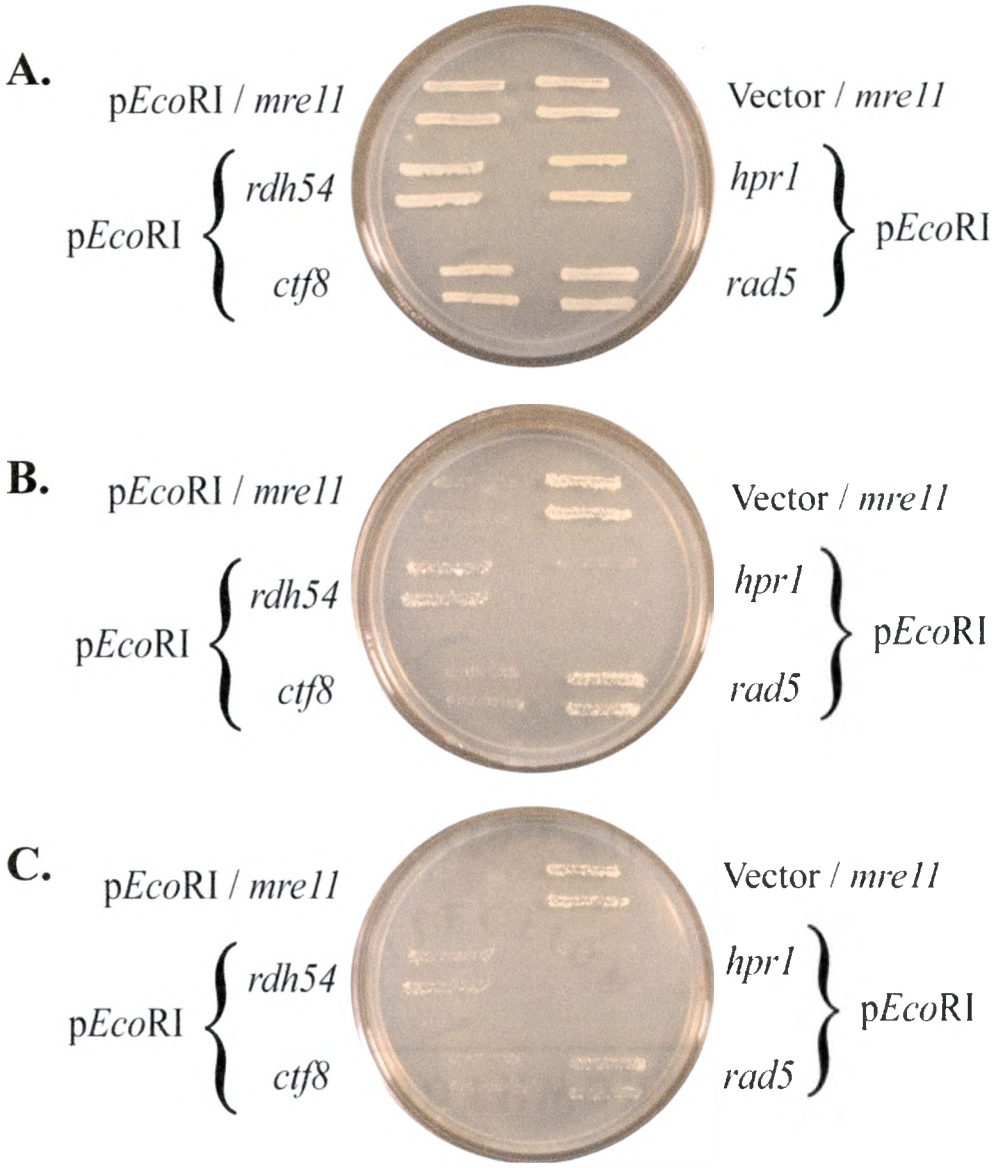

Figure 11. Double-imprint *Eco*RI assay of four new yeast mutants. Each plate contains two controls including YCpGal:RIb / *mre11* and pRS316 / *mre11* cells and four new mutants (*rdh54, hpr1, ctf8,* and *rad5*) double-imprinted onto Raff (**A**), Raff + Gal (**B**), and Gal (**C**) plates. In this assay, *rdh54* and *rad5* grew poorly only on Gal plates (SS), while *hpr1* and *ctf8* grew poorly on both Raff + Gal and Gal plates (SSS).

From these experiments, using the classification system described above, 20 mutants were tentatively identified as having severe sensitivity to *Eco*RI killing, and were categorized as SSS, 34 mutants exhibited moderate sensitivity, and were categorized as SS, and the remaining mutants showed little to no sensitivity (Table 2).

Table 2. *Eco*RI-sensitive mutants identified by double-imprint replica-plating

SSS Mutants	SS Mutants
anc1, akr1, bck1, cax4, clc1, cnm67, ctf4, ctf8, ddc1, grr1, hpr1, kre22, mms2, mms4, mms22, npl6, rad10, ume6, vph2, ybro99c	*adk1, apn1, bud32, bur2, dia4, dcc1, dhh1, exo1, hpr5, hfi1, htl1, mct1, mus81, not5, nup84, nup133, pol32, rad1, rad5, rad9, rem50, rdh54, rlr1, rpb9, rpl31a, rvs161, sae2, slx8, spt10, srv2, vid21, ybr100w, ygl218w, ylr235c*

Application of semi-quantitative dilution pronging cell survival assays

Dilution pronging is a more quantitative method for survival assays than conventional replica-plating or double-imprint replica-plating (5). The pronging method employed for this project involves harvesting cells and placing them in the first well of a microtiter dish at a specific concentration (Figure 12). The cells are then serially diluted 5-fold horizontally for six wells along the microtiter dish. A tool called a pronger is placed in the microtiter dish. Once removed, cells from the dish are suspended from individual pegs on the pronger. The pronger is then placed onto a plate, transferring the

cells onto the surface. This method allows quantification of cell growth rates and survival.

