DNA DOUBLE-STRAND BREAK REPAIR DEFICIENCY IS ASSOCIATED WITH
CHANGES IN CELL CYCLING AND CELL MORPHOLOGY

IN SACCHAROMYCES CEREVISIAE

by

Monica F. Weis, B.S.

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Committee Members:

L. Kevin Lewis, Chair
Karen Lewis
Liqin Du
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I. INTRODUCTION

Self-assembly using deoxyribonucleic acid (DNA) as a blueprint is ubiquitous in living systems. DNA is a polymer and the sequence of nucleotide monomers act as a code that regulates the expression of genes encoding proteins and functional RNAs in a cell. Nucleic acid monomers form a chain via phosphodiester bonds. The chains associate with each other to form a double helical structure with two deoxyribose phosphate backbones. Guanine, adenine, thymine, and cytosine bases vary on the backbones and form the actual code. Two complementary DNA strands arranged in opposite directions form the double helical structure held together by hydrogen bonding between bases on opposite strands and planar stacking between adjacent bases. Guanine base pairs with cytosine and adenine base pairs with thymine. The base pairs are strong enough to hold the molecules together in optimal biological conditions, yet can easily denature to enable transcription and replication. DNA and its encoded sequence are the instruction manual critical for all lifeforms to carry out the most basic of functions. Thus, it is crucial that chromosomal structure and sequence are conserved (1).

The genomic integrity of an organism is constantly being challenged. Chromosomal DNA lesions are continuously being introduced by both intracellular
metabolic processes and extracellular agents. It is estimated that a single human cell experiences ~ 50,000 - 80,000 DNA lesions per day that must be repaired (4). Exogenous sources that can introduce DNA insults include radiation and exposure to clastogenic (DNA strand breaking) chemicals, and endogenous sources include enzymes and the reactive oxygen species that are a natural consequence of aerobic metabolism (1-4).

Sources of exogenous DNA damage

Solar radiation is radiant energy emitted by the sun. Some of this energy is emitted in invisible rays called ultraviolet (UV) radiation and damages DNA by the absorption of photons. The predominant genotoxic photoproducts of UV radiation are dimers between adjacent pyrimidine bases, called cyclobutane pyrimidine dimers (CPDs) and 6,4-photoproducts (4).

Ionizing radiation is any electromagnetic wave or particle that can remove an electron from an atom or molecule in the medium through which it travels. When living material is ionized, it temporarily excites molecules into reactive species that are capable of causing direct DNA damage or indirect DNA damage through the generation of reactive oxygen species (ROS) (4).

DNA damage can also be chemically introduced by clastogenic chemicals such as bleomycin or methyl methanesulfonate (MMS). MMS is an alkylating agent that methylates the nitrogen and oxygen atoms in DNA bases as well as phosphodiester bonds. This can lead to secondary DNA damage due to stalled and unresolved replication forks upon encountering methylated bases (10). The toxicity of bleomycin, a chemotherapeutic glycopeptide antibiotic, rests primarily in the generation of single-
strand and double-strand DNA breaks. The exact mechanism of action is unknown although it is thought that bleomycin acts as a pseudo-enzyme that, upon chelation of Fe$^{2+}$, generates free radicals in the presence of O$_2$ (Fenton reaction) (5).

Higher level eukaryotic aerobic organisms cannot live without oxygen, yet it is inherently a threat to their existence due to the two valent electrons present in its molecular form. The superoxide anion radical, hydrogen peroxide and the extremely reactive hydroxyl radical are common products resulting from reduction of molecular oxygen to water during mitochondrial respiration. This is thought to be the primary source of intracellularly generated ROS, providing the basis for the so-called “oxygen paradox” (5): oxygen is required for survival yet is inherently detrimental.

Although each DNA damaging agent has a signature type of damage that it creates, some DNA damage types do not have mutually exclusive sources since secondary lesions commonly occur from different types of original damage sites. Secondary lesions that can occur within the chromosome include damaged bases, sugar alterations, abasic sites (AP sites), DNA-protein crosslinks, and single-strand or double-strand breaks (DSBs) (1,5).

**Repair processes for DNA damage in living systems**

Repair processes for the plethora of inevitable DNA lesions that occur have been found among cellular organisms investigated indicating that DNA damage is a ubiquitous threat to the conservation of the genetic code. Pathways that have been characterized include base excision repair (BER) for the repair of damaged bases or abasic sites, nucleotide excision repair (NER) for the removal of bulky lesions such as ultraviolet
light-induced pyrimidine dimers, and mismatch repair (MMR), which removes misincorporated bases that occur during replication and recombination. In response to double-stranded breaks two major pathways are used: nonhomologous end-joining (NHEJ) and homologous recombination (HR) (also called homology-directed repair or HDR) (2,4).

**Pathways for the repair of double-stranded breaks**

NHEJ is the simpler of the two pathways, involving the direct ligation of the broken chromosome ends (Figure 1). Minimal energy expenditure is required, but it is also associated with information loss at the break sites (2, 4). There are at least 8 genes whose products form three essential protein complexes to carry out NHEJ in cells of the budding yeast *Saccharomyces cerevisiae*: Yku70/Yku80, Mrx, and DNA Ligase IV. The Yku70/Yku80 complex is a key player in the initiation of the NHEJ repair pathway and is involved in binding the ends of broken chromosomes as a heterodimeric ring comprised of Yku70 and Yku80 to initiate NHEJ and recruit further complexes. It is thought that the complex also serves to protect damaged and exposed DNA ends from nucleases. The Mrx complex, comprised of Mre11, Rad50, and Xrs2, bridges the two ends of the broken chromosome, ensuring that they remain in close proximity for repair, as well as recruiting the DNA Ligase IV complex. In humans, Xrs2 is replaced with Nbs1. The Mre11 portion of this complex has a 3’-5’ double-stranded DNA exonuclease activity in the presence of manganese as well as single-stranded endonuclease and weak helicase activities (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 DNA Ligase IV complex to facilitate ligation of the two DNA strands (6). The DNA Ligase IV
complex, consisting of Dnl4, Lif1, and Nej1, completes the repair process. The Dnl4 subunit of the DNA Ligase IV complex is a DNA ligase that is ATP-dependent (2) and the Lif1 subunit is suggested to associate with the Xrs2 subunit in Mrx (6, 8, 38). Of the three protein complexes involved in NHEJ, Mrx is unique because it is involved in the HR pathway as well. NHEJ is the secondary pathway for the repair of DSBs in *Saccharomyces cerevisiae* (budding yeast) but the primary pathway in humans (2, 5).

![Figure 1. Schematic representation of the nonhomologous end-joining pathway for the repair of DNA double-strand breaks (8).](image)

HR is considered the more conservative of the two pathways for DSB repair because it uses homologous DNA regions as a template to repair the break and is highly accurate (Figure 2). Major genes involved with HR in yeast include *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54, MRE11*, and *XRS2*, which are all
members of the RAD52 group of repair genes (11, 12). The protein products of these
genes perform an intricate, multistep process that involves two known complexes, Mrx
(discussed above) and the Rad52 complex (comprised of Rad51-Rad52-Rad54-Rad55-
Rad57-Rad59) (9, 10). The Mrx complex nuclease, in conjunction with other nucleases,
creates long 3’ single-stranded overhangs that are hundreds of nucleotides long on both
broken DNA strands (Figure 2). This resection by Mrx takes place in HR but not NHEJ
and is regulated by cell cycle phase and by specific proteins such as Rif1 (37). Resection
is followed by binding of the single-stranded DNA binding protein complex, Replication
Protein A (Rpa), which protects the exposed DNA strands. Rpa recruits the Rad52
complex of proteins for homology searches, strand invasion and information exchange
with an unbroken homologous chromosome. This process is concluded with DNA
replication, branch migration, nucleosome assembly, and resolution resulting in a
repaired chromosome. Additional protein complexes are also recruited during this
process, such as those required for DNA damage checkpoint responses, nucleosome
remodeling, sister chromatid cohesion and other processes (3, 31). Although many
specifics of the HR mechanism remain unknown, functions of several of the proteins
involved have been characterized. Rad51 is a strand exchange protein that forms a helical
filament with long 3’ssDNA ends that searches for sequences with homology to the
broken DNA ends. Rad52 helps to stimulate strand exchange and facilitates Rad51
binding to ssDNA by displacing the single-stranded DNA binding protein Rpa. The
Rad52 protein has been shown to be most critical, as rad52 mutants often display more
severe phenotypes than rad51, rad54 or other mutants in the Rad52 group in many assays
(4, 11). The homologous recombination pathway is unable to be utilized in rad52
mutants (9). Rad54 is a DNA-dependent ATPase that stimulates strand exchange by modifying the topology of homologous dsDNA strands. Rad55 and Rad57 help to stabilize the binding of Rad51 to ssDNA. The role of Rad59 is unclear and rad59 mutants have very modest phenotypes, although the Rad59 protein has sequence homology to Rad52 (32). HR is the primary pathway for the repair of double-stranded breaks in yeast but the secondary pathway in humans (4, 6).
Figure 2. Schematic representation of the homologous recombination pathway for the repair of DNA double-strand breaks. Adapted from (34).

The reason for preferential use of NHEJ by human cells is likely to be the substantial amount of repetitive DNA in multicellular eukaryotes (2). Except after DNA replication when sister chromatids are close (late S phase and G2 phase) the search for homologous sequences in the chromosomes during HR would more than likely lead to
high levels of genetic exchange between repetitive elements, possibly leading to chromosomal translocations or deletions. For example, a break in an Alu repeat (a repetitive DNA sequence) has almost one million possible donors (14). Therefore, organisms with a highly repetitive genome may favor NHEJ, as a small deletion associated with NHEJ is less mutagenic than frequent aberrant recombination events. The small deletion events may also be better tolerated in higher organisms.

**Yku proteins are important for both NHEJ and telomere stability**

Telomeres are repetitive sequences found at the ends of linear eukaryotic chromosomes that are essential for protecting the genome from being compromised due to degradation by nucleases, end-to-end fusions, or recombination events during each cell division. They also solve the end replication problem, whereby every time the chromosome replicates a portion of the lagging strand cannot be replicated and is thus lost. In wildtype *S. cerevisiae*, telomeres are from 350-500 bp in length. Ku plays several roles at the telomere regions that are important for maintaining genomic stability including regulating telomere DNA replication, transcriptional silencing of telomere-proximal genes, and nuclear positioning of telomeres. Ku also protects telomeres from recombination or chromosomal end-joining as well as nucleolytic degradation (42).

