Summer 7-21-2016

The Effects of RAD1 and RAD10 on Mitochondrial Stability in the Saccharomyces cerevisiae

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The Effects of \textit{RAD1} and \textit{RAD10} on Mitochondrial Stability in the \textit{Saccharomyces cerevisiae}

Master’s Thesis
Presented to the Department of Biology
and the
Faculty of the Graduate College
State University of New York: The College at Brockport
In Partial Fulfillment of the Requirements for the Degree
Master of Science in Biology
by
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July 2016

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# Table of Contents

Abstract.................................................................................................................. 4  
Introduction.......................................................................................................... 5  
  *Saccharomyces cerevisiae* as a Model Organism........................................ 5  
  *Saccharomyces cerevisiae* Mating and Reproduction................................. 5  
Mitochondria: The Powerhouse of the Cell......................................................... 6  
Mitochondrial DNA............................................................................................... 7  
Mitochondrial DNA Repair................................................................................... 9  
Mitochondrial DNA Damage.............................................................................. 10  
  Mismatch Repair............................................................................................... 10  
  Base Excision Repair....................................................................................... 13  
  Nucleotide Excision Repair........................................................................... 16  
  Non-homologous End Joining......................................................................... 18  
  Single-Strand Annealing............................................................................... 19  
  Synthesis-Dependent Strand Annealing....................................................... 22  
  Crossing Over.................................................................................................. 24  
  Mitochondrial DNA Mutations Effect on Respiration............................... 26  
Mitochondrial Diseases....................................................................................... 26  
  *RAD1* and *RAD10* Nuclear Genes............................................................ 28  
    *RAD1* and *RAD10* in Nucleotide Excision Repair................................. 29  
    Flap Endonuclease Activity in Single-Strand Annealing........................... 30  
    XPF-ERCC1................................................................................................. 31  
Objectives........................................................................................................... 31  
Materials and Methods......................................................................................... 32  
  Media and Strains........................................................................................... 32  
  Respiration Loss Assay.................................................................................. 34  
  Cytoduction..................................................................................................... 35  
  Direct Repeat-Mediated Deletion Assay....................................................... 37  
  Lea Coulson Method of the Median............................................................... 38  
  Plasmid Isolation.............................................................................................. 39  
  Yeast Transformation of CSY002 with Plasmids E234 and E240............... 40  
  Induced Direct Repeat-Mediated Deletion.................................................... 42  
Results................................................................................................................... 45  
  Respiration Loss Assay.................................................................................. 45  
  Direct Repeat-Mediated Deletion Assay....................................................... 48  
  Induced Direct Repeat-Mediated Deletion Assay......................................... 56  
Discussion............................................................................................................ 62  
  *Rad10Δ* and *Rad1Δ* strains show increased rate of respiration loss compared to wildtype............................................................. 62
Rad1Δ and Rad10Δ strains show no significant difference in mitochondrial DRMD events. ................................................................. 64
Rad1Δ and RAD10Δ strains show a significant decrease in nuclear DRMD events. ......................................................................................... 65
Rad1Δ and Rad10Δ strains show no significant difference in induced DRMD events .................................................................................. 67
References ............................................................................................................................................................................................... 70
Abstract

The mitochondria have long been known as the powerhouse of the cell due to their essential role in oxidative phosphorylation to create energy. Mitochondria have their own genome separate from the nucleus, and may undergo mutations that lead to neuromuscular disease as well as account for some effects of aging. The effects and repair mechanisms of mutations to nuclear DNA have long been studied in order to map out the specific proteins and pathways involved. Nucleotide excision repair is a pathway involving a single-strand break which allows the template strand to be copied after removing the damaged bases. Rad1p and Rad10p are subunits of the nucleotide excision repair factor 1 (NEF1) which cleaves 5′ of the site of damage. Single strand annealing is a repair pathway in which a double-strand break is detected and regions of homology recombine creating 3′ flaps of nonhomology. Rad1p and Rad10p together form a flap endonuclease that specifically cuts at the 5′ incision during single-strand annealing (Bardwell et al. 1994). The goal of this research focuses on determining if the nuclear genes RAD1 and RAD10 play a role in mitochondrial stability. The results of an assay measuring spontaneous respiration loss showed a 1.5 fold increase in rad10Δ strains from wildtype strains and a 1.2 fold increase in rad1Δ strains. The increase in respiration loss of rad10Δ strains was significant with a p value of 0.00003 in a two-tailed t-test. The increase in rad1Δ strain respiration loss was insignificant with a p value of 0.068 in a two-tailed t test. A direct repeat-mediated deletion (DRMD) assay was performed and resulted in a 2.3 fold decrease in nuclear mutation rate in rad10Δ strains compared to wildtype and a 1.8 fold decrease in rad1Δ strains. A two-tailed t-test was performed and indicated that the 2.3 fold decrease in nuclear mutation rates was significant with a p value of 0.0003 for rad10Δ strains. The 1.8 fold decrease in rad1Δ strain mutations was significant with a p value of 0.0005 in a two-tailed t test. The DRMD assay also indicated that there was no significant change in mutation rates in mitochondrial DNA of rad10Δ, or rad1Δ strains compared to wildtype. A two-tailed t-test demonstrated the insignificance of the mitochondrial mutation rate changes, a p value of 0.44 was obtained for rad1Δ and a p value of 0.73 was obtained for rad10Δ. Results from an induced DRMD assay showed a 1.25 fold increase in mutation rates of rad10Δ strains compared to wildtype and a 1.20 fold increase in rad1Δ strains. The increase in both strains was found to be insignificant using a two-tailed t test with p values of 0.063 and 0.052 for rad1Δ and rad10Δ strains respectively. While the results from the respiration loss assay indicate that RAD1 and RAD10 may be involved in maintaining the integrity of the mitochondrial genome, further exploration cannot support this claim. RAD1 and RAD10 are not involved in maintaining the mitochondrial genome through the single-strand annealing pathway it is known to function in within the nucleus. The
genes may be involved in another pathway which was not tested in the assays used in this study such as nucleotide excision repair within the mitochondria.

**Introduction**

*Saccharomyces cerevisiae* as a Model Organism

Yeast is an ideal model organism to study scientifically. Yeast is eukaryotic and therefore many discoveries made using yeast are applicable to humans. Many genes and proteins discovered in yeast have homologs in humans and other model organisms. Along with homology between yeast and other organisms, yeast is also ideal due to their fast life cycle and the fact that they can utilize both aerobic and anaerobic respiration. Colonies arise from a single cell; therefore each cell from a single colony is genetically identical, homoplasmic. Homoplasmy is also present in mitochondrial DNA within cells of *Saccharomyces cerevisiae* (Dujon 1981). This homoplasmy along with its quick life cycle and abundance of human homologs makes it an ideal model organism for mitochondrial DNA research.