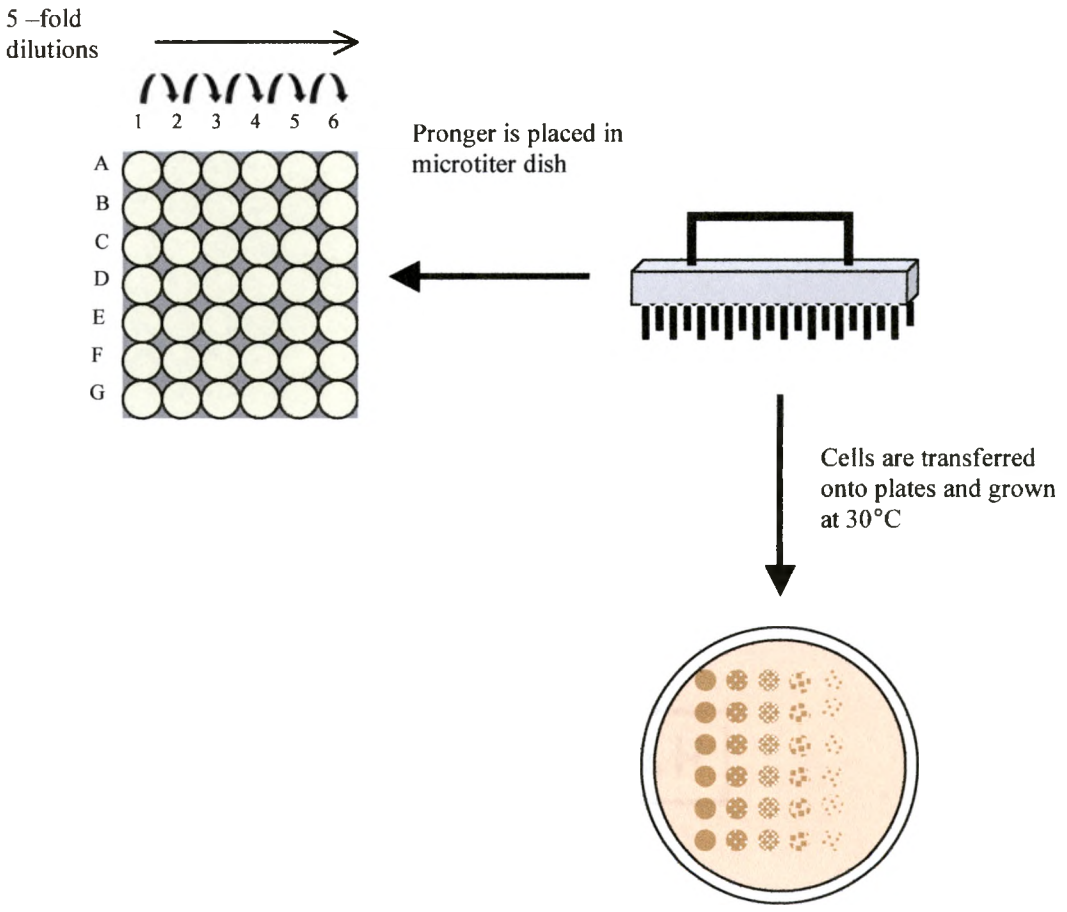

Figure 12. Diagram of the dilution pronging cell survival assay. Cells are diluted into each well of a microtiter dish at a specific concentration. The pronger is placed into the microtiter dish and then spotted onto plates, transferring cells that later become colonies, allowing for quantification of cell growth on various plate media.

For these assays, BY4742 cells containing the vector pRS316 or YCpGal:RIb were used as a WT control. The assays were performed first on several known HR and NHEJ mutants (Figure 13). 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 from left to right. For the experiment shown in the figure, 8 rows of cells were pronged for 5 columns of serial dilutions. The first 2 rows on each plate are WT with vector and with YCpGal:RIb (shown as p*Eco*RI in the figure). The six subsequent rows consisted of known HR or NHEJ mutants also containing YCpGal:RIb. Each set of 8 strains was pronged to Raff – Ura, Raff + Gal – Ura, and Gal – Ura plates. Wildtype cells showed only slight sensitivity to *Eco*RI on Raff + Gal and Gal plates, with colonies growing in the fifth column. *mre11, rad50, rad51, rad52* and *rad55* (*rad55* is not shown) mutants all exhibited severe sensitivity to *Eco*RI, displaying > 25-fold killing relative to p*Eco*RI / WT cells on Raff + Gal plates and > 125-fold killing on Gal plates. In contrast, *rad54* and *rad57* cells (*rad57* is not shown) exhibited only slight sensitivity. NHEJ-deficient *yku70, yku80, sir2,* and *sir3* mutants all exhibited growth equal to that of WT, and were therefore categorized as having no sensitivity to *Eco*RI. Sensitivity to *Eco*RI has been seen in Nhej$^-$ mutants in other strain backgrounds (3), but was not apparent in the BY4742 background under the conditions employed here.

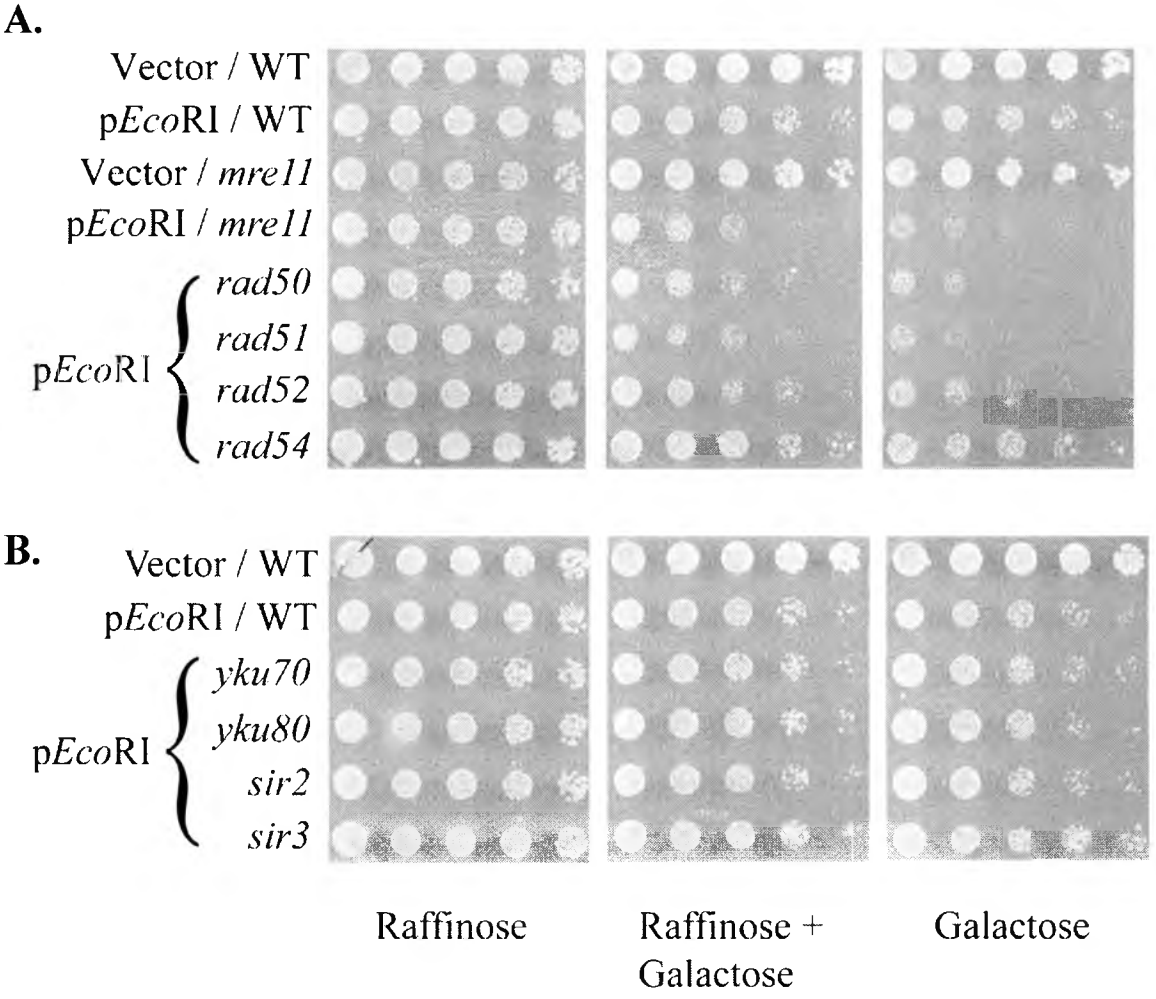

Figure 13. Dilution pronging assays of well-characterized DSB repair mutants. HR mutants (**A**) and NHEJ mutants (**B**) were pronged onto Raff, Raff + Gal, and Gal plates and grown at 30°C for 3 days. p*Eco*RI is YCpGal:RIb.

Application of dilution pronging cell survival assays to EcoRI-sensitive mutants originally identified by double-imprint replica-plating

Pronging assays were used to categorize the set of 54 *Eco*RI-sensitive mutants suggested by double-imprints. For these assays, each mutant was transformed with the

vector pRS316 to use as an internal control. A total of 2×10^7 cells was used as before. On each plate, the first two rows were WT cells containing vector and YCpGal:RIb. As shown in Figure 14, the next two rows were *mre11* cells with vector and YCpGal:RIb, which served as a control. The subsequent four rows in this particular plate consisted of two sets of mutants (*mms2* and *mms4*) containing vector and YCpGal:RIb. Both *mms2* and *mms4* displayed $\geq$ 25-fold more killing on Raff + Gal than WT cells and $\geq$ 125-fold more on Gal plates. These characteristics are similar to those of *mre11* cells and therefore these mutants were characterized as highly sensitive to *Eco*RI killing. Phenotypes of *mms4* cells were similar to those of *ybr099c* mutants, which is not unexpected because the open reading frames (ORFs) for the two genes overlap each other on opposite strands. The *Saccharomyces* Genome Database (yeastgenome.org) suggests that *MMS4* is a functional gene but *YBR099C* is not.