**The cell cycle regulates important cellular functions**

Faithful replication and propagation of genetic information is paramount for cell viability. Therefore cells have evolved complex regulatory systems to coordinate the order and timing of cell cycle events to allow for complete and accurate DNA replication (Figure 3). The eukaryotic cell cycle is divided into four phases, G₁, S, G₂, and M. G₁ is
the main growth phase, S phase is when DNA replication occurs, G2 is when the cell prepares for mitosis, and M is mitosis, where chromosome segregation, nuclear division, and cytokinesis occur. The two “G” phases, or gap phases, provide time for growth and for the cell to monitor the internal and external environment to ensure it is adequately prepared to commit to chromosomal replication or cell division. When levels of radiation or chemically induced lesions are high, DNA damage-responsive cell cycle checkpoints are activated, causing cells to pause in the G2 phase of the cell cycle. This arrest is the result of the DNA Damage Response (DDR) which provides time for repair of damaged DNA and prevents the passage of erroneous coding information to subsequent cells (4).

Figure 3. Schematic representation of the cell cycle and cell cycle checkpoints in S. cerevisiae (20).
The DDR results from the actions of many checkpoint and repair proteins which are evolutionary conserved from *S. cerevisiae* to vertebrates (4, 19). Thus, significant findings in yeast can be extrapolated to higher order systems. These proteins have various roles including as sensors, adaptors/mediators, and effector kinases. Sensing of the DNA damage results in a phosphorylation cascade that recruits many checkpoint proteins to the damage site and cell cycling machinery (Figure 4). The principal proteins in *S. cerevisiae* involved in the early steps of DSB checkpoint activation are listed in Table 1 along with their human homologues. The DNA damage response (DDR) pauses cell cycling to shift cellular priority to the rapid detection and repair of DNA damage in order to maintain genome integrity (4).

Figure 4. Schematic of cell cycle checkpoint proteins at a DNA damage site (20).
Table 1: Some of the relevant principal proteins in *S. cerevisiae* involved in the early steps of DSB checkpoint activation (20).

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<thead>
<tr>
<th>Class of protein</th>
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<th>Proposed function(s)</th>
<th><em>H. sapiens</em></th>
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<td>Sensors</td>
<td>Mec1-Ddc2</td>
<td>Principal PIKK complex involved in sensing DNA damage and transducing the checkpoint signal. Mec1 binds Ddc2, which mediates its recruitment to sites of damage</td>
<td>ATR-ATRIP</td>
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<td>Sensors</td>
<td>Rad24-Rfc2-5 (RFC-like complex)</td>
<td>Loading of Ddc1-Rad17-Mec3 (9-1-1) complex onto DNA. Role in DNA damage signaling.</td>
<td>RAD17-RFC2-5</td>
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<tr>
<td>Sensors</td>
<td>Ddc1-Rad17-Mec3 (PCNA-like complex)</td>
<td>Heterotrimeric complex, structurally related to PCNA, also called 9-1-1. DNA damage signal transduction and recruitment of other checkpoint proteins</td>
<td>RAD9-RAD1-HUS1</td>
</tr>
<tr>
<td>Adaptors/Mediators</td>
<td>Rad9</td>
<td>Molecular adaptor/mediator required for DNA damage signal transduction and DSB repair. Required for Rad53 and Chk1 activation.</td>
<td>53BP1; BRCA1; MDC1</td>
</tr>
<tr>
<td>Effector</td>
<td>Chk1</td>
<td>Effector kinase involved in signal transduction.</td>
<td>CHK1</td>
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**Disease can be a result of deficient DNA repair systems**

Many human genes related to disease have orthologs in *S. cerevisiae*, and high conservation of regulatory and metabolic systems in eukaryotes has contributed to widespread studies of these genes in lower, simpler systems. Several diseases have been identified that stem from deficient DSB repair (33). 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* (*NBS1* in humans), can have devastating effects such as promoting the development of Nijmegen breakage syndrome (NBS) (33). In humans a defect in *SGS1* gene homologs, helicases involved in the HR pathway, can lead to Werner’s or Bloom’s Syndrome. Both diseases increase patient risk for certain types of cancer (21). 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 (22). Certain cancers are associated with mutations in the BRCA1 and BRCA2 genes, which are responsible for assisting hRAD52 with its role in hRAD51 binding to ssDNA near DSB sites (39, 40). Mutant yeast cells defective in HR exhibit common characteristics such as slower growth and sensitivity to clastogenic chemicals. RAD52-deficient cells are categorized as extremely sensitive to X-rays and DNA damaging agents due to their loss in cell viability and increased rate of chromosome loss. rad55, rad57 and rad59 mutants are categorized as modestly sensitive to DNA damaging agents. A previous study has suggested that diploid rad51 mutants may spend more time in G2 phase, possibly because unrepaired DSBs in the cells are causing constitutive activation of the cell cycle DDR (45). Previous work in the L. K. Lewis lab also revealed that several RAD52 group mutants have elevated levels of G2 cells during growth in standard media under optimum conditions, without exposure to exogenous DNA damaging agents (6, 35, 36).

**Yeast as a model system**

There are several advantages to using yeast, *Saccharomyces cerevisiae*, as a simple eukaryotic model system. Yeast are commercially readily available and large quantities can be grown cheaply which is accommodating to a lab budget. Yeast cells are also non-pathogenic and can be handled with ease. The yeast genome contains a haploid set of 16 well-characterized chromosomes presenting a way to do sophisticated genetics assays in a eukaryotic system that is well-defined. Yeast are particularly well-suited for molecular genetics studies for a multitude of reasons. They have a rapid proliferation rate, which allows studies of phenotypic effects of mutations that affect senescence and future generation cell vitality to be performed with ease as compared to other eukaryotes.
The approximate doubling time of yeast during exponential phase, ~90 minutes in rich broth and ~140 minutes in defined synthetic media (glucose complete), allows these studies to be completed within a relatively quick time frame. Budding yeast cells also have the advantage of being morphologically different from other cells. Discerning cell phase (G₁, S, and G₂/M) is possible simply by visualizing the cells using a phase contrast microscope. In animal cells, G₁, S, and G₂ phases do not have an obvious effect on cell morphology and must be distinguished with staining techniques. Yeast cells have a high transformation efficiency (>10⁵ transformation events possible per µg of DNA) as well as a natural tendency to perform integrative recombination with transformed DNA via homologous recombination. Plasmids or DNA fragments with foreign DNA sequences flanked by cloned yeast sequences can be inserted into specific locations in the chromosome at will. Stable crosses can be carried out with ease by mixing haploid MATα and MATα strains with the genetic makeup of one’s choosing and complementary auxotrophic mutations. Diploid colonies are selected for by the resultant prototrophic phenotype. This enables the relatively easy construction of a diploid genome with heterozygous or homozygous alleles at gene loci of choice. All of these traits make yeast particularly receptive to gene cloning and genetic engineering as evidenced by the fact that mammalian genes are routinely introduced into yeast cells for analyses of their functions. The two sexes, MATα and MATalpha cells, present an opportunity to confirm study results in two similar haploid systems. Yeast strains that have a particular genetic trait of interest can be stored indefinitely for future study by being transferred into and kept in 15% (v/v) glycerol in -60°C temperature or lower. Yeast cells are later easily revived by transferring a small amount of frozen culture to a plate for growth (30).
Goals of this project

Major goals of this project were to quantitate changes in cell cycling and cell morphology in S. cerevisiae mutants that are deficient in DNA double-strand break repair, especially cells defective in HR, as well as cells that are deficient in both DNA repair and DNA damage checkpoint responses. In initial work, the observation that DNA repair-deficient rad52 mutants spend more time in G2 phase than wildtype cells was investigated. A high percentage of G2 cells were observed in both haploid and diploid rad52 mutants. In haploid cells, overall cell doubling times for rad52 mutants during log phase growth were found to be 150% longer than in wildtype (WT) cells. Quantitation of the time spent in each phase revealed that rad52 and WT cells spend similar amounts of time in G1 and S phase, but rad52 cells spend 2.7 times longer in G2.

To test our hypothesis that the increase in G2 cells is due to higher levels of unrepaired DSBs leading to constitutive activation of DNA damage checkpoints, mutations in seven known checkpoint genes were combined with rad52 mutations to create rad52 ddr double mutants. G2 cells were reduced to near WT levels in all seven rad52 DNA damage checkpoint double mutants tested.

The possibility that inactivation of other members of the RAD52 group might also lead to high levels of G2 cells was also examined. rad51, rad54, rad55, rad57, and rad59 mutants also exhibited large numbers of G2 cells and spent approximately 2 times as much time as WT cells in G2 phase during log phase growth.

Cells’ nuclei were stained with DAPI and analyzed microscopically to compare WT and rad52 cells’ nuclear morphologies. These experiments demonstrated that the
nuclear structures found in *rad52* cultures were consistent with the findings of past studies on Xray- and ultraviolet light-induced G_2_ arrest of yeast cells. Furthermore, many log phase *rad52* cells appeared to be enlarged when cell sizes were measured using phase contrast microscopy. Interestingly, only about half of the G_2_ phase *rad52* cells were enlarged. This result suggests that cells were pausing in G_2_ but continuing to grow in size before eventually entering M phase and undergoing cell division. The results of this research can be translated into higher eukaryotes such as humans in order to better understand repair systems and responses, and possibly help prevent the myriad of complicated diseases that stem from DNA damage.
II. MATERIALS AND METHODS