*Saccharomyces cerevisiae* Mating and Reproduction

*Saccharomyces cerevisiae* can reproduce by two means, asexually and sexually. In the absence of other strains of yeast, a colony will maintain a haploid nature. These cells asexually divide by budding and are more susceptible to high stress conditions than diploid cells. When pheromones from a different strain are detected, the colonies begin to grow towards each other in order to reproduce.
sexually. When the nuclei fuse, the cells may divide by mitosis until nutrient sources become depleted and the colonies begin to starve. Once the colonies experience a lack of nutrients, they undergo meiosis and form spores that will begin growing in areas with more resources available. If the spores do not become relocated in an area with enough nutrients for survival, the spores remain dormant until conditions become more favorable.

**Mitochondria: The Powerhouse of the Cell**

The mitochondria of a cell are essential to its functioning and survival. In budding yeast, *Saccharomyces cerevisiae*, energy can be created through oxidative phosphorylation or through fermentation. Oxidative phosphorylation is a more efficient method of producing adenosine triphosphate (ATP) by aerobically catabolizing carbon sources. Oxidative phosphorylation is the last step in cellular respiration which uses the electron transport chain to produce approximately 32 molecules of ATP. Only certain carbon sources can be used in oxidative phosphorylation due to the intermediates required in pyruvate formation.

Dextrose is the preferred carbon source in glycolysis; other sources may be modified before being utilized. Glycerol is a non-fermentable carbon source which yeast undergoes glycolysis using. Yeast are unable to undergo fermentation using glycerol. Fermentation occurs when there is no oxygen present and creates 2 molecules of ATP and a waste byproduct such as ethanol or lactic acid. Despite the
difference in efficiency, *Saccharomyces cerevisiae* preferentially undergo fermentation in the presence of high dextrose concentrations even in the presence of oxygen.

**Mitochondrial DNA**

Mitochondria are only found in eukaryotes and are inherited exclusively from the mother. The mitochondria are unique in that they contain their own genome which replicates and is activated separately from the nuclear genome. Mitochondrial DNA also differs from nuclear DNA in that there is only one copy of the nuclear genome per cell; mitochondrial DNA may contain thousands of copies of the genome per cell (Holt & Reyes 2012). Despite the abundance of copies of mtDNA, cells remain homoplasmic, meaning they propagate identical copies to each of their offspring. Even in the event of a mutation which creates heteroplasmy, the heteroplasmic state is transient. As the cell line ages and undergoes divisions the majority of cells will return to a homoplasmic state (Dujon 1981). The presence of a mitochondrial genome along with a membrane surrounding the organelle is supportive of the endosymbiotic theory of life. The endosymbiotic theory of life states that mitochondria and chloroplasts likely began as organisms that were engulfed by prokaryotic cells.

Although the proteins encoded by mitochondrial DNA (mtDNA) are used primarily in the electron transport chain, the majority of proteins localized to the
mitochondria are translated from nuclear DNA. Mutations in mtDNA cause respiration deficient mutants due to their inability to utilize the electron transport chain in oxidative phosphorylation. MtDNA is found in a form of nucleoids and is copied by DNA polymerase gamma. Despite the knowledge that DNA polymerase gamma is responsible for mtDNA replication, the mechanisms are not understood completely as of yet. Hypotheses of leading and lagging strand replication are the current model for mtDNA replication involving mitochondrial DNA polymerase gamma, Twinkle DNA helicase, mitochondrial RNA polymerase, single-stranded DNA binding protein, RNase H1, DNA ligase III, and topoisomerases (Holt & Reyes 2012).

The human mitochondrial genome is 16.5 kilobases (kb) long while the yeast mitochondrial genome has been found to be 85.8 kilobases long (Holt & Reyes 2012, Langkjaer 2003). The noncoding regions of human mtDNA are found primarily localized to one region, this region contains the majority of variances in human mtDNA sequences (Holt & Reyes 2012). The human mtDNA sequence is known to encode 13 proteins involved in oxidative phosphorylation along with transfer and ribosomal RNAs utilized in the transcription of those proteins (Holt & Reyes 2012). The yeast mtDNA encodes the three subunits of cytochrome c oxidase (COX1, COX2, and COX3) along with other proteins, 24 tRNAs, as well as the small and large ribosomal RNAs (Langkjaer 2003). Figure 1 shows similarities and differences between human and *Saccharomyces cerevisiae* mtDNA. The yeast genetic code varies from the universal code in three codons, one being that AUA is translated to
Seger instead of the typical AUG (Langkjaer 2003).

Mitochondrial DNA Repair

The repair pathways of nuclear DNA have been understood and mapped out previously to include mechanisms which repair single-strand breaks, double-strand breaks, and replication dependent events. Single-strand breaks may be repaired by a number of mechanisms such as nucleotide excision repair, base excision repair, and mismatch repair. Double-strand breaks may be repaired through non-homologous end joining, crossing over, single-strand annealing, and synthesis-dependent strand annealing. Replication dependent mutation events may be due to
DNA polymerase slippage or template switching. DNA repair mechanisms are not without error and may repair damage while still causing a mutation.

Mitochondrial DNA Damage

The repair pathways present in mtDNA are only more recently being discovered along with the genes encoding the required enzymes. The six main sources of mtDNA damage are alkylation, strand breaks, mismatched bases, hydrolytic damage, formation of adducts, and oxidative damage (Alexeyev et al. 2013). Oxidative damage is one of the most detrimental to the cell because it is likely to happen often in the mitochondria. The proximity to the electron transport chain which creates reactive oxygen species (ROS) causes oxidative damage to occur most frequently. Mitochondria have been shown to have a reduced number of repair pathways as evidenced by their inability to repair UV-induced pyrimidine dimers and some types of alkylation damage (Alexeyev et al. 2013).

Mismatch Repair

Mismatch repair involves detection of an incorrectly paired base, removal of the incorrect base and adjacent sequences, resynthesis, and ligation (see Figure 2). Mismatch repair mechanisms are conserved from the nucleus to the mitochondria and is involved in repairing errors due to slippage of DNA polymerase as well as spontaneous and induced base deamination (Alexeyev et al. 2013). Current research indicates that the proteins involved in mismatch repair in the mitochondria
are not the same as those required for the mechanism in the nucleus. The Y-box binding protein is responsible for detecting mismatches in the mitochondria rather than MSH2, MSH3, and MSH6 (MUTSα or MUTSβ) (Alexeyev et al. 2013). MLH (MUTL) initiates excision of the mismatched base and adjacent sequences for resynthesis. The mitochondria reduce the number of oxidized deoxyribonucleotide triphosphates (dNTPs) by means of MTH1 which hydrolyzes the oxidized dNTPs. By containing MTH1, the mitochondria prevent the incorporation of oxidized dNTPs into DNA and reduce the amount of mismatched bases created (Nakabeppu, 2001).
Figure 2. **Mismatch Repair Pathway.** The mismatch repair pathway is employed when DNA polymerase slippage, spontaneous base deamination, or induced base deamination occurs. The incorrectly based pair and adjacent sequences are removed following detection by MUTSα or MUTSβ. MUTLα initiates excision and recruits necessary proteins for resynthesis. The newly synthesized section is then ligated and mismatch repair is complete.