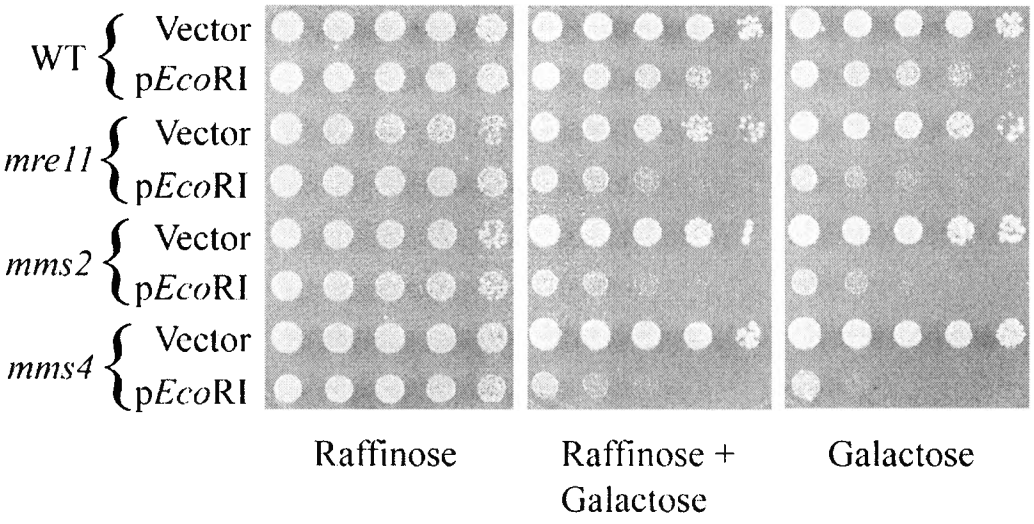

Figure 14. Dilution pronging assay of control and new mutants. Both WT and *mre11* cells containing vector and YCpGal:RIb were used as controls on each plate. Experimental procedures were performed as for Figure 13.

An example of a pronging experiment involving mutants categorized as SS by the double-imprint replica-plating method is shown in Figure 15. The first two rows are WT cells with vector and YCpGal:RIb. The next six rows consist of three sets of mutant strains containing vector and YCpGal:RIb (*hpr5, ylr235c,* and *dcc1*). From this assay, it was determined that *hpr5* was not more sensitive to *Eco*RI than WT on either Raff + Gal or Gal plates. It was also determined that *ylr235c* was consistently moderately sensitive and *dcc1* was actually one of the most sensitive mutants.

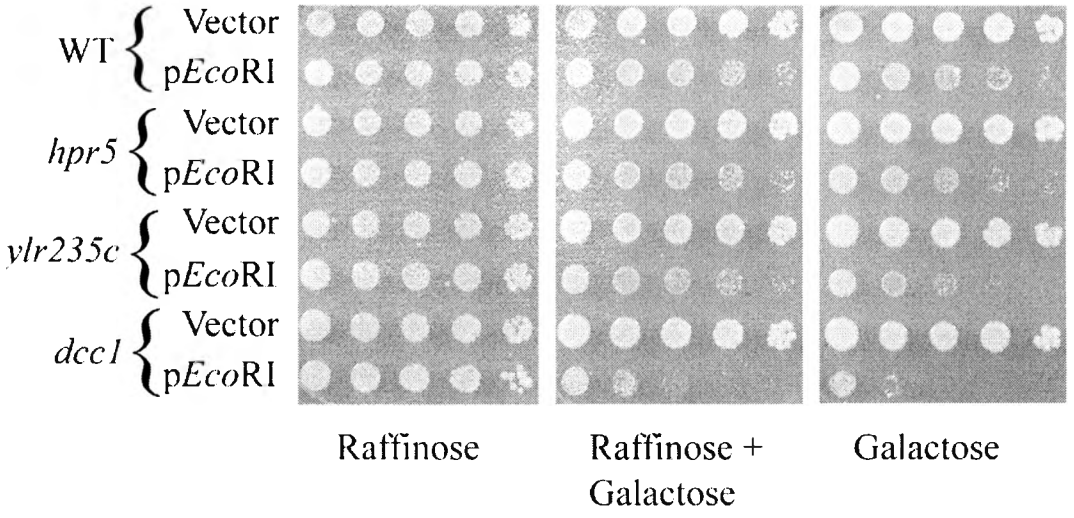

Figure 15. Dilution pronging assay of those mutants previously classified as SS. The control for this assay was WT cells containing vector and YCpGal:RIb. Also pronged in this assay were *hpr5, ylr235c,* and *dcc1* cells containing vector and YCpGal:RIb. Experimental procedures were performed as for Figure 13.

Out of all 102 mutants tested, including 7 known repair-deficient strains treated as controls (Table 1), plus 95 library strains considered unknowns, 22 mutants were categorized as highly sensitive to *Eco*RI-induced killing. This set included the well-characterized recombination genes *RAD50, MRE11, RAD51, RAD52* and *RAD55*, plus 17 other genes. These genes are listed with brief descriptions (yeastgenome.org) in Table 3.

Table 3. Most sensitive mutants

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
anc1	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,
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
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
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

Table 3-Continued. Most sensitive mutants

Gene Name	Name Description	Description
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
kre22	N/A	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
mms2	Methyl Methanesulfonate Sensitivity	Protein involved in error-free postreplication DNA repair; forms a heteromeric complex with Ubc13p that has a 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 Sensitivity	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
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
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

*Well-characterized recombination genes used as controls.

A total of 18 genes, including known recombination pathway genes *RAD54* and *RAD57*, were classified as being moderately sensitive to *Eco*RI. These genes are listed along with brief descriptions in Table 4.

Table 4. Moderately sensitive mutants

Gene Name	Name Description	Description
adk1	Adenylate Kinase	Adenylate kinase, required for purine metabolism; localized to the cytoplasm and the mitochondria; lacks cleavable signal sequence
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
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
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
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
rad5	Radiation Sensitive	DNA helicase proposed to promote replication fork regression during postreplication repair by template switching; contains RING finger domain
rem50	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

Table 4-Continued. Moderately sensitive mutants

Gene Name	Name Description	Description
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	Protein with a role in accurate meiotic and mitotic double-strand break repair; phosphorylated in response to DNA damage and required for normal resistance to DNA-damaging agents
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
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
vid21	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
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
*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
*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

* Well-characterized recombination genes used as controls.

Fifty-three of the 102 library strains did not have detectable sensitivity to *Eco*RI expression (Table 5). Ten of the mutants could not be tested, usually because of poor growth on Raff or Gal plate media. Mutants such as *mdm10* and *mdm20* were known to have mitochondrial defects, so these results were not unexpected (Table 6).