Reagents

Ethlenediaminetetraacetic acid (EDTA) and sodium hydroxide (NaOH) were purchased from EMD Chemicals, Inc. (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and lithium acetate (LiAc) were obtained from Alfa Aesar (Heysham, Lancashire). Sodium dodecyl sulfate (SDS) was obtained from J.T. Baker (Center Valley, PA). Sodium acetate anhydrous, and glacial acetic acid were purchased from Mallinckrodt-Baker, Inc. (Paris, KY). The sonicated salmon sperm carrier DNA was obtained from Agilent Technologies (Santa Clara, CA). Ethidium bromide (EtBr) was obtained from IBI Scientific (Peosta, IA). Sodium chloride, sodium citrate dihydrate, Tris-HCl, and the Qubit dsDNA BR Assay Kit were purchased from ThermoFisher Scientific (Fair Lawn, NJ). Ammonium sulfate, isopropanol, formamide, and Trizma base were purchased from VWR International (West Chester, PA). Boric acid, maleic acid, polyethylene glycol (PEG 4000), Sarkosyl (N-lauryl-sarcosine), and Tween 20 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Agarose LE was purchased from Gold Biotechnology (St. Louis, MO). TAE (50X) buffer was purchased from Omega (Cowpens, SC). Ethanol was obtained from Texas State University (San Marcos, TX). DIG High Prime DNA Labeling and Detection Starter Kit II, PCR DIG Labeling Mix, and DNA Molecular Weight Marker III were purchased from Roche Applied Science (Penzberg, Germany). RNase A was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Restriction enzymes, dNTPs, MgCl₂, Vent polymerase, Taq DNA polymerase, 6X loading dye, and 2 log DNA ladder were purchased from New England Biolabs (Beverly, MA). ExTaq DNA polymerase was obtained from Takara
(Madison, WI). All primers (Table 1) were created by Integrated DNA Technologies (IDT) (Coralville, IO). All amino acids, and ampicillin (Amp) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). D-(+)-glucose, soy peptone, and yeast nitrogen base were made by Amresco (Solon, OH). Bacto yeast extract was manufactured by Becton, Dickinson, and Company (Sparks, MD). Agar, molecular biology grade was obtained from Teknova (Hollister, CA). QIAprep Spin Miniprep Kits were purchased from Qiagen (Hilden, Germany). DAPI staining solution was purchased from GeneTex (Irvine, CA). Molecular Probes ProLong Gold antifade reagent was purchased from Life Technologies (Eugene, OR). Phosphate Buffered Saline 10X liquid concentrate was purchased from OmniPur (Gibbstown, NJ).

Equipment

Horizon 11-14 gel rigs were from LabRepCo (Horshan, PA). The Sorvall Lynx 6000 floor centrifuge, Savant DNA 120 SpeedVac Concentrator, Qubit 2.0 Fluorometer, replica-plating tool, and velvet squares were obtained from Thermo Fisher Scientific (Fair Lawn, NJ). The MXX-123 analytical balance was purchased from Denver Instruments (Bohemia, NY). Vortexes and all incubators were purchased from VWR Scientific Products (Radnor, PA). The model 5424 centrifuge was purchased from Eppendorf (Hamburg, Germany). The UV Stratalinker 2400 was purchased from Stratagene (La Jolla, CA). The T100 Thermal Cycler, Trans-Blot Turbo Blotting System, and ChemiDoc XRS+ instruments were obtained from Bio-Rad Laboratories (Hercules, CA). The RED imaging system was purchased from Alpha Innotech (San Jose, CA). HB-1000 Hybridizer was purchased from UVP Laboratory Products (Upland, CA). The E-
Class shaker, Orbit Environ Shaker, and Lab Rotator were made by Lab Line Instruments (Tripunithura, India). The orbital shaker and digital dry baths were purchased from BioExpress (Kaysville, UT). The microscope used for DAPI imaging was an EVOS FL digital LED-based transmission and fluorescence microscope purchased from Advanced Microscopy Group (Mill Creek, WA). The other microscope was a United Scope model M837T phase contrast microscope (Hopewell Junction, NY).

**Yeast Strains, Plasmids and Primers**

All DNA primers used in this study are listed in Table 1. PCR confirmation using primers RAD52E and LEU2A was done to verify the rad52 deletion in BY4742 strains not containing the RAD52 plasmid. PCR confirmation was done with both RAD52E + LEU2A and RAD52U1 + LEU2A primer sets in the double mutants. BY4741 rad52 knockouts were also double verified by PCR in this fashion. Primers used in this project are listed in Table 1. All single mutants and wild-type strains were available in the strain deletion libraries obtained from Open Biosystems (6).

All original library strains and new strains created for this project are listed in Table 2. All plasmids used in these experiments were isolated from *E. coli* cells by alkaline lysis (41) (Table 1). Most yeast strains used for the project were derivatives of BY4741 and BY4742 (57) (Table 2). Double mutant yeast strains were constructed by transforming seven different checkpoint single mutant strains from a deletion yeast strain library with a plasmid fragment containing the *RAD52Δ::LEU2* insertion derived from the plasmid pΔ52L (6). pΔ52L was cut with NotI and ApaI prior to transformation into cells. The seven checkpoint mutants included *rad9, rad17, rad24, mec3, ddc1, dun1* and
chk1 cells. Two versions of each rad52/ddr double mutant were made, one with a functioning RAD52 gene expressed via the galactose promoter on a URA3 plasmid (p316GalyR52) and one without. Checkpoint single mutant strain numbers are listed in Table 2. Cells were spread to either glucose minus leucine or galactose minus leucine minus uracil plates to select for cells with a LEU2 insertion and also to select for the plasmid and express Rad52 via the galactose promoter if applicable. Plates were placed at 30°C for colony growth. Colonies were patched to fresh glucose minus leucine or galactose minus leucine minus uracil plates for further purification.

<table>
<thead>
<tr>
<th>Table 2. List of PCR primer sequences. A complete list of primers used.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer Sequence</strong></td>
</tr>
<tr>
<td>5-rad52            CAGGAAACAGCTATGACC</td>
</tr>
<tr>
<td>3-rad52            GTAAATATTAATACGACACATGGAGGAA</td>
</tr>
<tr>
<td>tRad52A            CGCCAGCAGTGAAATCACCACAGTTGGATA</td>
</tr>
<tr>
<td>tRad52B            CTAAATGATCTATTGGTCTTTCCGAGTTGCCAT</td>
</tr>
<tr>
<td>tRad52C            CTCTTGACACGCTCTTAAATACGTAGTCTTTGGG</td>
</tr>
<tr>
<td>tRad52D            GACATCTTTCCAGAAAATTCATCTCCAAGACGAGGA</td>
</tr>
<tr>
<td>tRad52E            GTCAGCGTGCTAAGTCATGTGTGGTAGGTTGTA</td>
</tr>
<tr>
<td>tRad52F            AGTCATTAGTAAGTGTAATCAGAGTCAGGAAGACTGA</td>
</tr>
<tr>
<td>Rad52U1            ATCGTCAGTCTTTACCCTACGCTGTGGTG</td>
</tr>
<tr>
<td>Rad52U2            CCCAAGATCTTTGTACTGATCTTAGGTACTGCA</td>
</tr>
<tr>
<td>Leu2A              TCCAGCGCCTCATCTCGAGGTGGGACAC</td>
</tr>
<tr>
<td>Leu2B              TTTTGTTGCCCAGTCTGCTGCTTGCCCTCT</td>
</tr>
<tr>
<td>M13 forward primer</td>
</tr>
<tr>
<td>M13 reverse primer</td>
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Table 3. Yeast strain list. A complete list of *S. cerevisiae* strains used for this project.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype Source</th>
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<tr>
<td>YLKL568</td>
<td>BY4742, MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
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<tr>
<td>YLKL567</td>
<td>BY4741, MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
</tr>
<tr>
<td>YLKL890</td>
<td>rad9Δ::G418r</td>
</tr>
<tr>
<td>YLKL935</td>
<td>rad17Δ::G418r</td>
</tr>
<tr>
<td>YLKL942</td>
<td>rad24Δ::G418r</td>
</tr>
<tr>
<td>YLKL898</td>
<td>mec3Δ::G418r</td>
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<td>YLKL1563</td>
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<td>YLKL861</td>
<td>rad52Δ::G418r</td>
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<tr>
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</tr>
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<tr>
<td>YLKL1190</td>
<td>rad59Δ::G418r</td>
</tr>
<tr>
<td>YLKL864</td>
<td>rdh54Δ::G418r</td>
</tr>
<tr>
<td>YLKL860</td>
<td>rad51Δ::G418r</td>
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<td>YLKL1189</td>
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<tr>
<td>YLKL1579</td>
<td>rad52Δ::G418r</td>
</tr>
<tr>
<td>YLKL1581</td>
<td>BY4741 rad52Δ::LEU2</td>
</tr>
<tr>
<td>YLKL1582</td>
<td>BY4742 rad9Δ::G418r rad52Δ::LEU2 + p316GalyR52</td>
</tr>
<tr>
<td>YLKL1583</td>
<td>BY4742 rad17Δ::G418r rad52Δ::LEU2 + p316GalyR52</td>
</tr>
<tr>
<td>YLKL1584</td>
<td>BY4742 rad24Δ::G418r rad52Δ::LEU2 + p316GalyR52</td>
</tr>
<tr>
<td>YLKL1585</td>
<td>BY4742 chk1Δ::G418r rad52Δ::LEU2 + p316GalyR52</td>
</tr>
</tbody>
</table>
VL6α = MATα ura3-52 leu2-3,112, trp1-289 his7-2

S1 = MATα ura3-52 leu2-3,112 trp1-289 his7-2 ade5-1 lys2::insE-4A

BGW1-7a = MATa ura3-52 leu2-3,112 his4-519 ade1-100

YLKL477 = YPH102 = MATα ura3-52 leu2·Δ1 his3·Δ200 lys2-801(amber) ade2-101 (ochre)

Table 4. Plasmid list. A complete list of plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pΔ52L (rad52Δ::LEU2 deletion plasmid)</td>
<td>Ed Perkins</td>
</tr>
<tr>
<td>p316GalyR52 (GAL1p::RAD52 URA3 CEN/ARS)</td>
<td>Lab Plasmid</td>
</tr>
</tbody>
</table>

Cell Culture Solutions and Media

Non-selective YPDA yeast plate growth media contained 1% bacto yeast extract, 2% soy peptone, 2% D-(+)-glucose, 2% agar, and 0.001% adenine. YPDA broth was prepared as YPDA media but without agar. Synthetic dropout media was used for plasmid selection. Dropout mix was composed of 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% D-(+)-glucose, 2% agar, and all essential amino acids and bases used for strain selection. In some cases, yeast synthetic growth media contained 2% galactose, instead of 2% glucose.
E. coli cells were grown for the isolation of plasmids using TB + ampicillin (amp) broth (1.2% bacto tryptone, 2.4% yeast extract, 0.5% glycerol, 10% 0.017 M \( \text{KH}_2\text{PO}_4 \) + 0.072 M \( \text{K}_2\text{HPO}_4 \), 100 \( \mu \text{g/mL} \) amp) or for sustained growth on LB + amp plates (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 100 \( \mu \text{g/mL} \) amp + 1.5% agar).