**Base Excision Repair**

Single-strand break repair pathways are present in the mitochondria and are often considered a subclass of base excision repair (BER) (Alexeyev et al. 2013). Base excision repair is the preferred pathway for the most common oxidative damage occurring in the mitochondria. The proficiency of the mitochondria in BER is vital to its ability to repair damage caused by ROS. The mitochondria are more efficient at repairing 8-oxodG, the most prominent oxidative damage, than the nucleus (Alexeyev et al. 2013). Base excision repair is divided into two types, short-patch and long-patch.

Both short-patch BER and long-patch BER have three steps: damage recognition/strand scission, gap tailoring, and DNA synthesis/ligation. In short-patch BER, a DNA glycosylase excises the damaged base and creates an abasic site. An apurinic/apyrimidinic endonuclease incises the abasic site to create a 5' deoxyribosephosphate (5'-dRp). XRCC1 along with AP endonuclease (APE) create a 5' nick removing the 5'-dRp. DNA polymerase β synthesizes a new complementary segment and DNA ligase ligates the new segment, (see Figure 3) (Alexeyev et al. 2013).

Long-patch BER is used most commonly to repair 5' blocking groups such as 5' deoxyribonolactone (5'-dL). Creation of this lesion may be as high as 72% of total oxidative sugar damage in DNA upon exposure to radiation including a base loss.
The 5' blocking group is removed by DNA polymerase to create a flap. The flap is removed by two nucleases to create the nicked DNA duplex. The nicked duplex can be ligated by DNA LIG3 (Alexeyev et al. 2013).
Figure 3. **Base Excision Repair.** Base excision repair is initiated by a DNA glycosylase (G) which excise the damaged base creating an abasic site. An apurinic/apyrimidinic endonuclease incises the abasic site creating a 5’ deoxyribosephosphate (5’-dRp). DNA polymerase β excises the 5’-dRp and synthesizes a new complementary segment. DNA ligase ligates the new segment completing base excision repair.

Nucleotide Excision Repair

The nucleotide excision repair pathway has yet to be documented in the repair of mitochondrial DNA. Nucleotide excision repair is typically employed to repair bulky lesions caused by UV exposure. These lesions may be repaired by another mechanism in the mitochondria but no alternative pathway has been described to date (Alexeyev et al. 2013). Nucleotide excision repair begins by detection of lesions by a nucleotide excision repair factor composed of multiple proteins. The DNA is unwound to form an open complex of the lesions and surrounding bases. The Rad1p/Rad10p complex makes an incision 5' to the damage; Rad2p makes an incision 3' to the damage. A portion of the DNA 25-30 bases long is excised, DNA polymerase resynthesizes the excised portion, and DNA ligase ligates the newly synthesized portion (see Figure 4).

Single-strand break repair is especially crucial to mtDNA integrity. When exposed to ROS, mtDNA experiences single-strand breaks at 1000 fold increased frequency compared to double-strand breaks (Alexeyev et al. 2013).
Figure 4. **Nucleotide Excision Repair Pathway.** Nucleotide excision repair removes bulky lesions such as those created by exposure to UV light. The thymine dimers are recognized by nucleotide excision repair complexes and a portion of the DNA is unwound. Rad1p/10p are involved in nucleotide excision repair factor 1, acting as endonucleases which cleave the 5’ side of the lesion. Rad2p performs the incision on the 3’ region of the lesion. The damaged portion plus 25-30 bases are removed and the gap is resynthesized.

Non-homologous End Joining

Double-strand breaks are less common than single-strand breaks when mtDNA is exposed to ROS. In the nucleus, double-strand breaks may be repaired through non-homologous end joining, synthesis-dependent strand annealing, crossing over, and single-strand annealing. Non-homologous end joining begins when a double-strand break is detected such as those in collapsed replication forks. The break is connected when the Ku70/Ku80 heterodimer attaches to the ends of the breaks and recruits DNA-dependent protein kinase, catalytic subunit (DNA-PKcs). This mechanism ligates ends bound by Ku70/Ku80 regardless of whether the bound ends are from the same chromosome (see Figure 5). If portions of one strand have been degraded, the ends will join and excise the bases not paired which can lead to deletions (Sancar et al. 2004). Non-homologous end joining has been observed in Ku deficient cells which signifies other genes must be involved in binding the ends of the double-strand breaks for ligation (Coffey et al. 1999).
Single-Strand Annealing

Single-strand annealing is a repair pathway associated with information loss.

When a double-strand break occurs in or near regions of homology, the regions of
non-homology are digested to create an overlap of the regions of homology. The regions of homology align and create 3' flaps of non-homology. The flaps of non-homology are removed by an endonuclease such as the Rad1p/Rad10p complex. The Rad1p/Rad10p complex removes the 3' tails of non-homology, DNA polymerase fills gaps, and then DNA ligase attaches the fragments (see Figure 6). The digestion of regions of non-homology makes this mechanism not conservative (Ivanov and Haber 1995).

This form of homologous recombination differs from others. Single-strand annealing does not require two separate molecules of DNA; instead it repairs the DNA by recombining two sequences on the same strand of DNA. By contrast, synthesis-dependent strand annealing and crossing over require exchange between two molecules of DNA. This distinction of only needing one DNA duplex potentially make SSA more conservative depending on the distance of the regions of homology utilized compared to crossing over.
Figure 6. **Method of action of single-strand annealing involving Rad1 and Rad10.** The grey shaded portions represent repeat regions of homology. When a double-strand break occurs in between two regions of homology an exonuclease may degrade portions of the DNA until flanking regions are adjacent. A flap of nonhomology is formed on each strand, this flap is cleaved by the Rad1p/10p endonuclease complex. Synthesis is performed by DNA polymerase to join the breaks and maintain a functional double-stranded DNA.

Synthesis-Dependent Strand Annealing

Synthesis-dependent strand annealing is a form of homologous recombination in which the regions flanking the double-strand break are conserved. A double-strand break is detected and end resectioning is performed by MRN, BLM, CtIP, EXO1, and DNA2. End resectioning reveals 3' single-stranded portions of DNA which RPA binds to prior to RAD51 paralog recruitment by BRCA1/BRCA2. RAD51 filament formation proceeds strand invasion and D-loop formation. A D-loop is formed by invasion of the double-strand break without forming Holliday junctions. The D-loop provides a template for synthesis of missing sections of DNA which causes gene conversion (see Figure 7). The newly synthesized strand dissociated from the template strand without crossing over occurring (Panier and Boulton, 2014). This conservative model does not cause deletions or exchange of fragments of DNA (Miura et al. 2012).
Figure 7. Synthesis-Dependent Strand Annealing. Double-strand DNA breaks may be repaired through synthesis-dependent strand annealing (SDSA). SDSA begins similarly to homologous recombination with or without crossing over. Exonuclease activity resects the ends to create 3' ssDNA for RPA to bind to. Once RPA is bound, RAD51 paralogs along with BRCA1 and BRCA2 replace RPA with RAD51. A D-loop is formed by strand invasion, resynthesis occurs, and the invading strand is displaced.