Table 5. Mutants categorized as not sensitive to *in vivo* expression of *Eco*RI

apn1	*hpr1*	*rad1*	*sgs1*
asf1	*hpr5*	*rad6*	*sir2*
bdf1	*jem1*	*rad9*	*sir3*
bem1	*lif1**	*rad10*	*sir4*
cbc2	*mec3*	*rad17*	*slx8*
chl1	*mus81*	*rad18*	*srb5*
dia4	*nat3*	*rad24*	*vid31*
dhh1	*nej1***	*rad27*	*yaf9*
*dnl4***	*not4*	*rad59**	*ybr100w*
dun1	*npl6*	*rad61*	*yku70***
fab1	*nup120*	*ref2*	*yku80***
fun12	*nup133*	*rdh54*	
gos1	*pat1*	*rlr1*	
hof1	*pol32*	*rsc1*	

*Well-characterized recombination genes.
**Well-characterized end-joining genes

Table 6. Mutants not tested

Mutant	Reason Not Tested
bud32	Grows poorly
cdc40	*URA3*$^{+}$
clc1	Does not grow on raffinose
hfi1	Grows poorly
mdm10	Mitochondria problem. YPG^{-}, Gal^{-}
mdm20	Mitochondria problem. YPG^{-}, Gal^{-}
rvs167	Absent from library
xrs2	Absent from library
ygl218w	Grows poorly

Fifteen of the previously categorized SSS were consistently categorized as the most sensitive mutants (Table 3). Five of the previously categorized SSS mutants were placed in a new category. *hpr1, npl6,* and *rad10* were found to have no sensitivity to *Eco*RI, and *clc1* and *grr1* were not testable due to growth problems with the vector strain (Table 6).

These results also produced 17 mutants that were categorized differently than the double-imprint *Eco*RI assay, primarily because vector and *Eco*RI plasmid-containing cells could be placed side-by-side for comparison and because of the more quantitative nature of the pronging assays. Two mutants, *not5* and *htl1* were placed in the most sensitive category (Table 3), 12 mutants were found to have no sensitivity to *Eco*RI (Table 5), and *bud32* and *ygl218w* were not testable due to growth problems with the vector strain (Table 6). Seventeen mutants were categorized as having some sensitivity to *Eco*RI (Table 4). Inconsistencies may be due to isolate differences and require further testing using multiple isolates.

As seen in Tables 3 and 4, many genes required for resistance to *Eco*RI have previously been implicated in processes linked to DSB repair. Exo1 has previously been shown to be a backup nuclease for the Mrx complex in HR (5). Genes shown to be involved in sister chromatid cohesion or modification include *CTF4, CTF8, HTL1, DCC1* and *ANC1*. The genes *MMS2, MMS22,* and *MMS4* have been implicated in post-replication or recombinational DNA repair, though their precise roles are uncertain. Finally, Ddc1 is a DNA damage response checkpoint protein.

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

All of the mutants examined in this study had previously been found to be sensitive to two or more physical and chemical DNA damaging agents. Comparison of those sensitivities from earlier the work to the new *Eco*RI experiments are difficult because some of the previous experiments involved haploid mutants and others used diploid cells (22, 32, 33). All of the experiments performed for the current project used haploid cells. In an effort to try to compare the effects of these general clastogens to *Eco*RI, the sensitivity of each new mutant to MMS and bleomycin was determined.

To test for MMS sensitivity, the 33 *Eco*RI-sensitive mutants plus seven control DSB repair mutants that were sensitive to *Eco*RI were pronged to synthetic Glu plates and Glu plates containing 1 mM or 2 mM MMS. Each plate contained WT BY4742 cells as a control (Figure 16). This figure shows a set of pronged plates with control mutants (A), a set of highly *Eco*RI-sensitive mutants (B), and a set of moderately *Eco*RI-sensitive mutants (C). The parameters of characterization for these assays were as follows: > 125-fold killing (SS), 125-fold killing (M), < 125-fold killing or much smaller colony

diameter than WT (S), and killing equal to that of WT (R). For the controls in Figure 16A, 2 mM MMS caused too much damage and the cells were unable to recover. However, 1 mM MMS allowed some mutants to exhibit more sensitivity than others. For example, *mre11, rad50, rad52,* and *rad54* cells were severely sensitive to 1 mM and were categorized as SS.

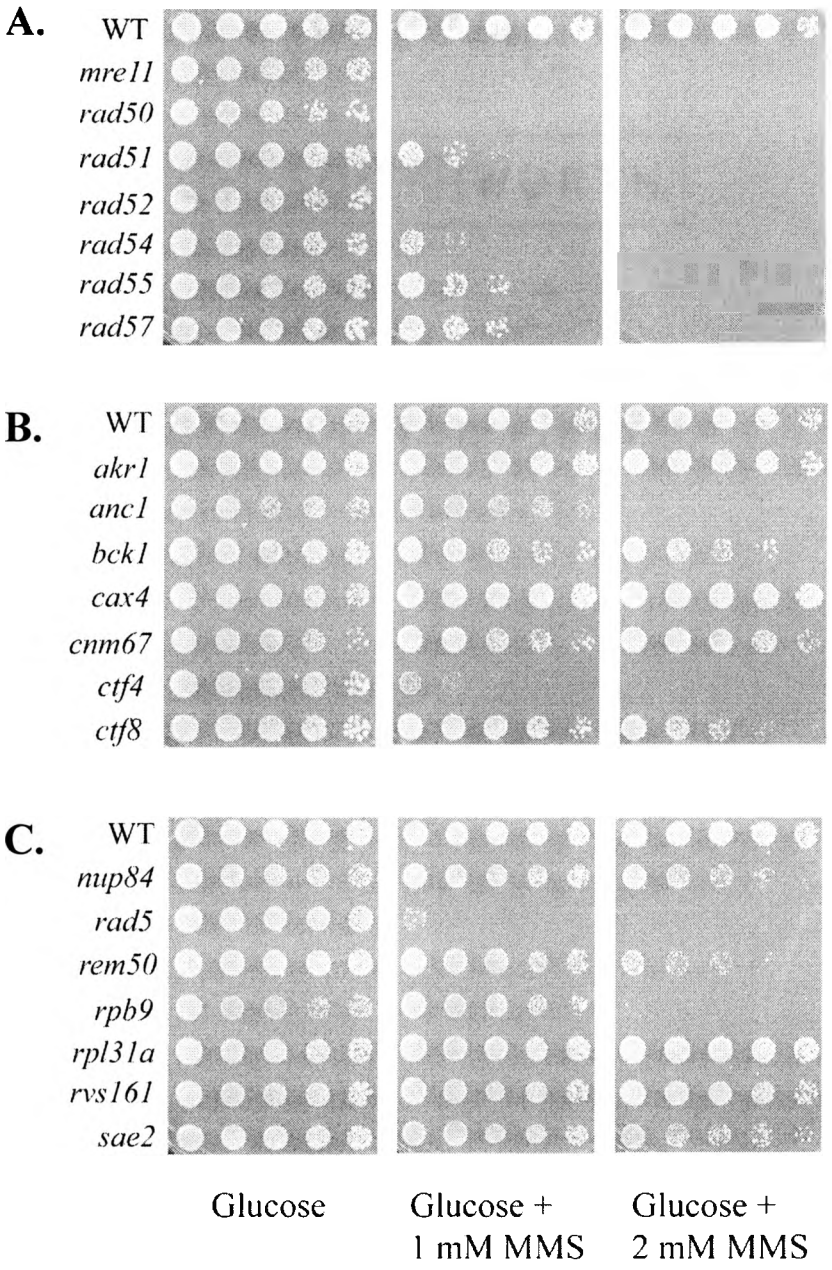

Figure 16. Dilution pronging assay testing resistance of *Eco*RI-sensitive mutants to MMS. Wildtype cells, control DSB repair mutants (**A**), highly *Eco*RI-sensitive mutants (**B**), and moderately *Eco*RI-sensitive mutants (**C**) were pronged to Glu, Glu + 1 mM MMS, and Glu + 2 mM MMS and grown for 3-4 days at 30°C.