**Gel Electrophoresis**

Gel electrophoresis was performed in Horizon 11-14 gel rigs using 0.7 – 2% agarose gels in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at ~120 - 150 V or 0.5X TB buffer (45 mM Tris, 45 mM boric acid) at ~250 V. Ethidium bromide (0.5 \( \mu \text{g/ml} \)) was used to stain agarose gels and images were captured on the Alpha Innotech Red Imaging system.

**Chromosomal DNA Purification – Mini preps for PCR verification of gene knockout**

An optimized protocol was designed by the Lewis laboratory (52) as follows. Three mL of yeast cells were pelleted the supernatant was removed, and 300 \( \mu \text{L} \) of 6% SET solution (6% SDS, 10 mM EDTA, and 30 mM Tris pH 8.0) was added. This mixture was incubated at 65°C for 15 minutes and transferred to a water/ice bath for 5 minutes. 150 \( \mu \text{L} \) of cold potassium acetate solution (3.0 M potassium acetate and 2.0 M acetic acid) was added, then the tube was repeatedly inverted for 10 s. The mixture was centrifuged at 21000X g for 10 minutes to pellet protein and cell debris. The supernatant (containing the nucleic acid component) was combined with 500 \( \mu \text{L} \) isopropanol and mixed for 30 seconds before being spun at 21,000X g for 2 minutes. The supernatant was
removed and the pellet was washed with 500 µL 70% ethanol, then incubated at room temperature for 1 minute before the ethanol was removed. The remaining ethanol was removed through the use of a speedvac for 10 – 15 minutes until dry. The pellet was suspended in 50 µL TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA), and 1 µL of 2 mg/mL RNase A was added to degrade RNA contaminants. This mixture was incubated at room temperature for 10 minutes. The pellet was allowed to dissolve at 4°C then stored at -20°C.

**Plasmid DNA Isolation**

An improved alkaline lysis protocol for *E. coli* plasmid DNA minipreps method from the Lewis laboratory was used that is very similar to the alkaline lysis method described by Green and Sambrook (55). Bacterial colonies were grown in 2-5 mL of TB broth + Amp in a tall tube overnight in a 37°C shaker. One-and-a-half milliliters of the culture was added to a microfuge tube and quick spun at 21,000X g for 15-20 seconds and the supernatant was removed. The bacterial pellet was resuspended in 100 µL of Solution I (50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0) by scraping the bottom of each tube against a wire test tube rack until the pellet was thoroughly dispersed. The mixture was kept on ice to reduce the amount of nicked open circular plasmid DNA (51). Two hundred µL of freshly prepared solution II (0.2 M NaOH + 1% SDS) kept at room temperature was added. Tubes were closed and inverted rapidly five times to mix, and stored on ice for 1 minute. One hundred and fifty µL of ice-cold Solution III (3 M KOAc) were added and the tubes were inverted rapidly several times to
mix. The samples were stored on ice for 3 minutes before centrifugation at 21,000X g for 10 minutes. The supernatant was transferred to a new tube except for the last 4-5 mm above the cell debris pellet. The DNA was precipitated by adding 400 µL of isopropanol, inverting several times to mix, and then spinning for 5 minutes at 21,000X g. The supernatant was removed using a 1000 µL pipettor, and the pellet was washed with 500 µL of 70% EtOH without disturbing the pellet. The sample was then centrifuged for 2 minutes at 21,000X g with pellets facing the same direction as in the first centrifugation so they were not dislodged. The supernatant was removed with a 1000 µL pipettor and put in the speedvac for ~10 minutes or until the pellet was dry. The pellet was resuspended in 50 µL of TE plus 2 µL of 2 mg/mL RNase A and vortexed. The pellet was incubated at room temperature for 10 minutes and then stored at -20°C.

**PCR Amplification of rad52Δ::LEU2 Insert for Verification of Gene Disruption**

Reactions were done in 0.2 mL PCR tubes with 1 µM LEU2A and 1 µM Rad52U1 or rad52E primers, 1X Taq Polymerase Thermopol buffer, 0.25 mM dNTPs, Taq Polymerase (5 units), ddH2O and 3 µL chromosomal DNA used as a template in a final volume of 50 µL. Five µL (10 µL for weak primer sets) of each sample were combined with 5 µL of ddH2O and 2 µL 6X loading dye, mixed, and loaded onto a 0.8% agarose gel. Gels were stained with ethidium bromide for 15 minutes and imaged. PCR cycles were performed using 94°C for 30 seconds, annealing temperatures from 50°C – 56°C for 40 seconds, and an extension temperature of 72°C for 120-180 seconds (1 minute for the first kb with an additional 30 seconds added for each kb after). PCR was
conducted in an Applied Biosystems 2720 Thermal Cycler or a Bio-Rad T100 for 34 cycles.

**Ethanol Precipitation to Clean Up Chromosomal DNA and DIG-labelled PCR Fragments**

One twentieth volume of 3 M sodium acetate was added to samples (one tenth if suspended in ddH₂O as opposed to a buffer such as TE with salt). Two and a half volumes of cold 100% ethanol was then added to samples followed by vortexing. Samples were put on wet ice or kept at 4°C for greater than or equal to 15 minutes and then spun at 21,000 x g for 15 minutes. The supernatant was pulled and discarded. The pellet was rinsed with an appropriate volume (200-500 µL) 70% ethanol, spun for 3-5 minutes, and the supernatant again removed and discarded. Samples were then put in the speedvac for 5-10 minutes until visibly dry and resuspended in an appropriate volume of ddH₂O or TE.

**Transformations of DNA into yeast cells**

Yeast strains were transformed using a modified rapid lithium acetate/DMSO transformation method described by Tripp *et al.* (56). Cells were shaken overnight in 4 mL YPDA broth. One-and-a-half mL of the liquid culture was spun down and the growth media was removed. Cells were incubated in 0.2 M DTT at 42°C for 20 minutes. Cells were spun down and the supernatant was removed. Carrier DNA (50 µg) and transforming DNA (50-300 ng) were added to the pellet and the solution was mixed. A
master mix composed of 40% PEG 4000, 0.1 M LiAc, 1.0 mM EDTA, 0.1 M Tris (pH 7.5) was added to the pellet and DNA. A volume of DMSO was then added equivalent to 56 μL per transformation. This mixture was shaken for 15-30 minutes at 30°C, then at 42°C for 15 minutes. The mixture was spun down, the supernatant discarded, and 1 mL of YPDA broth was added. The samples were mixed and then incubated in a shaker for 30 minutes at 30°C. Cells were spun down and the broth removed. The pellet was resuspended in 200 μL ddH₂O and aliquots of the cells were plated to selective media at 30°C for colony formation.

**Double imprint replica plating to find isolates with MMS sensitivity**

At least 2 independent PCR-confirmed isolates from each rad52 mutant were patched onto fresh synthetic dropout media plates and grown for 2 days at 30°C. The first plate with patches of cells grown on it would then be used as a master that was used to transfer the same pattern of cells onto to a sterile velvet. A second plate was placed on the velvet so cells could be transferred. A new velvet was obtained and the second plate was used as a master plate for cells to transfer to the new velvet. A third and fourth plate were placed on the new velvet and cells were transferred. The plates were put into an incubator at 30°C for 3 days to observe MMS sensitivity.

**Streaking survival assay**
Cells were streaked for single colonies in individual quadrants of plates and allowed to grow for 2-5 days at 30°C and compared. Images were taken of the plates using a Canon Powershot G3 digital camera and saved as JPEG files.

**Qubit Fluorometry for Quantification of DNA**

Two assay tubes for the standards and one tube per sample that was tested were labeled. The Qubit working solution was prepared by diluting Qubit BR dsDNA reagent 1:200 in Qubit BR dsDNA buffer and vortexing. Two hundred μL of Working Solution was made for each standard and sample, plus an additional 200 μL. The standards were prepared by adding 190 μL of Working Solution to 10 μL of Standard #1 (0 ng/μL dsDNA) and 10 μL of Standard #2 (100 ng/μL dsDNA). Samples were prepared by adding 2 μL of DNA to 198 μL of Working Solution. One sample with a previously measured concentration was always included as well. Once standards and samples were prepared, all tubes were vortexed for 2-3 seconds and incubated at room temperature for 2 minutes. Tubes were inserted into the Qubit 2.0 Fluorometer and readings were taken.

**Doubling time experiments**

To determine the doubling time of a strain of yeast, 1 mL of YPDA broth was inoculated at 3x10^6 cells/mL in 2 mL Eppendorf tubes and placed into a shaker in a 30°C incubator. Each strain was tracked over a period of 8 hours starting at hour 3 using a hemocytometer and phase contrast microscopy. The following serial dilution scheme was used to make counting of cells easier and more timely. Starting at hour five, a 1:5 dilution was made by taking 60 uL aliquots from each isolate and mixing them with 240
uL ddH₂O before vortexing, sonicking, and loading onto the hemocytometer. At hours six and seven, 1:10 dilutions were made by diluting 30 uL cells into 270 uL ddH₂O. At hour eight, a 1:20 dilution was made by mixing 30 uL into 570 uL ddH₂O. Strains were immediately placed back into shaker after taking aliquots so as to not disturb doubling times. Four isolates from each strain were tracked so averages and standard deviations could be computed. Averages and standard deviations were then plotted on a semi-log scale in Microsoft Excel (y axis = the log of cells/mL x10⁶, X axis = time in hours) and the best fit trendline plotted. Doubling times were obtained from the equation of the trendline.

**DAPI Staining of Log Phase Yeast Cells to View Nuclear Morphologies**

**Innoculation of log phase cultures**

Cultures were inoculated in a 2 mL tube containing 1 mL glucose complete broth at 3x10⁶ cells/ml. Cells were harvested in mid-log phase after 4 hours. Glucose complete broth was used because YPDA often produces substantial background during the staining process.

**Formaldehyde Fixation prior to centrifugation to avoid displacement of nucleus**

Cells were vortexed and sonicated at 22% amplitude for 10 seconds. One tenth volume of 36% formaldehyde per 1 mL yeast was added and tubes were left to sit 1-2 hours at room temperature. Cells were then washed twice with 1X Phosphate Buffered
Saline (PBS) before resuspending in original culture volume of PBS. Cells were spun at 10K x g for 3 minutes between washes. It is an option to leave cells at 4°C at this point for use later in the day/week.

**Before Staining**

Cells were spun down and resuspended in an appropriate volume 70% EtOH, then incubated at room temp for 30-40 min. Following incubation, cells were spun down, the supernatant was removed, and they were resuspended in PBS. Cells could be stored at 4°C at this point for use later in the day.