Crossing Over

Crossing over is a form of homologous recombination in which homologous regions on chromosomes recombine and may exhibit chromosomal crossover or gene conversion. A double-strand break may be resolved by the formation of a D-loop, strand displacement, and a Holliday junction formation. A Holliday junction occurs when double strand displacement creates two junctions between homologous chromosomes. A double-strand break repaired through crossing over follows the same initial steps as synthesis-dependent strand annealing. Synthesis-dependent strand annealing and crossing over diverge following the D-loop formation.

Crossing over involves strand invasion and D-loop formation followed by formation of a double Holliday junction. Following synthesis based on template strands the junctions may resolve to create regions of crossover between the two chromosomes (see Figure 8). In the event that crossover does not occur between the two chromosomes, gene conversion occurs where sections of DNA are replaced with identical sequences to the homologous chromosome leading to loss of heterozygosity (Miura et al. 2012).
Figure 8. Homologous Recombination with and without crossing over. Homologous recombination occurs following a double-strand break in the DNA. Endonucleases are recruited to resect the ends for RPA to bind. RAD51 paralogs facilitate the assembly and stabilization of the RAD51 filament complex. A double Holliday junction is formed, the junction may be dissolved, resolved with crossover, or resolved without crossover.

Mitochondrial DNA Mutations Effect on Respiration

In the mitochondria, double-strand breaks can be repaired by nonhomologous-end joining (NHEJ) or homologous recombination (HR). Although Drosophila melanogaster have an efficient pathway for repairing bleomycin-induced double strand breaks in the mitochondria, a similar pathway in mammalian cells has not yet been discovered (Alexeyev et al. 2013). The current understanding of NHEJ and HR in mitochondria is incomplete but there is evidence that supports their presence. Homologous recombination has been implicated in experiments in which double strand breaks were repaired and large mtDNA deletions were observed (Alexeyev et al. 2013).

Petite colonies may arise from two main forms of deletions which cause respiration deficiency. Petite colonies are notable by their smaller than average size. One form of mutation common among petite colonies is the loss of the mitochondrial genome (rho₀). The other form of mutation is a combination of point mutations or deletions (rho⁻). Studies have found that a subset may arise through direct repeat mediated deletions (Dujon 1981). The redundancy of mtDNA allows the repair pathways to be less critical, the cell can still live if the damage is not repaired; if damage is not repaired the mtDNA molecule is frequently degraded (Alexeyev et al. 2013).

Mitochondrial Diseases
The mitochondrial genome is very small compared to the nuclear genome. The mitochondrial genome is inherited maternally and each cell contains thousands of copies rather than one. Genetic mutations in the mitochondrial genome have been linked with aging, cancer, and many other diseases. Mitochondrial diseases may be caused by nuclear DNA defects in genes encoding mitochondrial metabolism or mtDNA maintenance (Taylor and Turnbull 2007).

Symptoms associated with mitochondrial disease are extremely variable but many are found in organs with high energy demands. Both Alzheimer's disease and Parkinson's disease have been linked to damage caused by reactive oxygen species. It has been difficult to prove a higher disposition to such diseases based on mitochondrial mutations. Current studies have shown both a significance and insignificance in expression of clinical symptoms. The inconsistency of different studies makes it difficult to pinpoint what effects mitochondrial mutations have in diseases (Taylor and Turnbull 2007).

The homoplasmic nature of mtDNA along with its polyploidy account for variable differences observed in symptom presentation. A certain threshold of mutations must be reached before wildtype activity is diminished by mutant activity (Zeivani and Di Donato 2004). The first maternally inherited disease to be linked to an mtDNA point mutation was Leber Hereditary Optic Neuropathy (LHON). LHON is a disease which occurs in mostly young males in which a rapid painless loss of vision
in both eyes occurs. The vision loss is not simultaneous but progressive from one
eye to the other. Of the worldwide patients with LHON, approximately 90% contain
one of 3 most prevalent point mutations. The pathogenic mutations in LHON have
all been shown to be a part of complex I of the electron transport chain (Zeivani and
Di Donato 2004).

These mutations in mtDNA and nuclear DNA responsible for the stability of
mtDNA have been associated with multiple diseases and symptoms. The
redundancy of mtDNA makes it easier for cells to continue functioning while some
copies contain these mutations. Once the level of mutants reaches a certain
threshold symptoms displayed increase and are often localized.

*RAD1* and *RAD10* Nuclear Genes

Some nuclearly encoded proteins are known to be present in the
mitochondria; in fact, the majority of proteins localized to the mitochondria are
encoded by nuclear genes. Two nuclear genes of interest are *RAD1* and *RAD10*
which work in a complex during single-strand annealing and nucleotide excision
repair. *RAD1* and *RAD10* were discovered in a UV irradiation experiment of
*Saccharomyces cerevisiae*. The experiment by B.S. Cox and J.M. Parry concluded
that radiation sensitivity experienced by cells which lost the function of 22-30
proteins made cells more susceptible to UV damage. Along with *RAD1* and *RAD10*,
Cox and Parry also mutated loci within *RAD2, RAD3, RAD4, RAD5, RAD6, RAD7,*
Seger

RAD9, RAD10, RAD14, RAD16, and RAD17 which caused cells to display radiation sensitivity (Cox and Parry, 1968). The protein encoded by RAD1 is 1100 amino acids long and located on the short arm of chromosome 16 (RAD1/YPL022W, SGD). The protein encoded by RAD10 is 210 amino acids long and located on the long arm of chromosome 13 (RAD10/YML095C, SGD). The Rad1p-Rad10p complex is homologous to the XPF-ERCC1 complex in humans (Prakash & Prakash 2000).

**RAD1 and RAD10 in Nucleotide Excision Repair**

There are two classes of genes involved in nucleotide excision repair in yeast: Class 1 and Class 2. Class 1 genes such as RAD1 and RAD10 confer a very high UV radiation sensitivity when their function is lost. There are four complexes involved in nucleotide excision repair, nucleotide excision repair factors 1-4 (NEF1-4). Rad1p and Rad10p are subunits of NEF1 along with Rad14p (Prakash and Prakash 2000).

NER begins when a bulky lesion such as a thymine dimer is created by UV radiation. Nucleotide excision repair factors 1 and 4 along with RPA are involved in damage recognition. The damaged DNA is then unwound and an incision is created on either side of the lesion and the adjacent bases removing approximately 25-30 bp (see Figure 4). DNA unwinding creates a bubble including the damage site, the Rad1p-Rad10p complex is able to recognize this bubble and remove the damaged bases (Prakash & Prakash 2000). Rad1p and Rad10p make the incision 5' to the
damage while Rad2p makes the incision 3' to the damage. DNA polymerase fills in
gaps and DNA ligase connects the fragments (Davies et al. 1995).