Four of the 17 highly *Eco*RI-sensitive mutants and 5 of the 16 moderately *Eco*RI-sensitive mutants were found to have no sensitivity to either concentration of MMS (Table 7). This result is in contrast to a previous study (32). The difference may be that the previous study used diploid cells, while this study used haploid cells, which might cause a difference in sensitivity to certain clastogens. Interestingly, only 6 of the 17 highly *Eco*RI-sensitive mutants were SS on 2 mM MMS and only 3 out of 16 moderately *Eco*RI-sensitive mutants were SS. However, the seven control recombination-deficient strains (Figure 16A) were all highly sensitive (SS) at 2 mM MMS and all but *rad55* and *rad57* were SS when 1 mM MMS was used.

To test for bleomycin sensitivity, the same set of 33 *Eco*RI-sensitive and seven controls were pronged to Glu, Glu + 2 μg/ml bleomycin, and Glu + 4 μg/ml bleomycin plates (Figure 17). Each plate contained WT BY4742 cells as a control. The parameters of characterization were used as described above. All control mutants showed strong sensitivity to high concentrations of bleomycin (Figure 17A). As with MMS, some mutants did not show sensitivity to either concentration of bleomycin, with 9 of 17 highly *Eco*RI-sensitive and 5 of 16 moderately *Eco*RI-sensitive mutants classified as resistant (R).

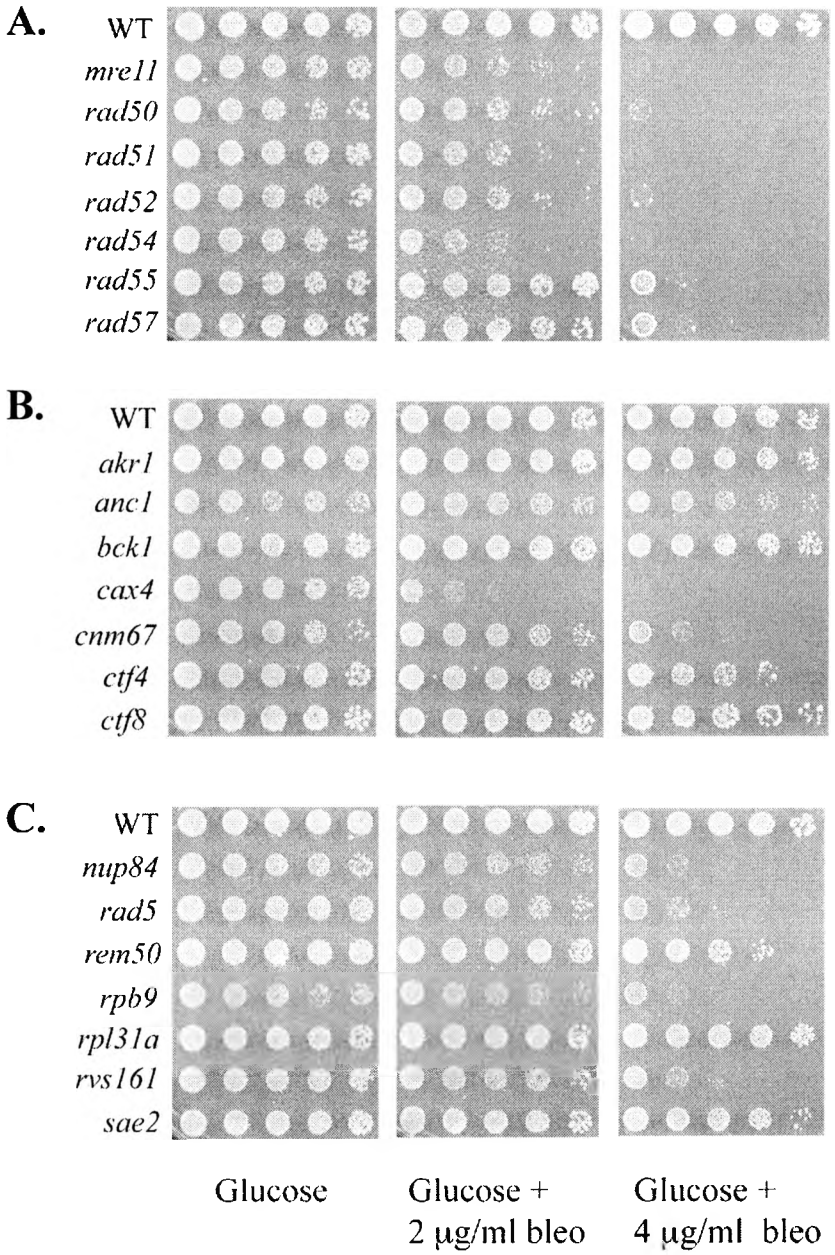

Figure 17. Dilution pronging to test sensitivity of control and new *Eco*RI-sensitive mutants to bleomycin. Wildtype cells, control mutants (**A**), highly *Eco*RI-sensitive unknown mutants (**B**), and moderately *Eco*RI-sensitive mutants (**C**) were pronged to Glu, Glu + 2 µg/ml bleomycin, and Glu + 4 µg/ml bleomycin.

Results obtained in all MMS and bleomycin resistance assays are summarized in Table 7. Two strains (*akr1* and *not5*) were not sensitive to either MMS or bleomycin, but exhibited strong sensitivity to *Eco*RI. It is possible that these mutants do not have DNA repair defects, but instead may express *Eco*RI at higher levels than normal or may transport EcoRI protein into the nuclease more effectively than normal cells. Surprisingly, two mutants (*cax4* and *adk1*) were sensitive to *Eco*RI and to bleomycin, but resistant to MMS. Three other mutants (*mms2, mms4,* and *ybr099c*) were sensitive to *Eco*RI and strongly sensitive to MMS, but not to bleomycin. It is currently unclear how a cell can be sensitive to *Eco*RI-induced DSBs and to one chemical clastogen, yet be resistant to another chemical clastogen. It is conceivable that transport or intracellular processing of some chemicals, but not others, is altered in the mutants. It is also possible that there are differences in the specifics involved in the repair mechanism needed for each type of clastogen.

Table 7. Characterization of *Eco*RI-sensitive mutants to MMS and bleomycin

	Mutant	MMS		Bleomycin	
Controls		1 mM	2 mM	2 μg/ml	4 μg/ml
	WT	R	R	R	R
	mre11	SS	SS	S	SS
	rad50	SS	SS	S	SS
	rad51	SS	SS	S	SS
	rad52	SS	SS	S	SS
	rad54	SS	SS	S	SS
	rad55	M	SS	R	SS
	rad57	M	SS	R	SS
Most *Eco*RI Sensitive					
	akr1	R	R	R	R
	anc1	S	SS	R	S
	bck1	S	S	R	R
	cax4	R	R	SS	SS
	cnm67	R	S	R	M
	ctf4	SS	SS	R	S
	ctf8	R	M	R	R
	dcc1	S	M	R	R
	kre22	R	M	M	SS
	mms2	S	SS	R	R
	mms4	S	SS	R	R
	mms22	SS	SS	R	S
	not5	R	R	R	R
	ume6	S	S	R	R
	vph2	R	R	SS	SS
	ybr099c	S	SS	R	R
Moderately *Eco*RI sensitive					
	akd1	R	R	M	SS
	bur2	R	S	R	S
	ddc1	R	S	R	R
	exo1	R	R	R	R
	mct1	S	M	R	S
	nup84	R	S	S	SS
	rad5	SS	SS	S	M
	rem50	R	M	R	S
	rpb9	R	SS	S	SS
	rpl31a	R	R	R	R
	rvs161	R	R	R	M
	sae2	R	S	R	R
	spt10	R	S	SS	SS
	srv2	R	R	R	R
	vid21	R	S	R	S
	ylr235c	SS	SS	R	S

Note: *htl1* was not analyzed in this assay due to technical problems with the isolate.