**Staining**

Cells were spun down at 10,000xg for 3 minutes, the supernatant was removed and 500 uL ddH₂O was added. Cells were then spun down again and the supernatant was removed. Two hundred microliters of 1X DAPI solution (Gene Tex Mix) was added to the pelleted cells and they were lightly vortexed to mix. Cells were then incubated 10 min at room temperature, spun down again and DAPI was pipetted out. Cells were washed with 1X PBS 2 times and ddH₂O once for 5 min each before spinning down to remove unbound DAPI. Cells were then resuspended in 3/10th of the original culture volume of ddH₂O.

**Mounting**
Prior to pipetting onto a slide, cells were lightly sonicated (using at least 300 uL volume in a 1.5 mL microfuge tube) (7 pulses at 20% duty cycle) and roughly 4 uL was pipetted at a time onto a slide. Then slides were passed rapidly through a bunsen burner flame a few times every five seconds until dry. Dilutions were made (1:5 and 1:10) and 4 uL aliquots were pipetted onto slides as well to be sure to obtain optimal imaging cell densities. Four microliters of ProlongGOLD antifade solution was pipetted on top of the dried sample before adding the cover slip. Cells must fix in the dark for 24 hours before imaging. The DAPI treated cells were exposed to as little light as possible, including when staining, before the 24 hour designated fixing period had passed. If the edges are sealed with a non-specific type of fingernail polish after fixation, slides will keep for several months.
III. RESULTS AND DISCUSSION

The primary focus of this project was to investigate the observation that mutant yeast cells defective in DNA double-strand break repair have a larger percentage of G₂ cells in liquid cultures than wild type cells. This indicates that the cells are spending longer amounts of time in G₂ phase. Previous work found that rad52 and rad51 cells, both defective in DSB repair, have a higher number of G₂ cells than wild type cells (43, 45-47). These mutants have the same phenotype that normal, healthy cells exhibit after being exposed to DNA damaging agents. We speculate that these DSB repair-deficient cells are pausing in G₂ phase due to the inability to repair endogenous DNA damage caused by reactive oxygen species (ROS) that are a normal byproduct of oxygen metabolism and also by other mechanisms. This damage could potentially lead to a constitutively activated DNA damage response. Major goals of this project were to quantitate the changes in cell cycling and cell morphology in *S. cerevisiae* mutants that are deficient in DNA double-strand repair, especially cells defective in HR, as well as cells that are deficient in both DNA repair and DNA damage checkpoint responses.

Initial experiments by undergraduate Paige Hall tested the percentage of large budded (G₂) cells in *rad52* cell cultures from different strain backgrounds isolated from around the world to test if this high percentage was conserved. Cells were inoculated into YPDA broth at 3x10⁶ cells/mL and shaken at 30°C for 4 hours to get the cells into mid log phase. Cells were then examined using phase contrast microscopy. An example of the types of images analyzed for these experiments that were taken with the phase contrast microscope is shown in Figure 5.
Cells were classified as being in G₁, S, or G₂/M based on their cellular morphologies. Cells in G₂ and M have large buds and are defined as cells in which the bud is ≥ 50% of the size of the mother cell (43). G₂ and M phase cells are grouped together due to the fact that they can only be distinguished by analyzing their nuclear morphologies, which cannot be done with standard phase contrast microscopy. M phase happens so rapidly that the vast majority of large-budded cells visualized are in G₂ phase (Figure 6). G₂ and M cells can be distinguished by applying nuclear stains that probe internal morphologies such as DAPI to visualize nuclear separation.
Figure 6. Timeline of the cell cycle phases G₁, S, G₂ and M. Length of line segments corresponds to the amount of time spent in each phase during log phase growth at 30°C. M happens very quickly with respect to the rest of the cell cycle.

The experiments showed that rad52 mutants in each of the 5 backgrounds had about the same increase in percentage of G₂/M cells, many of them with a distended, or enlarged phenotype (Figure 7). WT cell cultures contained approximately 30% G₂/M cells, but each rad52 mutant culture had ~ 50% G₂/M cells. This result indicated that the phenotype is conserved across different strain backgrounds.
Figure 7. Yeast strain backgrounds BY4742, BWG1-7a, YPH102, S1, and VL6alpha were grown to log phase to investigate the phenomenon of an increase in the amount of G$_2$ cells found in cell cultures of DNA repair-deficient rad52 mutants. The increased number of G$_2$/M cells was conserved across all strain backgrounds. Four cultures of each strain were analyzed. Averages and standard deviations are shown.

In addition to haploid yeast cells, diploid rad52$^{-/-}$ cells from two different strain backgrounds were also investigated. A high percentage of G$_2$ cells (~60%) was observed in each of the diploid rad52 mutants as well.

Figure 8. Diploid rad52$^{-/-}$ yeast strains exhibit high levels of G$_2$/M cells during log phase growth.
The first objective of this new project was to reproduce Paige Hall’s findings. WT and \textit{RAD52}-deficient \textit{BY4742} cells harvested from plates were counted and inoculated into YPDA broth at $3 \times 10^6$ cells/mL, shaken for 4 hours at 30°C, and analyzed by phase contrast microscopy. It was observed that WT cell cultures had about 28\% G₂/M cells while \textit{rad52} cells had 50\% G₂/M cells (Figure 9). This was consistent with the results that were previously observed.

![Figure 9](https://via.placeholder.com/150)

\textbf{Figure 9.} WT and \textit{rad52} cultures were grown to mid-logarithmic phase and \% G₂/M phase cells quantified. Four replicates of each strain were tested. Error bars indicate standard deviations.

Cell cycle doubling times during logarithmic phase growth were then calculated for WT and \textit{rad52} cells. Cells were inoculated at $3 \times 10^6$ cells/mL into YPDA broth and
cell titer was tracked using a hemocytometer over a period of eight hours, starting at hour three, when cells are growing exponentially. The time was then plotted against a logarithmic scale of cell titer. The equation of the best fit trendline was then used to compute the doubling time for each haploid strain (Figure 10). To calculate the doubling time, a value was plugged in to the Y variable of the equation and the resultant X value recorded. The first Y value was then doubled and plugged into the equation and the second X value recorded. The doubling time is the difference between X₂ and X₁. rad52 mutants had 50% longer doubling times than WT cells (128 minutes to complete each cell cycle versus 83 minutes for WT cells).
Figure 10. Plot of cell titer versus time for BY4742 WT and rad52 cells. Four cultures of each strain were analyzed and results were averaged. Wild type cells had a doubling time of 83 minutes and rad52 mutant cell cycles required 128 minutes.

The observation that DNA repair-deficient rad52 mutants spend more time in G2 phase than WT cells was then investigated in more detail. The percentage of cells in each cell cycle phase during growth in cell culture is proportional to the amount of time spent in that phase. Total cell cycling time (doubling time) and the percentages of cells in G₁ (unbudded), S (small-budded), and G₂/M (large-budded) during logarithmic phase growth were used to calculate the minutes spent in each phase (Figure 11). M phase is very short so it was assumed most large-budded cells were in G₂. rad52 cells spend 2.7 fold more time in G₂ phase than WT cells (63.8 minutes as compared to 23.5 minutes). By contrast, minutes spent in G₁ and S were similar in the two strains.
Figure 11. Time spent in each cell cycle phase for WT and rad52 cells. rad52 mutants spend approximately the same amount of time in G₁ and S phase as WT cells, but they spend about 2.7 fold more time in G₂ phase.

We wanted to investigate how the percentage of G₂/M cells changes as cells transition from stationary phase, through logarithmic phase, and back to stationary phase growth. WT and rad52 cells were inoculated at 3x10⁶ cells/mL into YPDA broth and shaken for 48 hours. Aliquots were removed at 0, 4, 8, 12, 24, and 48 hours. Both percent G₂/M cells and cell titer (cells per mL) were calculated from aliquots (Figure 12). The percentage of large-budded cells continued to increase in rad52 cells until hour 8, when they reached 67%. WT cells reached a peak percentage of 34% at hour 4.
Figure 12. The percentage of G2/M cells (top) and cell titer (bottom) were tracked for a period of 48 hours for 4 isolates of WT and rad52 cells.
Rad52 protein works with several other proteins to repair DSBs via the homologous recombination pathway. Many of these proteins are members of the RAD52 group, which includes Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, Mre11, Xrs2, Rdh54 and the Rpa complex. The possibility that inactivation of other members of the RAD52 epistasis group might also lead to high levels of G2/M cells was also examined. RAD52 epistasis group members that were tested are listed in Table 5 below with their corresponding protein function.

<table>
<thead>
<tr>
<th>Rad52 Epistasis Group Member</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad52</td>
<td>catalyzes assembly of Rad51 onto DNA</td>
</tr>
<tr>
<td>Rad51</td>
<td>binds ssDNA; performs homology search</td>
</tr>
<tr>
<td>Rad54</td>
<td>strand invasion</td>
</tr>
<tr>
<td>Rad55</td>
<td>stabilizes Rad51 and ssDNA</td>
</tr>
<tr>
<td>Rad57</td>
<td>stabilizes Rad51 and ssDNA</td>
</tr>
<tr>
<td>Rad59</td>
<td>removes non-homologous ends from ssDNA</td>
</tr>
<tr>
<td>Rdh54</td>
<td>unwinds DNA to promote strand opening</td>
</tr>
</tbody>
</table>

Strains were inoculated into YPDA broth at 3x10^6 cells/mL and grown to log phase before quantifying the percentage of G2/M cells using a hemocytometer. *rad51, rad54, rad55, rad57,* and *rad59* mutants also exhibited higher percentages of G2/M cells
than WT cells, with non-overlapping standard deviation error bars (Figure 13). *rdh54* mutants were the only cells that did not exhibit a strong increase. Rdh54 plays a role in homologous recombination in diploid cells and during meiosis (44). Haploid cells were tested in this experiment and *rdh54* haploids may not exhibit a phenotype for that reason. This hypothesis was addressed in further experiments described later.

**Figure 13.** Quantification of G₂/M cells in recombination-defective RAD52 epistasis group mutant cell cultures. All strains had strongly elevated levels of G₂/M cells except *rdh54*. Four replicates of each strain were tested and averages and standard deviations are shown.
The next experiment measured how long each RAD52 epistasis group mutant was spending in $G_1$, $S$, and $G_2$ phase. To do so, doubling times were determined by inoculating each strain into YPDA broth at $3 \times 10^6$ cells/mL followed by shaking at 30°C. Cell titer was tracked using a hemocytometer and phase contrast microscopy for a period of 8 hours starting at hour 3 as before. The log of the cell titer was then plotted against growth time and the data fit to an exponential curve. The resulting equation of the best fit trendline was used to find the doubling time (Figure 14).
Figure 14. Graph of the log of cell titer tracked with a hemocytometer vs time. The equations of the best fit trendlines were used to find the doubling times of the RAD52 epistasis group mutants.