*RAD1* and *RAD10* are involved in damage recognition and endonuclease
activity of NER. Cells with decreased function or loss of function of these genes
experience a greater sensitivity to UV radiation due to their decreased ability to
repair lesions.

**Flap Endonuclease Activity in Single-Strand Annealing**

Rad1p and Rad10p function in a complex as a flap endonuclease which
cleaves the 3' tails of nonhomology in single-strand annealing. Spontaneous
deletions commonly occur in direct repeat sequences in DNA, these sequences are
sites where a double-strand break (DSB) may occur. Single-strand annealing (SSA) is
a process of double-strand break repair. After degradation by an exonuclease
exposes regions of homology, annealing occurs. The annealing creates a duplex
region as well as 3' single stranded flaps of nonhomology. To repair the
recombination site, flanking direct repeats are removed as well as intervening
sequences (Ivanov and Haber 1995). Nicking to remove the 3' tail occurs exclusively
on the 3' strand in duplex region of duplex single-stranded junctions (Bardwell et al.
1994). After removal of the 3' tail, extension by DNA polymerase occurs indicating
that the Rad1p-Rad10p complex creates a 3’-OH group in its products (Bardwell et
al. 1994).
Single-strand annealing is a repair pathway which may cause large deletions between regions of homology. Although these deletions may be disparaging if too large, they are superior to not repairing the break if other mechanisms are unable to.

**XPF-ERCC1**

The XPF-ERCC1 complex in humans is homologous to the Rad1p-Rad10p complex in yeast which is required for single-strand annealing and nucleotide excision repair. Although XPF-ERCC1 is homologous to Rad1p-Rad10p, it does not act in the same pathways. NER is conserved among yeast and humans but SSA has not yet been identified in humans. XPF is the catalytic domain of the complex which cleaves the 3’ strands of nonhomology (Ahmed et al. 2008). ERCC1 is required in the complex in order to bind DNA and maintain stability while catalytic cleavage is occurring. The incision in NER occurs 5’ to the damage; the product contains a 3’-OH group which allows extension by DNA polymerase (Ahmed et al. 2008). The second repair pathway XPF-ERCC1 is involved in is an error-prone Ku86p (XRCC5) independent pathway utilized to repair oxidative stress and ionizing radiation (Ahmed et al. 2008). The mechanisms of this Ku86p independent pathway are not yet known but are thought to be similar to microhomology-mediated end joining.

**Objectives**
The objective of this project is to determine if RAD1 and RAD10 are integral to maintaining mitochondrial DNA stability. The gene products are known to work in both nucleotide excision repair and single-strand annealing to maintain nuclear DNA integrity in Saccharomyces cerevisiae. The human homologs XPF and ERCC1 work similarly in nucleotide excision repair and a pathway similar to microhomology-mediated end joining. Through a series of assays involving strains with loss of function of either RAD1 or RAD10 this project aims to determine their role in mitochondrial DNA repair and stability. Subsequently from the results regarding mitochondrial DNA stability in these cells, potential DNA repair pathways utilized in the mitochondria may be deduced.

**Materials and Methods**

**Media and Strains**

Media used in this study includes YPG+0.2% dex, YPG, YPD, SD-ARG-URA, SD-ARG, SD-TRP, and SRaff-ARG-URA. YPG is made with 20 g agar, 20 g bacto-peptone, 10 g yeast extract, and 900 mL water. The solution is autoclaved and 100 mL of 20% glycerol is added following removal from the autoclave. YPD is also made with 20 g agar, 20 g bacto-peptone, 10 g yeast extract, and 900 mL water. Following removal from the autoclave, 100 mL 20% dextrose is added to YPD media. Alternately, 20 g of dextrose may be added prior to autoclaving and 1L of water added rather than 900 mL. The synthetic medias SD-ARG, SD-ARG-URA, SD-TRP, and SD-URA only differ
in the amino acid mixture used. All the aforementioned synthetic medias contain 5 g ammonium sulfate, 1.7 g yeast nitrogen base without amino acids and ammonium sulfate, 20 g agar, 20 g dextrose, and appropriate amounts of complete supplement mixture (CSM) with desired amino acids omitted. One liter of water is added to the ingredients and autoclaved. SRAff-ARG-URA is made with 2.5 g ammonium sulfate, 0.85 g yeast nitrogen base without amino acids and ammonium sulfate, the appropriate amount of CSM-ARG-URA, and 10 g of Raffinose. The above ingredients are mixed with 500 mL of water and autoclaved. Raffinose is a trisaccharide composed of galactose, glucose, and fructose.

Wildtype, rad1Δ, and rad10Δ strains used in each assay are listed below in Table 1. DRMD rad10Δ strains were created through cytoduction of LKY797 which contained the nuclear reporter and NRY066 which contained the mitochondrial reporter (see Figure 14 for reporter). Similarly, rad1Δ DRMD strains were created through cytoduction of LKY793 with NRY066. Induced DRMD strains were created by transforming DRMD strains with the plasmids E234 and E240 which has a pYES2.1 backbone as shown in Figure 17.

<table>
<thead>
<tr>
<th>Strains Used</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>Respiration Loss</td>
<td></td>
</tr>
<tr>
<td>DFS188</td>
<td>MATa ura3::52 leu2-3,112 ly2 his3 arg8::hisG</td>
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<tr>
<td>NRY201</td>
<td>DFS188 rad10Δ::KANMX</td>
</tr>
<tr>
<td>NRY203</td>
<td>DFS188 rad1Δ::KANMX</td>
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<tr>
<td>DRMD</td>
<td>DFS188 Rep96::ARG8::cox2 Rep96::URA3::trp1</td>
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<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------</td>
</tr>
<tr>
<td>CSY001</td>
<td>DFS188 rad10Δ::KANMX Rep96::ARG8Δ::cox2 Rep96::URA3::trp1</td>
</tr>
<tr>
<td>CSY002</td>
<td>DFS188 rad10Δ::KANMX Rep96::ARG8Δ::cox2 Rep96::URA3::trp1</td>
</tr>
<tr>
<td>CSY012</td>
<td>DFS188 rad1Δ::KANMX Rep96::ARG8Δ::cox2 Rep96::URA3::trp1</td>
</tr>
</tbody>
</table>

**Induced DRMD**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSY080</td>
<td>LKY196 Rep96::ARG8Δ::cox2 E234</td>
</tr>
<tr>
<td>CSY086</td>
<td>LKY196 Rep96::ARG8Δ::cox2 E240</td>
</tr>
<tr>
<td>CSY064</td>
<td>LKY196 rad10Δ::KANMX Rep96::ARG8Δ::cox2 E234</td>
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<tr>
<td>CSY066</td>
<td>LKY196 rad10Δ::KANMX Rep96::ARG8Δ::cox2 E234</td>
</tr>
<tr>
<td>CSY068</td>
<td>LKY196 rad10Δ::KANMX Rep96::ARG8Δ::cox2 E240</td>
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<td>LKY196 rad1Δ::KANMX Rep96::ARG8Δ::cox2 E234</td>
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</tr>
<tr>
<td>CSY102</td>
<td>LKY196 rad1Δ::KANMX Rep96::ARG8Δ::cox2 E240</td>
</tr>
</tbody>
</table>

Table 1. Strains listed by name and assay for wildtype, rad1Δ, and rad10Δ.