Cell Cycle Analysis of EcoRI-Sensitive Mutants

Cells that are defective in DNA repair have a constant, increased level of DNA damage in their chromosomes during normal growth. In yeast cells, this 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 (46).

To screen for potential repair mutants with constant unrepaired damage, the most highly sensitive *Eco*RI mutants along with thirteen known HR, NHEJ, and checkpoint mutants as controls were analyzed for spontaneous and induced cell cycle arrest in G_2 phase. Cells in G_2 cannot be distinguished from M phase cells in the light microscope, but the majority of large-budded cells are in G_2. Cells were harvested and inoculated into liquid YPDA cultures at a starting concentration of 1×10^6 cells/ml and grown at 30°C for 5 hours to reach mid-log growth phase. Three cultures of each strain were counted using light microscopy for percentage of G_2/M phase cells out of 100 cells. G_1 cells are a single cell with no bud, S phase cells have a bud < 50% of the size of parent cell, and G_2/M cells have a bud > 50% of the size of parent cell (Figure 18).

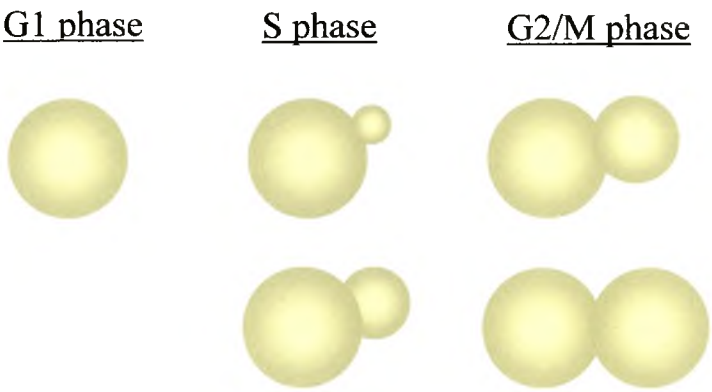

Figure 18. Representation of cells and bud size for each phase of the cell cycle. G_1 cells are a single, un-budded cell, S phase cells have a parent cell and a small bud < 50% of the size of the parent cell, and G_2/M phase cells have a parent cell and a large bud ≥ 50% of the size of the parent cell.

The percentage of G_2/M phase cells in each set of three cultures was averaged for each mutant and graphed with the standard deviation. In Figure 19A, the average percentage of G_2/M cells is shown for WT cells and for two NHEJ pathway mutants (*dnl4* and *sir2*). Each of these strains had an average of ~30-35% G_2/M cells during log phase. This result reinforces the idea that NHEJ is not the primary pathway of DSB repairs in yeast, as cells did not have enough spontaneous unrepaired damage to cause an increase in G_2 cells. In contrast, all of the recombination mutants tested (*mre11* and *rad50-rad59*) exhibited higher spontaneous levels of G_2/M cells than WT (Figure 19B).

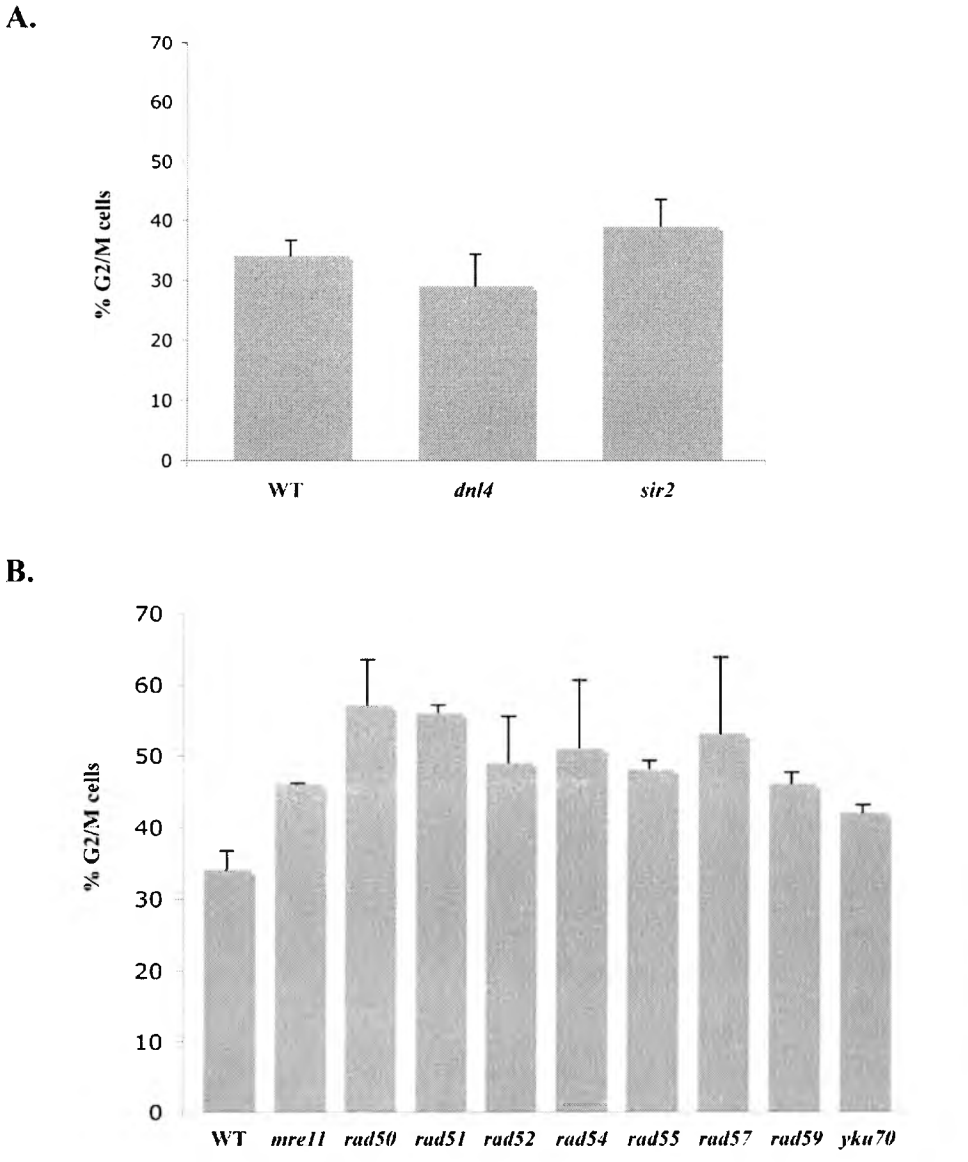

Figure 19. Characterization of G_2/M cells in control strains that are defective in NHEJ or HR. Percentages of cells that were large-budded (primarily G_2 cells) were determined by light microscopy. **A.** Mutants that had percentages of G_2/M cells similar to that of WT cells (with overlapping standard deviations). **B.** DNA repair mutants that had percentages of G_2/M cells greater than that of WT cell cultures.

The subset of 17 highly *Eco*RI-sensitive mutants was then analyzed for spontaneous G_2/M cell cycle arrest as described above. Seven of the mutants had a higher spontaneous level of G_2/M cells than WT (non-overlapping standard deviations) (Figure 20A) and 10 mutants had G_2/M cell levels overlapping with that of WT cells (Figure 20B). It is likely that many of the mutants in Figure 20A have high spontaneous levels of unrepaired DNA damage or other type of chromosome instability resulting in constantly activated checkpoints.