All of the RAD52 epistasis group mutants displayed elevated doubling times (~90 minutes as compared to ~83 min for WT cells). *rad52* cells differed from the others and exhibited a more extreme phenotype, with a doubling time of 128 minutes. Results are as listed in Table 6 below.
Next, using the doubling times and cell cycle percentages, the length of time RAD52 epistasis group members were spending in each phase of the cell cycle was calculated, as done previously for *rad52* cells. While WT cells spent 24 minutes in G$_2$ phase, *rad51, rad54, rad55, rad57*, and *rad59* cells spent nearly twice as long in G$_2$ (ranging from 39.2 to 46.0 minutes).

<table>
<thead>
<tr>
<th>Rad52 Epistasis Group Member</th>
<th>Doubling Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>83</td>
</tr>
<tr>
<td><em>rad52</em></td>
<td>128</td>
</tr>
<tr>
<td><em>rad51</em></td>
<td>88.9</td>
</tr>
<tr>
<td><em>rad54</em></td>
<td>93.6</td>
</tr>
<tr>
<td><em>rad55</em></td>
<td>91.2</td>
</tr>
<tr>
<td><em>rad57</em></td>
<td>92.1</td>
</tr>
<tr>
<td><em>rad59</em></td>
<td>93.1</td>
</tr>
<tr>
<td><em>rdh54</em></td>
<td>90</td>
</tr>
</tbody>
</table>
Figure 15. Time spent in each phase of the cell cycle by several RAD52 epistasis group mutants.

Most of the experiments discussed in this work thus far have involved the use of haploid BY4742 MATα strains. Haploid yeast cells are classified into two mating types called MATα and MATa. Experiments with the MATα mutants showed that rad52 mutant phenotypes were much more extreme than in the other DSB repair mutants (e.g., 128 minute doubling time versus ~90 min for other mutants). New experiments were performed to see if this extreme phenotype that rad52 mutants exhibit would also be observed in BY4741 MATa rad52 mutants. A new homemade rad52Δ::LEU2 BY4741
MATα strain was created by transformation of WT cells with a fragment of the plasmid pΔ52LEU2. RAD52 was inactivated by exploiting the fact that yeast cells undergo high rates of homologous recombination via inserting a functioning LEU2 gene into the RAD52 gene. A schematic of the approach used is shown below (Figure 16). The plasmid was cut with NotI and ApaI endonucleases and the resulting fragment was transformed into BY4741 cells using the LiAc and heat shock method as listed in chapter II. The flanking rad52 regions surrounding LEU2 underwent genetic exchange with the RAD52 gene on chromosome XIII. This deletes a portion of the RAD52 gene and inserts LEU2 (Figure 16).

Figure 16. RAD52 inactivation via transformation with a fragment of the plasmid pΔ52LEU2.
After transformation, cells were grown on leucine-deficient synthetic media to select for cells that underwent an exchange event and were successfully expressing the \textit{LEU2} gene. Colonies that grew on leucine-deficient media were then streak-purified and confirmed to have the \textit{RAD52} gene knocked out via polymerase chain reaction (PCR) using the chromosomal DNAs as templates. One primer for the PCR reaction corresponded to the \textit{RAD52} gene (RAD52E) and the other to \textit{LEU2} (LEU2A). There should only be a PCR product if the \textit{LEU2} gene was inserted into the \textit{RAD52} locus (illustrated in Figure 17).
Figure 17. Logic behind verification of RAD52 gene knockout via PCR. The selection of primers ensured that the 1200 bp product is only seen if the LEU2 gene is inserted into the RAD52 locus.

Several isolates exhibited a band at 1200 bp, which therefore confirmed that they had the LEU2 gene inserted into the RAD52 locus, inactivating the RAD52 gene and thus making cells homologous recombination-deficient. Four confirmed isolates (5, 6, 7, and 8) were streak-purified and inoculated into YPDA broth at 3x10^6 cells/mL and grown at 30°C for 4 hours to reach mid log phase as before to assess levels of G_2/M cells in each
culture. $MATa$ WT, $rad51$, and a $rad52$ mutant from a $MATa$ yeast deletion strain library were also analyzed. Both $rad52$ strains, as well as the $rad51$ strain, exhibited higher levels of large-budded cells. The range of G$_2$/M percentages was similar to that seen in the earlier $MATa$ strain experiments (45-55%). Results are depicted in the graph below (Figure 18).

Figure 18. Percentage of G$_2$/M cells in BY4741 ($MATa$) WT, $rad51$, and two different $rad52$ strain cell cultures. Four replicates were tested. Error bars indicate standard deviations.
The doubling times of the BY4741 (MATa) strains were determined to see how they compared to the doubling times in the BY4742 (MATa) strain background. Cells were inoculated at 3x10⁶ cells/mL into YPDA broth and shaken at 30°C as before. The log of the cell titer was plotted against time as shown in Figure 19. The equation of the best fit trend line was used to determine the doubling time of each strain. As seen previously in BY4742 cells, the BY4741 rad52 mutants had a more extreme phenotype than rad51 cells, with a doubling time of 115 minutes versus 85 minutes.

![Image](image.png)

**Figure 19.** Growth curves used to calculate doubling times of BY4741 (MATa) strains. Cell cycle doubling times were calculated to be 85, 85, and 115 minutes for WT, rad51, and rad52 cells, respectively.
Doubling times were multiplied by the percentage of G2 cells present during logarithmic phase growth to obtain an estimate of the average time cells were spending in G2 phase (Figure 20).

BY4741 rad52 cells spent ~2.5 times the amount of time that WT cells spend in G2. This is comparable to the ~2.7 times the amount of time BY4742 rad52 cells spend in G2 phase as compared to WT. G2 phase in the rad51 cells was also increased, but much less so than in rad52 mutants. These experiments demonstrate that DSB repair-deficient rad51 mutants exhibit high G2 cells in both MATa and MATα strains. They also confirm that rad52 cell phenotypes are more extreme than those seen in rad51 cells in both cell types.
Figure 20. Time spent in G₂ phase for BY4741, rad51, and rad52 cells. rad52 cells spend ~2.5 x more time in G₂ than WT cells.

rdh54 cells were the only RAD52 group members whose time in G₂ was not statistically different than WT. However this gene is known to affect recombination in diploids, not haploids. We created WT and rdh54/rdh54 diploids by mating MATα and MATa strains according to the mating protocol seen below (Figure 21).
Figure 21. Schematic of mating protocol used for crossing BY4741 (rdh54) and BY4742 (rdh54) cells to obtain rdh54−/− diploid strains. BY4741 cells are ura3 leu2 his3 met15 and BY4742 cells are ura3 leu2 his3 lys2. The MATa and MATα cells were mixed together in the middle rectangle of the glucose complete plate, grown for 2 days at 30°C to allow mating to occur, and were then replicated to a plate lacking lysine and methionine to select for diploid cells (met15+/− lys2+/− ura3+/− leu2+/− his3+/−).
After streak purification of successfully mated colonies, levels of G2/M cells during mid log phase were quantified. Diploid WT cells did not have a statistically different level of G2/M cells as compared to \textit{rdh54} \textsuperscript{-/-} cells, with each strain displaying ~30\% G2/M cells (Figure 22).

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{G2M_levels.png}
\caption{Levels of un budded (G1), small budded (S), and large budded (G2) diploid WT and \textit{rdh54} \textsuperscript{-/-} cells during mid-logarithmic phase growth. \textit{rdh54} \textsuperscript{-/-} mutants exhibited WT levels of G2/M cells.}
\end{figure}
These experiments have indicated that *rad52* cells and cells with other genes of the RAD52 epistasis group inactivated are spending extended amounts of time in G₂ phase, but the mechanism involved has not been examined. We hypothesized that unreppaired DNA DSBs in homologous recombination-deficient *rad52* cells may be causing activation of the DNA damage response (DDR) system, which causes cells to pause in G₂ phase in an attempt to repair the damage before moving on to M (mitosis) phase. The thought follows the fact that *rad52* cells grown under normal conditions are exhibiting a phenotype that wildtype cells exhibit after being exposed to DNA damaging agents such as X-rays or UV radiation. When log phase cultures of yeast (a mixture of G₁, S, and G₂/M cells) are exposed to radiation, many of the cells pause in G₂, causing G₂ cells to accumulate to higher levels in the culture temporarily (Figure 23).
Figure 23. When exposed to DNA damaging agents, WT cells pause in G₂ phase due to a DNA damage response (DDR). This causes an increase in the number of G₂ cells found in culture. This phenomenon is similar to that exhibited by rad52 cells in log phase growth, even though these cells have not been exposed to an exogenous DNA damaging agent.

To test our hypothesis that rad52 mutants have high levels of G₂/M cells due to unrepaired DNA damage and thus a constitutively activated cell cycle checkpoint response, new double mutants were created. The RAD52 gene was inactivated by insertion of LEU2 in seven different MATα library strains that had DNA damage response genes knocked out. The strains in which RAD52 was inactivated are listed in Table 4 with their corresponding gene functions. The strains created by this procedure were rad52 rad9, rad52 rad17, rad52 rad24, rad52 mec3, rad52 ddc1, rad52 dun1 and rad52 chk1 double mutants.
After transformation with rad52::LEU2 DNA fragments as previously described, Leu⁺ colonies were streak-purified and chromosomal DNA was extracted from several of them for each strain. The genomic DNAs were then tested by PCR with the test primers RAD52E and LEU2A as before. An example of PCR products that underwent agarose electrophoresis is shown in the figure below (Figure 24). The PCR products were run on a 0.7% agarose gel. As shown in the figure, rad17 rad52 isolates 3 and 5, dun1 rad52 isolate 1, rad9 rad52 isolates 1, 3, and 4, and mec3 rad52 isolate 2 produced 1200 bp bands, confirming proper integration of LEU2 into RAD52.