**Respiration Loss Assay**

The strains used in this assay are the wildtype respiring DFS188, rad10Δ strain NRY201, and rad1Δ strain NRY203. The assay begins with cells cultured on agar containing yeast extract, peptone, and glycerol (a non-fermentable carbon source) for one day at 30°C. From this patch of cells, streak plating is performed on an agar plate containing yeast extract, peptone, and dextrose (a fermentable carbon source) for three days at 30°C. The use of glycerol in the patched plate ensures each colony is capable of undergoing oxidative phosphorylation. The use of dextrose in the streaked plate allows for non respiring cells to grow if the originally respiring cells lose function. Fifteen individual colonies undergo a serial dilution in sterilized deionized water (DI) creating a final dilution factor of $10^{-5}$. From the serial dilution, 50μL of each colony is spread on an agar plate containing yeast extract, peptone, glycerol, and 0.2% dextrose (YPG+0.2%dex) and grown for three days at 30°C. After

Seger 34
the three day grown period two phenotypes are visible, smaller colonies (petite) and larger colonies (grand), see Figure 9. The petite colonies arise from non-respiring cells which use the dextrose available to them and then arrest growth. The grand colonies are respiring cells which are able to ferment the dextrose as well as use glycerol in oxidative phosphorylation. The percent respiration loss is measured as the number of petite colonies divided by the total colonies on each plate. The median is used for each trial instead of the average of respiration loss. Multiple trials were performed with both the mutant and wildtype strains; the averages of the medians were calculated and the standard deviation described the amount of error between trials of each strain.

![Figure 9. Respiration Loss assay protocol.](image)

A cytoduction between LKY797 and NRY66 was required to create a rad10Δ strain containing both the nuclear and mitochondrial reporters. For rad1Δ strains, LKY793 is used in place of LKY797. LKY797 was grown on a synthetic dextrose
medium lacking uracil (SD-URA) and NRY66 was grown on a synthetic dextrose medium lacking arginine (SD-ARG) at 30°C for one day. Cells from each patch were inoculated into a broth containing yeast extract, peptone, and dextrose (YPD) and grown overnight at 30°C. An equal amount of cells were taken from the overnight cultures of NRY66 and LKY797 and grown for 6 hours at 30°C on YPD medium. By growing the cells together on YPD they were able to detect the opposite pheromones and grow towards each other. Once opposite genotypes were next to each other they were able to fuse nuclei; the YPD media ensured that both LKY797 and NRY66 were able to grow.

After the six hour growth period, cells were taken from the YPD media and streaked for singles on a synthetic dextrose medium lacking adenine and arginine (SD-ADE-ARG) and grown for three days at 30°C. The streaked SD-ADE-ARG plate was replica plated onto a synthetic dextrose medium lacking uracil and arginine (SD-URA-ARG) and a synthetic dextrose medium lacking lysine (SD-LYS). The desired phenotype of the colonies was URA+ARG+LYS+, therefore colonies that grew on SD-URA-ARG and not on SD-LYS were patched onto a synthetic dextrose medium lacking arginine and uracil (SD-URA-ARG). The patched cells grew for two days at 30°C before being frozen down in 20% sterile glycerol. The rad10Δ strains created through cytoduction containing the mitochondrial and nuclear reporter were named CSY001-CSY008 and are used in the DRMD assay.
Direct Repeat-Mediated Deletion Assay

The strains used in this assay are the wildtype LKY196, the rad10Δ strains CSY001 and CSY002, and the rad1Δ strain CSY012. The assay begins with patched cells which are grown for one day on SD-URA-ARG at 30°C. From the patches, cells are streaked for singles onto YPD and allowed to grow for three days at 30°C. Fifteen individual colonies are diluted in serial dilutions in DI water to a final concentration of 10⁻⁵. From the first dilution, 95μL of cells are plated onto synthetic dextrose medium lacking tryptophan (SD-TRP). From the second dilution, 100μL of cells are plated onto a medium containing yeast extract, peptone, and glycerol (YPG). Finally, 50μL of cells and 50μL of DI water are plated onto YPD medium. There are three plates that contain the cells from each individual colony. The plates are allowed to grow for three days at 30°C before counting. The total number of colonies on the YPD plates and SD-TRP plates are counted while only the larger colonies are counted on the YPG plates, see Figure 10. The YPG plates contain a lot of background noise which must be excluded while counting.

From the YPD plates a total cell count can be calculated by multiplying the average by the dilution factor of 20000. The SD-TRP plates allow the calculation of the rate of nuclear recombination with the use of the Lea Coulson method of the median. Cells that grow on SD-TRP plates have undergone nuclear recombination. The nuclear reporter contains the URA3 gene which when removed leaves a
functional TRP1 gene. Similarly, from the YPG plates, the rate of mitochondrial recombination can be calculated using the Lea Coulson method. Colonies that grow on the YPG plates have undergone recombination to remove the ARG gene from the mitochondrial reporter. When the ARG gene is removed, a fully functional COX2 gene is remaining and therefore those cells which have undergone this recombination can grow on YPG by utilizing respiration. Rates for each trial were averaged; the error was determined using the standard deviation.

Lea Coulson Method of the Median

The spontaneous mutation theory of parallel cultures allows a qualitative analysis; a quantitative analysis was first established by D. E. Lea in 1949 (Lea & Coulson 1949). Luria and Delbruck originally hypothesized that mutants in bacterial
cultures arise before contact with a bacteriophage. The hypothesis was supported by the wide distribution of mutants in parallel cultures caused by the difference in when the mutations occurred in each parallel culture (Luria, Delbruck, Anderson 1943). The Lea Coulson method of the mean incorporates information about both new mutants in a culture as well as the descendents of old mutants. A median is taken of the number of mutants in each parallel culture to estimate the distribution. From the calculated median ($r_0$), the mean number of mutations ($m$) can be calculated using the equation $r_0/m - \log (m) = 1.24$. After calculating $m$, calculations involving the table created by Lea give a rate of mutations derived from the median (Lea & Coulson 1949). The table created by Lea expands upon the qualitative analysis provided by Luria and Delbruck to create a quantitative analysis. Mutation rates may be inferred from parallel cultures and these mutation rates can be further analyzed by statistical methods such as the t-test to determine their significance.