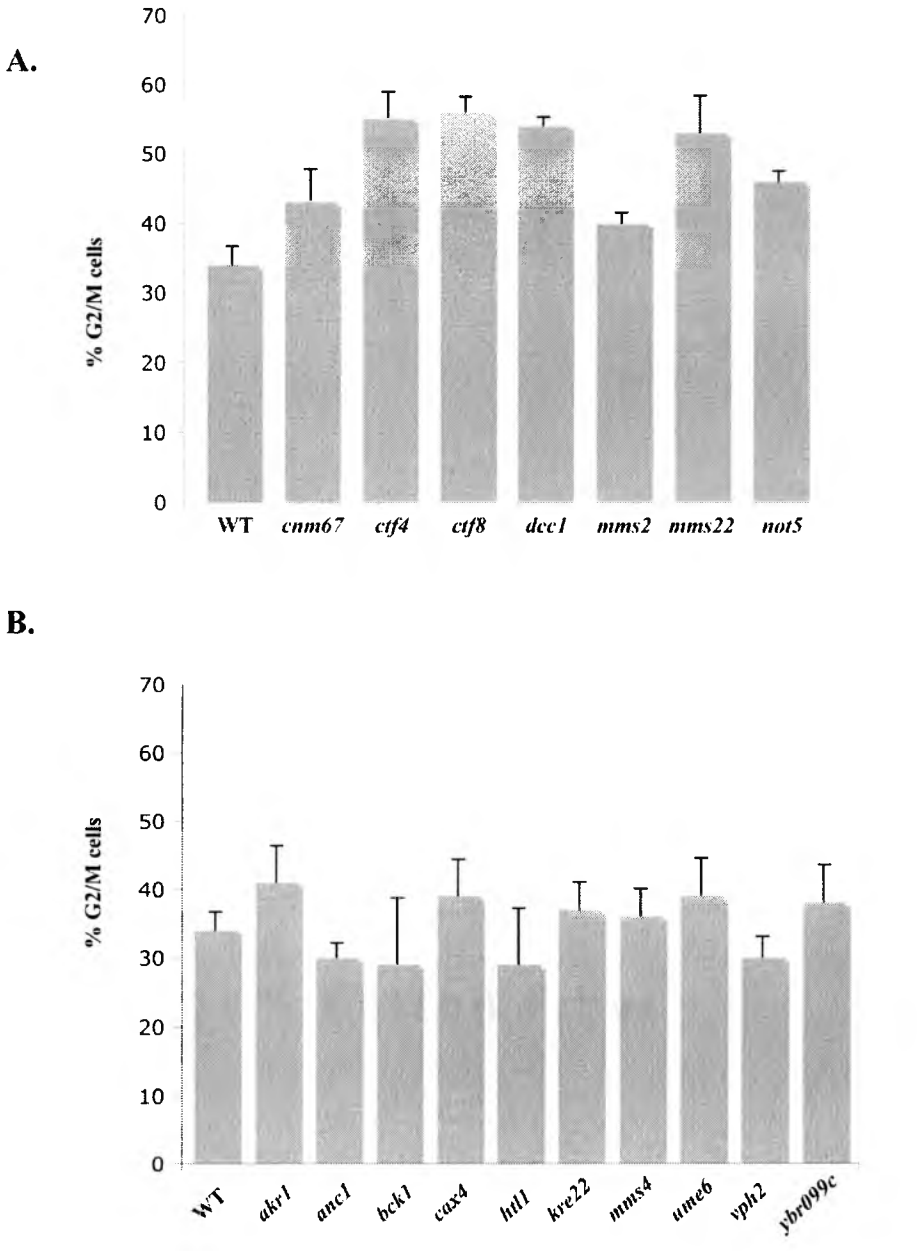

Figure 20. Characterization of spontaneous levels of G_2/M cells in mutants that were highly sensitive to *Eco*RI. Percentages of cells that were large-budded (primarily G_2 phase) were determined for each of the mutants. **A.** Mutants that exhibited percentages of G_2/M cells greater than that of WT. **B.** Mutants that had percentages similar to that of WT.

When normal cells incur large amounts of DNA damage from exposure to agents such as X-rays or MMS or bleomycin, they transiently arrest growth in G_2 phase, repair the damage, and then resume cycling. When recombination-defective mutants such as *rad51* or *rad52* are exposed to the same dose of mutagen, the cells arrest strongly in G_2 and many of the cells die at this stage because they cannot fix all of the lesions in their DNA. Thus, *rad51* and *rad52* cell cultures have more G_2 cells than WT cell cultures during normal growth, and also are induced to higher levels than WT after exposure to the same dose of a DNA damaging agent (3, 4). In contrast, yeast mutants that lack a gene needed to initiate the DNA damage checkpoint in G_2 (e.g., *mec3* or *rad24* cells) exhibit a *reduced* number of G_2-arrested cells after exposure to DNA damaging agents relative to WT cells (46).

It is possible that the 10 *Eco*RI-sensitive mutants that had WT levels of G_2 cells during normal growth included one or more mutants that are checkpoint response-deficient (like *mec3* and *rad24*), which could also be the cause of their sensitivity to *Eco*RI. To test this idea, seven control strains and new *Eco*RI-sensitive mutants with G_2/M phase cell percentages similar to WT were chosen to analyze bleomycin-induced cell cycle arrest in G_2 phase. Cells of each mutant were inoculated into six cultures at 1 x 10^6 cells/ml in YPDA broth. Cultures were then shaken vigorously at 30°C. After two hours, 0.06 μg/ml bleomycin was added to three of the cultures for each mutant. Cultures were subsequently shaken for 5 hours at 30°C, sonicated, and counted as described above. Percentages were averaged for each mutant and standard deviations were calculated. The difference in % G_2/M cells with and without the drug was compared to that of WT (Figure 21).