### Table 7. DDR mutant strains in which RAD52 was inactivated with their corresponding gene functions.

<table>
<thead>
<tr>
<th>Deletion Strain</th>
<th>Protein Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>rad9</td>
<td>adaptor/mediator (required for chk1 activation)</td>
</tr>
<tr>
<td>rad17</td>
<td>sensor (part of 911 complex)</td>
</tr>
<tr>
<td>rad24</td>
<td>loads 911 complex onto DNA; signaling role</td>
</tr>
<tr>
<td>mec3</td>
<td>sensor (part of 911 complex)</td>
</tr>
<tr>
<td>ddc1</td>
<td>sensor (part of 911 complex)</td>
</tr>
<tr>
<td>dun1</td>
<td>serine-threonine kinase</td>
</tr>
<tr>
<td>chk1</td>
<td>effector kinase</td>
</tr>
</tbody>
</table>

After transformation with rad52::LEU2 DNA fragments as previously described, Leu⁺ colonies were streak-purified and chromosomal DNA was extracted from several of them for each strain. The genomic DNAs were then tested by PCR with the test primers RAD52E and LEU2A as before. An example of PCR products that underwent agarose electrophoresis is shown in the figure below (Figure 24). The PCR products were run on a 0.7% agarose gel. As shown in the figure, rad17 rad52 isolates 3 and 5, dun1 rad52 isolate 1, rad9 rad52 isolates 1, 3, and 4, and mec3 rad52 isolate 2 produced 1200 bp bands, confirming proper integration of LEU2 into RAD52.
Figure 24. PCR products analyzed by agarose gel electrophoresis to confirm knockout of *RAD52*. The presence of a 1200 bp band indicates transformants that are *RAD52* deficient. Yellow arrows indicate the 1200 bp band.
Confirmed rad52 ddr double mutant isolates were inoculated into YPDA broth at 3x10^6 cells/mL, grown to mid log phase at 30°C and levels of G2/M cells quantified. G2/M cells were reduced to WT levels in 5 of the rad52 DNA damage checkpoint double mutants (with knockouts of mec3, ddc1, rad9, rad17, and rad24) and to near WT levels in 2 others (dun1 and chk1) (Figure 25). These results demonstrate that the high G2 phenotype observed in rad52 cells is dependent on DNA damage checkpoint genes.

Figure 25. Quantification of the percentage of G2/M cells in 7 different rad52 checkpoint double mutants. Inactivation of the DNA damage checkpoint returned homologous recombination-deficient rad52 cell cultures to near WT levels of G2/M cells.
We hypothesized that DSB repair-deficient strains that were coupled with a checkpoint mutation might exhibit stress or slow growth effects when grown over many generations due to an accumulation of unrepaired DNA damage. The idea is that cells are progressing into M phase regularly with damaged chromosomes, which may be further damaged by the forces applied by the mitotic spindle in metaphase and anaphase. To test this hypothesis, four rad52⁻ checkpoint⁻ double mutant strains (rad9 rad52, rad17 rad52, rad24 rad52, chk1 rad52) were created that contained a plasmid (pGAL::RAD52) expressing RAD52 via a galactose-regulated promoter. This design allowed the expression of RAD52 to be turned on and off depending on whether or not the growth media contained galactose or glucose (Figure 26).
Figure 26. Design schematic of rad52- ddr- double mutants that were transformed with a plasmid expressing RAD52. Depending on the growth medium, cells will either be expressing Rad52 or not.

After being transformed with the pGAL::RAD52 plasmid, checkpoint mutant strains were transformed with the RAD52::LEU2 plasmid fragment to inactivate the chromosomal copy of RAD52 and spread onto galactose minus leucine and uracil plates. Colonies were PCR verified to ensure the fragment underwent recombination with the

Cells make Rad52 and are DNA repair proficient

Cell are rad52- ddr- and may enter mitosis with broken chromosomes

...
correct locus on chromosome XIII, knocking out \textit{RAD52} and replacing it with \textit{LEU2} as described previously. Isolates were streak purified on galactose media that was deficient in uracil and leucine. The uracil deficiency selected for cells that contained the pGAL::RAD52 plasmid while galactose drove the expression of \textit{RAD52} via the galactose promoter on the plasmid. The leucine deficiency selected for cells that have the \textit{RAD52} locus disrupted with, and are actively expressing, the \textit{LEU2} gene. When plated on plates containing 5-FOA, which is toxic to \textit{URA3} cells, cells that had naturally lost the pGAL::RAD52 plasmid could be selected for and would grow to form colonies. This design allowed study of \textit{rad52} checkpoint double mutant cells without the worry of accumulation of DNA damage over generations of growth before the study took place.

After constructing the new double mutants, we wanted to observe the effects on growth that being \textit{rad52}-deficient and checkpoint-deficient had on these strains. Cells were repeatedly streaked to either galactose or glucose plates for a series of 6 streaks as shown below (Figure 27). In the left pathway, cells have an actively transcribed plasmid \textit{RAD52} gene and are checkpoint-deficient only. In the right pathway, cells are \textit{RAD52}-defective and checkpoint-defective, potentially causing them to pass through M phase repeatedly with unrepaired DNA damage. Each medium-sized colony on a plate has undergone ~21 generations or cell cycles, starting from a single cell.
Figure 27. Diagram of the two pathways checkpoint \textit{rad52 ddr} double mutants underwent during plate streaking assays. Colonies forming on earlier plates were picked and restreaked to new plates, forcing the cells to undergo many generations of growth with unrepaired chromosomes.
The four double mutant strains were streaked repeatedly, for a total of 6 streak plates, corresponding to ~126 generations, but growth rates and colony sizes did not change during the course of the experiment (data not shown). These results indicate that lack of \textit{RAD52} and a functional DNA damage response does not affect viability strongly in most of the cells.

We observed that many \textit{rad52} cells appeared to be enlarged compared to WT cells when analyzed using phase contrast microscopy. The next aim intended to quantify the large, distended phenotype observed in populations of \textit{rad52} cells. Cells were grown for four hours at 30°C to mid log phase as before prior to being analyzed and measured via phase contrast microscopy. Cell sizes across the largest diameter of the mother cell were measured using a micrometer scale provided by the Scope Image-Pro software package. A total of 150 WT and 150 \textit{rad52} cells were analyzed. The average sizes of unbudded, small-budded and large-budded cells are depicted in Figure 28. These cell diameters were ~ 5.25, 5.25, and 5.75 μm in WT cells. In contrast to this, \textit{rad52} cells in all three categories were larger with sizes of 6.1, 6, and 7.25 μm, respectively. This result indicates that unbudded and small-budded \textit{rad52} cells are 16.2% and 14.3% larger than the equivalent types of WT cells. Large-budded (G2/M) cells exhibited an even larger difference, with \textit{rad52} cells 26.1% larger than the equivalent WT cells.
Figure 28. Unbudded, small-budded, and large-budded cell size averages after 4 hours of growth in YPDA at 30°C for WT (A) and rad52 cells (B).
Large-budded homologous recombination-deficient rad52 cells were found to be, on average, 1.5 µm larger than WT cells of the same strain background. Average sizes were 5.8 µm and 7.3 µm for WT and rad52 cells, respectively (Figure 29, dashed line). Figure 28 shows the distribution of sizes among WT and rad52 cells. All WT cells were between 4 and 8 µm, with a relatively narrow distribution (blue line). In contrast, rad52 cells ranged from 4 to 10 µm in size (red line), indicating much greater heterogeneity in size. The plot distribution seen in Figure 29 was generated in excel. Four column headers were made: Data, Distribution, Average, and Standard Distribution in columns A-D. Data was sorted in column A from lowest to highest using the sort command. The average and the standard deviation of the data set were found by entering excel formulas into cell C2 and D2, =AVERAGE(A2:AX) and =STDDEV(A2:AX). Next, The distribution of each data point was found by entering the following formula into cell B2: = NORMDIST(A2,$C$2,$D$2,FALSE). The formula was then dragged down the B column labelled Distribution beside every data point in the “Data” A column. The data and distribution columns were then selected and a scatter plot was formed from the selected cells.
Figure 29. Cell size distributions for WT (blue) and rad52 (red) large-budded cells.

Homologous recombination-deficient rad52 cells were on average ~1.5 µm larger than WT cells of the same strain background. Dashed lines indicate average sizes of WT and rad52 cells, which were 5.8 and 7.3 µm, respectively.

To gain a better perspective of the distribution on cell sizes for large-budded WT and rad52 cells, the cells were broken down into three size categories, 4.0 – 5.9 µm, 6.0 – 7.9 µm, and ≥ 8 µm (Figure 30). WT cultures had no cells that fell into the ≥ 8.0 µM category, whereas almost 50% of rad52 cells were ≥ 8.0 µM. Interestingly, only 50% of the large-budded rad52 cells were ≥ 8.0 µM and the remaining 50% were the same size as WT cells. If cell enlargement is a response to elevated levels of unrepaired DNA damage, this result implies that only about half of the large-budded rad52 cells have unrepaired damage above the threshold for inducing a cellular response.
Figure 30. Size distributions of large budded homologous recombination-deficient rad52 cells vs. WT cells of the same strain background. Cells were broken down into the following size categories: 4.0-5.9, 6.0-7.9, ≥ 8.0 µm. WT cultures had no cells that fell into the ≥ 8.0 µm category, whereas almost 50% of rad52 cells were ≥ 8.0 µm. One hundred and fifty cells were analyzed for both rad52 and WT cultures.

The previous experiments revealed that rad52 cell cultures have high levels of large-budded cells. Cells’ nuclear morphologies and their proportion of G2 phase vs M phase cells were investigated next. The difference between G2 and M cannot be observed by phase contrast microscopy, but instead has to be visualized with a nuclear stain such as DAPI (4’,6-diamidino-2-phenylindole). DAPI is a blue fluorescent stain that binds
strongly to AT-rich regions of DNA via minor groove binding. It is a planar conjugated molecule excited by light of ~400 nm wavelength (48).

Both WT and rad52 BY4742 cells were grown for four hours so they would be in logarithmic phase growth before being sonicated and formaldehyde-fixed for two hours to preserve nuclear morphologies. After being rehydrated with 70% ethanol for an hour, nuclei were stained with DAPI and treated with ProLong Gold Antifade mountant to prevent the fluorescent signal from photobleaching. After a 24 hour dark fixation period, cells were analyzed microscopically to compare WT and rad52 cells’ nuclear morphologies. In the seminal paper by Weinert and Hartwell (43), treatment with X-rays or UV light caused an increase in large-budded G2 cells with a medial nuclear division (mND) morphology. Large-budded cells were classified into two distinct groups, mND (medial nuclear division morphology) and M (mitosis). Cells at the mND stage of G2 exhibit three possible nuclear structures whereby the nucleus is found near or across the neck of the bud (Figure 31).
Figure 31. Classification of large-budded cells as mND or M cells based on observed nuclear morphologies using DAPI staining. Cartoon example (top) actual DAPI stained cells (bottom) from the current project.