**Plasmid Isolation**

*E. coli* cells containing each of the desired plasmids were grown overnight at 30°C in 1.5 mL of lysogeny broth containing the antibiotic ampicillin (LB+AMP). Cells from the overnight culture were centrifuged for 11 seconds to pellet and resuspended in 100µL of GTE buffer (50mM glucose, 25mM Tris-HCl, pH 8, 10mM EDTA). To the resuspended cells, 200 µL NaOH/SDS lysis solution (0.2M NaOH, 1% SDS) was added and vortexed, this mixture sat at room temperature for five minutes
before proceeding. After the five minutes, 150µL of KOAc lysis solution was added (5M KOAc, 2M Acetic acid). This new mixture was vortexed and allowed to sit for five minutes at room temperature before proceeding. After the five minutes, 400µL of phenolchloroform solution, pH 7, was added and the new mixture was vortexed. The mixture was centrifuged for eight minutes to pellet chromosomal DNA and cell debris.

After centrifugation, approximately 700 µL of the supernatant was removed and transferred to a new tube. To the supernatant, 1 mL of 100% ethanol was added and vortexed. The supernatant and ethanol were left at -20°C for fifteen minutes. After the fifteen minute period, the supernatant and ethanol were centrifuged at room temperature for eight minutes to pellet the DNA. After centrifugation, the DNA was washed once with 200 µL of 70% ethanol. The mixture was centrifuged again for three minutes after which the 70% ethanol was removed by aspiration. Following aspiration, the pellet of DNA was dried in a speedy vac for five minutes. The dried pellet was resuspended in 50 µL of 100 µg/mL RNAse in TE, and then incubated at 37°C for fifteen minutes. After incubation, the resuspended pellet of plasmids was stored at -20°C until needed.

Yeast Transformation of CSY002 with Plasmids E234 and E240

To begin the transformation of strains required for the induced DRMD assay, individual colonies were selected from SD-TRP plates of a DRMD assay using CSY002
and LKY196. These colonies were grown for one day at 30°C on a plate containing a synthetic dextrose medium lacking arginine (SD-ARG). A small amount of cells from each patch plate were inoculated separately into 3 mL of YPD broth and grown at 30°C overnight. Each overnight culture was diluted 1:50 in YPD broth and grown to mid-log phase at 30°C (approximately three hours). For each transformation, 50 mL of culture was spun down for 10 minutes in a centrifuge and the supernatant was removed. After removing the supernatant, the cultures were resuspended in 1 mL DI water and transferred to a new tube.

After resuspension, the cells were spun down in a microfuge for 7 seconds and the supernatant was removed by aspiration. The pellet of cells was resuspended in 500 μL of 0.1M LiOAC in TE. For each transformation, 100 μL of cells, 15 μL salmon sperm DNA (10mg/mL), and 5 μL of either E240 or E234 were added to a new tube. The cells were incubated on a shaker set at 150 RPM for thirty minutes at 30°C. After the incubation period, 1 mL of 40% PEG, 0.1 M LiOAC in TE solution was added and the mixture was vortexed. The new mixture was incubated on a shaker set at 150 RPM for thirty minutes at 30°C. After the thirty minute incubation period, the cells were heat shocked at 42°C for fifteen minutes. Following the heat shock, the cells were spun down in a microfuge for seven seconds. The supernatant was removed by aspiration and the pellet was resuspended in 1 mL DI water. The resuspended pellet was spun again as before and the supernatant removed. Finally the cells were resuspended in 400 μL DI water; 200μL aliquots were plated on SD-
URA-ARG and grown for three days at 30°C. Individual transformants were patched on SD-URA-ARG and frozen down in 300 µL 20% glycerol.

**Induced Direct Repeat-Mediated Deletion**

The assay begins by patching strains including plasmids E234 and E240 (see Figure 17) of wild-type, rad1Δ, or rad10Δ. These patches are grown on SD-URA-ARG for two days at 30°C. After two days growth, 10 mL of SRaff-URA-ARG broth is inoculated with cells from the patches. These inoculations are incubated at 30°C overnight on a shaker at 200 RPM. After the overnight inoculations, a volume of approximately 1-1.5 mL is diluted in 50 mL of SRaff-URA-ARG. The volume added is determined by measuring to obtain an OD₆₀₀ between 0.07 and 0.08. Diluted overnight cultures are incubated at 30°C for 5-6 hours until an OD₆₀₀ between 0.1 and 0.2 is obtained. A serial dilution is created using 50 µL of the culture and 50 µL of the dilution is plated on YPD (between 5 and 8 plates per strain). The plates were incubated at 30°C for two nights (refer to Figure 11).

Following the 5-6 hour inoculation, a post-induced culture is created by adding 22.5 mL of pre-induced culture, 2.5 mL of 20% galactose and 25 µL of 100X L-Arginine (20 mg/mL stock) to an Erlenmeyer flask. The post-induced culture is incubated in a water bath shaker at 19°C for 16-18 hours. The OD₆₀₀ of the 16-18 hour cultures should be below 0.8 for optimal results. A serial dilution was created from the 16-18 hour culture using 5 µL and 50 µL of the dilution was plated on YPD.
(between 5 and 8 plates per strain). The plates were incubated at 30°C for two nights (refer to Figure 12).

Following the two night incubation periods, YPD plates are replica plated using velvets onto YPG and SD-ARG plates. The replica plates and original plates are incubated overnight at 30°C. Colonies which exhibited sectoring on SD-ARG plates were considered ARG⁻, those which exhibited sectoring on YPG plates were considered YPG⁺. Growth is quantified as follows; total growth (YPD), ARG⁻, YPG⁺, and ARG⁻YPG⁻.

Calculations were performed to determine the frequency of recombination. Post-induced rates were determined by subtracting the total number of ARG⁻YPG⁻ colonies for each strain from the total number of colonies grown on YPD for that strain and dividing the total number of colonies grown on YPG by the difference.

If spontaneous respiration occurred on pre-induced YPG plates, the same calculation would be performed on both pre and post-induced. The post-induced rate would be determined by subtracting the adjusted pre-induced rate. Three trials were performed with wild-type, rad1Δ, and rad10Δ strains (both plasmids would be run at the same time for one assay). Rates for each trial were averaged; error was described by standard deviation.
Figure 11. **Pre-Induced DRMD.** Strains containing the plasmids E234 and E240 are patched on SD-URA-ARG for 2 days and grown at 30°C. These patches are inoculated into SRaff-URA-ARG for one day and diluted until they reach an OD_{600} of approximately 0.07-0.08. The diluted cultures are grown for 5-6 hours until they reach an OD_{600} of approximately 0.1 to 0.2. A serial dilution is performed on the pre-induced culture and plated on YPD for two days at 30°C. The YPD plates are replica plated onto SD-ARG and YPG, all three are grown for one additional day before growth is quantified.