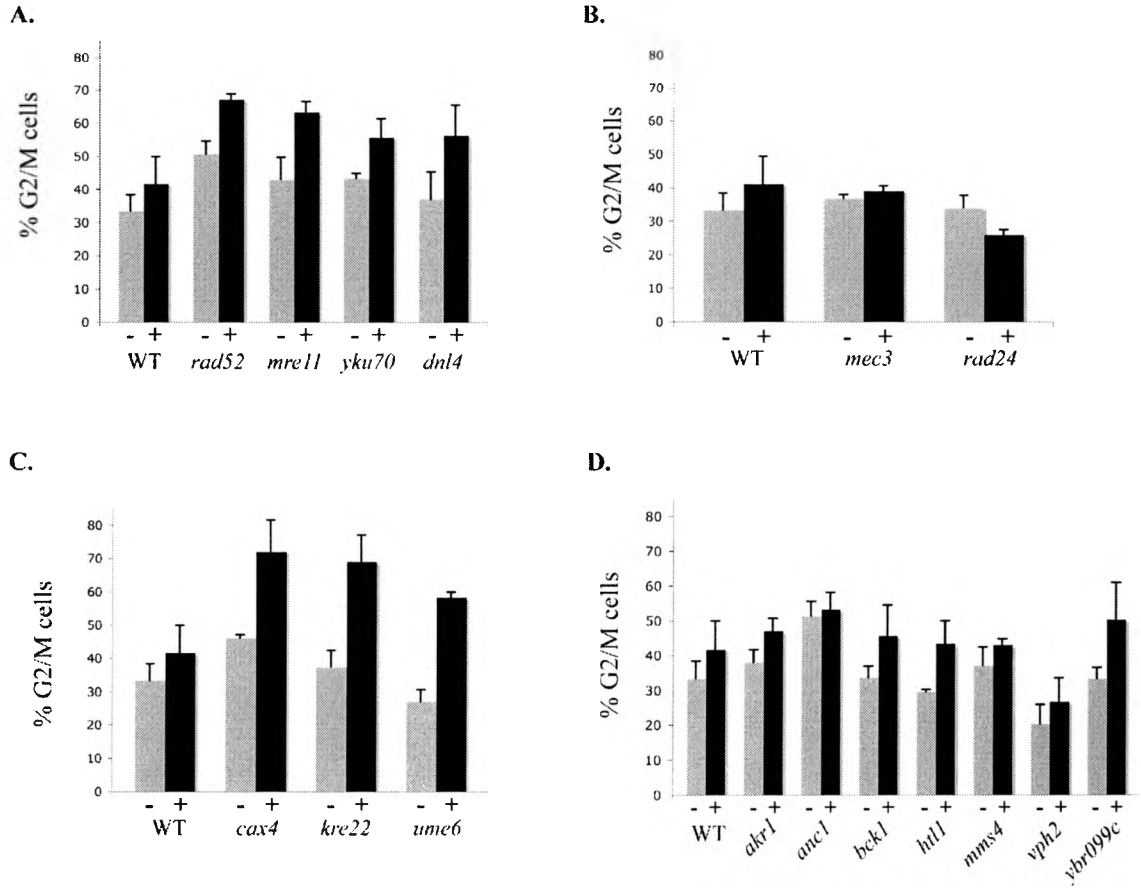

Figure 21. Analysis of bleomycin-induced G_2/M phase cell cycle arrest. The percentage of G_2/M cells before and after 5-hour induction with bleomycin for known HR, NHEJ, and checkpoint-deficient mutants was determined. Four control recombination- and NHEJ-defective mutants showed a large increase in arrest after addition of bleomycin (**A**) and two control checkpoint-defective strains exhibited only a slight increase or decrease in arrest (**B**). Three of the new mutants exhibited a large arrest after addition of bleomycin (**C**) and seven others had arrest not significantly different from that of WT (**D**).

rad52 cells (Rec$^-$), *mre11* cells (Rec$^-$ Nhej$^-$), and *yku70* and *dnl4* strains (Nhej$^-$) controls showed a large increase in arrest after addition of bleomycin compared to the increase in WT cells (Figure 21A). Two control checkpoint mutants (*mec3* and *rad24*) exhibited only a slight change in arrest. Three of the new mutants exhibited a large arrest response. These mutants exhibited an increase of at least 15% after treatment and had a final fraction of G_2 cells $\geq$ 50% of the cell population. These parameters were arbitrarily chosen based on analysis of the responses of the repair-deficient control mutants in Figure 21A. The remaining seven new mutants had cell cycle arrest percentages similar to that of WT cells.

The three mutants showing a large increase in G_2/M cell arrest similar to the recombination- and NHEJ-deficient mutants (*cax4, kre22,* and *ume6*) may suggest a role in DSB repair (3). Interestingly, the *anc1* mutant had a high level of spontaneous G_2 cells, but had almost no increase in G_2/M cell cycle arrest after the addition of bleomycin. Mutants displaying this behavior were also identified in a previous study (32), though the nature of the defect in DNA damage cell cycle checkpoints is unclear.

Summary and Conclusions

Many of the genes involved in both DSB repair pathways, HR and NHEJ, are well-known and characterized. A previous genome-wide screen of 5000 yeast mutants identified a large collection of genes exhibiting sensitivity to several clastogens, with phenotypes that are similar to those of known DSB repair genes. This evidence suggested that at least some of these new genes may also be involved in DSB repair.

A novel replica-plating assay using a *GAL1* promoter to express the endonuclease *Eco*RI was developed to screen this collection of mutants for sensitivity under conditions where wildtype cells exhibit no *Eco*RI-sensitivity. A total of 102 strains, including some well-known DSB repair-deficient mutants, were analyzed for *Eco*RI sensitivity using this assay. Thirty-three of the new mutants were found to have either severe or moderate sensitivity to *Eco*RI expression, suggesting that they had reduced ability to repair the DSBs created by this nuclease. Sensitivity was quantified using dilution pronging assays and from these assays, 17 new mutants were found to have strong sensitivity to *Eco*RI, and 16 were classified as having moderate sensitivity. In addition, 48 mutants did not have detectable *Eco*RI sensitivity, and 9 of the strains were found to be un-testable due to growth problems on the raffinose or galactose plate media.

All 33 *Eco*RI-sensitive mutants were screened for MMS and bleomycin resistance using dilution pronging assays. Nine mutants were resistant to MMS and 14 mutants were resistant to bleomycin, though past studies had suggested that these genes are needed for resistance to both drugs (22, 32, 33). The difference in sensitivity may be because the previous studies used diploid cells, while this study used haploid cells, and differences in testing methodologies may also have contributed. Interestingly, 4 of the

mutants were bleomycin-sensitive but MMS-resistant, while 9 others were MMM-sensitive, but bleomycin-resistant, though each was hyper-sensitive to *Eco*RI expression. Five strains were not sensitive to either MMS or bleomycin, but exhibited sensitivity to *Eco*RI, and may not have a defect in DNA repair. Instead, they may express *Eco*RI at higher levels than other cells or may transport *Eco*RI into the nuclease more effectively than normal cells.

Fourteen of the 33 *Eco*RI-sensitive mutants were sensitive to both MMS and bleomycin. These 14 genes (*anc1, cmn67, ctf4, kre22, mms22, bur2, mct1, nup84, rad5, rem50, rpb9, spt10, vid21, ylr235c*) are most likely to have important roles in DSB repair. Interestingly, 10 of these 14 genes appear to be involved in chromatin structure, including genes affecting sister chromatid cohesion (*ctf4*), meiotic chromosome segregation (*mms22*), and histone acetylation (*rem50*).

Seventeen of the most sensitive *Eco*RI mutants along with thirteen known HR, NHEJ, and checkpoint mutants as controls were analyzed for spontaneous and induced cell cycle arrest in the G_2 phase. This assay was performed to screen for phenotypes normally associated with either DNA repair deficiency or defects in cell cycle checkpoint responses. HR control mutants had high spontaneous G_2 cell arrest as well as a large increase in arrest after addition of bleomycin compared to WT cells. NHEJ mutants exhibited WT spontaneous G_2 levels, but also displayed a large increase after addition of bleomycin compared to WT cells. In contrast, control checkpoint-defective mutants had lower initial percentages and only a slight change in arrest compared to WT cells. Seven of the 33 *Eco*RI-sensitive mutants exhibited high levels of G_2/M cells during normal growth, suggesting that they have elevated levels of spontaneous DNA damage. Three

new mutants (*cax4, kre22,* and *ume6*) exhibited strong bleomycin-induced arrest responses similar to that of HR mutant controls.

anc1 mutants had high spontaneous levels of G_2/M cells that could not be induced higher by exposure to bleomycin. *ANC1* has previously been implicated in chromatin remodeling, which may explain why a defect in the ability to remodel chromatin may affect the checkpoint response for repair. Other genes such as *CTF4* and *CTF8* are described to be involved in chromatid cohesion, and *DDC1* is known to be involved in DNA damage checkpoint responses.

In conclusion, this study has identified new genes required for efficient repair of DSBs caused by *Eco*RI. Some of the mutants displayed characteristics similar to that of well-known HR-deficient mutants for the four assays of this study including *Eco*RI sensitivity double imprint replica-plating and dilution pronging, MMS and bleomycin sensitivity, and cell cycle checkpoint analysis. Out of the total mutants screened, the 14 genes required for resistance to *Eco*RI, MMS, and bleomycin gave similar results to that of *rad52* and *mre11* mutants suggesting their possible involvement in homologous recombination. Cultures of 3 of these 14 strains (*cmn67, cft4,* and *mms22*), also exhibited high spontaneous levels of G_2/M cells, similar to *rad50 – rad59* mutants. These three mutants may be a good place to start future studies to identify specific roles of these proteins in DSB repair. The results of this project may help to understand the intricate mechanisms involved in DSB repair and help to understand diseases caused by defects in these mechanisms.

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