Each imaged field of cells was first analyzed to report on the total number of cells in each field. Example of fields are shown below (Figure 32).
Figure 32. Example of DAPI and transmitted images of WT and rad52 cells. A and B are the DAPI-stained images of WT and rad52 cells, respectively. C and D are the same fields imaged with transmitted light.

All large-budded cells were identified using both the transmitted and DAPI stained images of each field. Nuclear morphologies were then determined and quantified and results are summarized in Table 8. Over 90% of the large-budded rad52 cells exhibited an mND phenotype. These experiments demonstrated that the nuclear structures found in most large-budded rad52 cells were consistent with the findings of past studies on X ray- and ultraviolet light-induced G2 arrest of yeast cells (43).
Table 8. Summary of DAPI staining analysis of *rad52* and WT cells.

<table>
<thead>
<tr>
<th></th>
<th># of fields</th>
<th>Total cells analyzed</th>
<th>Total large-budded cells</th>
<th>Average large-budded cells/field</th>
<th>Average M cells/field</th>
<th>Average mND cells/field</th>
<th>mND cells among large-budded cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>21</td>
<td>298</td>
<td>143</td>
<td>6.5</td>
<td>1.6</td>
<td>4.9</td>
<td>75.4 %</td>
</tr>
<tr>
<td><em>rad52</em></td>
<td>10</td>
<td>297</td>
<td>152</td>
<td>11.7</td>
<td>0.7</td>
<td>11.0</td>
<td>94.0 %</td>
</tr>
</tbody>
</table>
IV. SUMMARY AND CONCLUSIONS

The primary focus of this project was to investigate the observation that mutant yeast cells defective in DNA double-strand break repair have a larger percentage of G\textsubscript{2} cells in liquid cultures than wild type cells. This indicates that these cells are spending more time in G\textsubscript{2} phase. Previous work suggested that \textit{rad52} and \textit{rad51} cells, both defective in DSB repair, have a higher number of G\textsubscript{2} cells than wild type cells (43, 46). These mutants have the same phenotype that normal, healthy cells have after being exposed to DNA damaging agents. We concluded that these DSB repair-deficient cells pause in G\textsubscript{2} phase due to the inability to repair endogenous DNA damage. We speculate that a major source of this damage is caused by Reactive Oxygen Species (ROS) which are a normal, unavoidable byproduct of oxygen metabolism. The unrepaired damage then results in a constitutively activated DNA damage response. This project successfully quantitated the changes in cell morphology and cell cycling in \textit{S. cerevisiae} mutants that are deficient in DNA double-strand break repair via the HR pathway as well as cells that are deficient in both DSB repair and DNA damage checkpoint response.

The first objective of this project was to reproduce Paige Hall’s findings that \textit{rad52} cells have a higher number of G\textsubscript{2} cells than WT cells. It was re-analyzed and observed that WT cell cultures had about 28% G\textsubscript{2}/M cells while \textit{rad52} cells had 50% G\textsubscript{2}/M cells. These results were consistent with her previous findings.

Cell cycle doubling times were calculated for WT and \textit{rad52} cells during logarithmic phase growth. \textit{rad52} mutants had doubling times that were 50% longer than WT cells (128 minutes as compared to 83 minutes). Total cell cycling time (doubling
time) and the percentages of cells in G₁ (unbudded), S (small-budded), and G₂/M (large-budded) during logarithmic phase growth were multiplied to calculate the minutes spent in each phase. rad52 cells spent 2.7 fold more time in G₂ phase than WT cells (63.8 minutes as compared to 23.5 minutes). In contrast minutes spent in G₁ and S were similar in the two strains.

How the percentage of G₂/M cells changes as cells transition from stationary phase, through logarithmic phase, and back to stationary phase growth was investigated. The percentage of large-budded cells continued to increase in rad52 cells until hour 8 where they reached 67%, but WT cells reached a peak of 34% at hour 4.

The possibility that inactivation of other members of the RAD52 epistasis group might also lead to high levels of G₂ cells was also examined. rad51, rad54, rad55, rad57, and rad59 mutants also exhibited higher percentages of G₂ cells than WT cells, with non-overlapping standard deviation error bars. rdh54 mutants were the only cells that did not exhibit a strong increase in G₂ cells. Rdh54 plays a role in homologous recombination in diploid cells and during meiosis (44). Neither haploid nor diploid rdh54 strains exhibited high G₂ cells, suggesting that high levels of unrepaired DNA lesions are not present when this repair protein is absent.

Time spent in G₁, S, and G₂ phase was investigated for RAD52 epistasis group mutants. All mutants displayed elevated doubling times (~90 minutes as compared to ~83 min for WT cells). rad52 cells were different from the others and exhibited a much longer doubling time of 128 minutes.
The length of time RAD52 epistasis group members were spending in each phase of the cell cycle was calculated as done previously for rad52 cells. WT cells spent 24 minutes in G2 phase, rad51, rad54, rad55, rad57, and rad59 cells spent nearly twice as long in G2 (between 39.2 and 46.0 minutes).

Experiments with the MATα mutants showed that rad52 mutant phenotypes were much more extreme than in the other DSB repair mutants (e.g., 128 minute doubling time versus ~90 min for other mutants). New experiments were performed to see if this extreme phenotype that rad52 mutants exhibit would also be observed in BY4741 MATα rad52 mutants. As seen previously in BY4742 cells, the BY4741 rad52 mutants had a more extreme phenotype than rad51 cells, with a doubling time of 115 minutes versus 85 minutes. Doubling times were multiplied by the percent of G2 cells present during logarithmic phase growth to obtain an estimate of the average time cells were spending in G2 phase. BY4741 rad52 cells spent ~2.5 times the amount of time that WT cells spend in G2. This is comparable to the ~2.7 times the amount of time BY4742 rad52 cells spend in G2 phase as compared to WT. These experiments demonstrate that DSB repair-deficient rad51 and rad52 mutants exhibit high G2 cells in both MATa and MATα strains. They also confirm that rad52 cell phenotypes are more extreme than those seen in rad51 cells in both cell types.

To test our hypothesis that rad52 mutants have high levels of G2/M cells due to unrepaired DNA damage and thus a constitutively activated cell cycle checkpoint response, new double mutants were created. The strains created by this procedure were rad52 rad9, rad52 rad17, rad52 rad24, rad52 mec3, rad52 ddc1, rad52 dun1 and rad52 chk1 double mutants. G2/M cells were reduced to WT levels in 5 of the rad52 DNA
damage checkpoint double mutants and to near WT levels in 2 others. These results demonstrate that the high G₂ phenotype observed in rad52 cells is dependent on DNA damage checkpoint genes.

We hypothesized that DSB repair-deficient strains that were coupled with a checkpoint mutation might exhibit stress or slow growth effects when grown over many generations due to an accumulation of unrepaired DNA damage. To test this hypothesis, four rad52⁻ checkpoint⁻ double mutant strains (rad9 rad52, rad17 rad52, rad24 rad52, chk1 rad52) were created that contained a plasmid (pGAL::RAD52) expressing RAD52 via a galactose-regulated promoter. This design allowed the expression of RAD52 to be turned on and off depending on whether or not the growth media contained galactose or glucose (Figure 26). The 4 double mutant strains were streaked repeatedly, for a total of 6 streak plates, corresponding to ~126 generations, but growth rates and colony sizes did not change during the course of the experiment (data not shown). These results indicate that lack of RAD52 and a functional DNA damage response does not affect viability strongly in most of the cells.

The next aim intended to quantify the large, distended phenotype observed in populations of rad52 cells. Homologous recombination-deficient rad52 cells were on average 1.5 µm larger than WT cells of the same strain background. All WT cells were between 4 and 8 µm but rad52 cells range from 4 to 10 µm in size, indicating much greater heterogeneity in size.

The distribution of cell sizes for large-budded WT and rad52 cells were broken down into size categories, 4.0 – 5.9 µm, 6.0 – 7.9 µm, and ≥ 8 µm. WT cultures had no cells that fell into the ≥ 8.0 µm category, whereas almost 50% of rad52 cells were ≥ 8.0
µm. Thus 50% of the large-budded rad52 cells were ≥ 8.0 µm and the remaining 50% were the same size as WT cells. If cell enlargement is a response to elevated levels of unrepaired DNA damage, this result implies that only about half of the large-budded rad52 cells have excessive levels of unrepaired damage.

DAPI (4',6-diamidino-2-phenylindole) staining was used to differentiate between M and G₂ cells in logarithmic phase WT and rad52 cultures. Large-budded cells were classified into two groups, mND (medial nuclear division morphology) and M (mitosis). mND cells were identified by three possible nuclear structures whereby the nucleus is found near or across the neck of the bud. These experiments revealed that 95% of the large-budded rad52 cells exhibited the mND phenotype. Thus, the vast majority of the large-budded cells in log phase rad52 cell cultures are in G₂ phase, not M phase. Half of these G₂ cells are enlarged with a distended phenotype. These observations in rad52 cultures were consistent with the findings of past studies on X-ray- and ultraviolet light-induced G₂ arrest of yeast cells (43).

These experiments lead us to speculate that WT yeast cells are exhibiting a basal level of DNA damage checkpoints that are constitutively activated. We believe the main antagonist of this activation is aerobic metabolism and the natural production of ROS. Instead of taking a set amount of time, G₂ phase could innately consist of a growth phase with an additional varied amount of time for DNA damage repair based on the intensity of DDR signal received from the cell.

Further experiments include growing WT BY4742 cells in YPDA broth in the presence of a strong antioxidant such as N-acetylcysteine. If cells spend less time in G₂ phase than the normal ~24.5 minutes, this could indicate that the ROS species that are a
normal part of aerobic metabolism are influencing the magnitude of checkpoint activation based on DDR response. Thus, the pause in $G_2$ due to DDR checkpoint response could be thought of as an adjustable response instead of an outcome that is on or off.

Humans and other higher level eukaryotes defective in DNA repair may also be exhibiting a constitutive activation of DNA damage checkpoints. Humans that are deficient in their main DSB repair pathway, NHEJ, may have a measurable $G_2/M$ checkpoint similar to yeast.
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