Figure 12. **Post-Induced DRMD.** The pre-induced culture following the 5-6 hour incubation period is diluted 22.5 mL into 2.5 mL 20% galactose and 25 μL 1000X L-Arginine 16-18 hours 19°C. The new culture is incubated at 19°C for 16-18 hours without surpassing an OD_{600} of 0.8. A serial dilution is performed and plated on YPD for two days at 30°C. Replica plating is performed onto YPG and SD-ARG, all plates are incubated for one day at 30°C following replica plating before quantifying growth.
Results

RAD10 in *Saccharomyces cerevisiae* is known to be involved in single strand annealing and nucleotide excision repair. The respiration loss assay was performed to determine the spontaneous mutation rates among wild type and mutant strains. The direct repeat-mediated deletion assay was performed to determine the rate of nuclear and mitochondrial recombination by plating on selective media.

Recombination rates were determined using the Lea Coulson method of the median along with standard deviations. The significance of the results was calculated using a two-tailed t test. The induced DRMD was performed to determine rates of mitochondrial recombination events following a double-strand break in the DNA. The significance of the results was calculated using a two-tailed t test for post induced data corrected for spontaneous respiration loss events.

Respiration Loss Assay

This assay is used to determine the rate of spontaneous respiration loss in wildtype, *rad1Δ*, and *rad10Δ* strains. Cells may become respiration deficient through a complete degradation of the mitochondrial genome, point mutations, or recombination events. If the mitochondrial genome is completely degraded the cell will be unable to respire and will be considered rho⁰. Cells which undergo a point mutation or recombination event which causes them to lose the ability to respire are rho⁻. Cells in which respiration is unaffected are rho⁺. The assay does not
distinguish between which respiration deficient cells are rho⁻ and which are rho⁰. By
determining the rate of spontaneous respiration loss it is possible to determine
whether cells lacking the genes RAD1 and RAD10 have a higher rate of mutations
due to the inability to repair the mitochondrial genome. In this event it would aid in
determining the activity level of these proteins in repair mechanisms in the
mitochondria.

If a higher than average respiration loss is observed in strains lacking RAD1 or
RAD10, it could implicate them as integral proteins in the repair of mitochondrial
DNA. Cells would become respiration deficient due to the inability to repair
mutations occurring and the DNA could potentially degrade altogether depending
on the severity of mutations. These respiration deficient cells are classified as rho⁻
and rho⁰. This assay is nonselective in repair mechanisms which could be tested,
therefore the results may be due to any one of the pathways genes tested work in.
Serially diluted colonies were plated on YPG + 0.2% dextrose and grown for three
days at 30°C. After three days, petite and grand colonies were counted to calculate
a respiration loss frequency. Yeast preferentially undergoes fermentation; therefore
all cells will grow until the 0.2% dextrose is depleted. Once there is no longer
dextrose in the media cells that cannot undergo respiration will arrest at a smaller
size. This smaller size denotes mitochondrial DNA mutations involving genes used in
respiration which prevents transcription or causes translation errors. These
translation errors may cause improper protein folding and a lack of function.
Colonies which are able to utilize glycerol will deplete dextrose in the media but continue to grow and physically appear larger, these are known as grand colonies. Colonies unable to utilize glycerol physically appear smaller and are known as petite colonies (Figure 9).

The respiration loss frequency for rad10Δ strains was 1.5 times higher than that for the wildtype strain DFS188. The calculated frequencies were 2.49% with a standard deviation of 0.07% for wildtype and 3.75% with a standard deviation of 0.075% for rad10Δ strains, see Figure 13. The 1.5 fold increase in respiration loss of the knockouts compared to wildtype was found to be statistically significant using a two-tailed t-test with a p value of 0.00003. The 1.5 fold increase in respiration loss shows a mild effect of loss of RAD10 in Saccharomyces cerevisiae regarding mitochondrial function to utilize oxidative phosphorylation. The loss of RAD10 indicates significant mtDNA mutations affecting respiration of the cell.

The respiration loss frequency for rad1Δ strains was 1.2 times higher than that for the wildtype strain DFS188. The calculated frequency for three trials was 3.05% petites with a standard deviation of 0.28% for rad1Δ strains. This 1.2 fold increase was found to be insignificant using a two-tailed t test with a p value of 0.068. The lack of a significant difference in respiration loss in the absence of RAD1 in Saccharomyces cerevisiae could be due to the larger standard deviation in trials of NRY203 strains, see Figure 13.
**Respiration Loss in Wild-type, rad1Δ, and rad10Δ Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Respiration Loss Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFS188</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>NRY203</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td>NRY201</td>
<td>4 ± 0.2</td>
</tr>
</tbody>
</table>

Figure 13. **Spontaneous respiration loss in wild-type, rad1Δ, and rad10Δ strains.** The 1.5 fold increase in spontaneous respiration loss experienced by rad10Δ knockouts (NRY201) compared to wildtype (DFS188) strains is illustrated along with the 1.2 fold increase experienced by rad1Δ strains (NRY203). 1.5 fold increase in spontaneous respiration loss was found to be significant using a two-tailed t-test with a p value of 0.00003. The 1.2 fold increase in rad1Δ strains was found to not be significant using a two-tailed t-test with a p value of 0.068.

**Direct Repeat-Mediated Deletion Assay**

The Direct Repeat-Mediated Deletion (DRMD) assay measures the rate of mutations in both the mitochondria and nucleus. This assay measures DRMD events caused by DNA polymerase slippage, non-homologous end joining, single-strand annealing, and crossing over. This more targeted approach limits the results to mutations which would only be repaired by the aforementioned mechanisms. Other homologous repair mechanisms could lead to a DRMD event but it is much less common. The cells which become respiration deficient in this assay are due to a
mutation or degradation of the mitochondrial genome which could not be repaired via one of these mechanisms. This allows a more definitive determination of the effect of genes on the ability to repair the mitochondrial genome while also comparing it to the ability to repair the nuclear genome. A lower than average DRMD rate would imply the gene involved in the assay is involved in repairing the DNA in the mitochondria or nucleus (based on plates being analyzed).

The DRMD assay involves the wildtype LKY196 which contains two unique reporters that allow the rate of DRMD in both nuclear and mitochondrial events to be measured. The NRY201 strain cannot be used to measure DRMD and therefore a new rad10Δ strain must be created incorporating the nuclear and mitochondrial reporters. LKY797 has a nuclear reporter containing a 96 basepair (bp) casette and can synthesize uracil and adenine when grown on media not containing them. In addition to a nuclear reporter, LKY797 also has a mitochondrial reporter which allows it to synthesize lysine when grown on media not containing it. NRY66 has a 96 bp cassette and can synthesize arginine when grown on media not containing it. For rad1Δ strains, LKY793 is used in place of LKY797.

The DRMD assay is used to measure the rates of nuclear and mitochondrial recombination that occurs during spontaneous DRMD events. Spontaneous DRMD events may be due to double-strand breaks or may be due to replication dependent events such as DNA polymerase slippage or template switching. The double-strand
breaks may be repaired by non-homologous end joining, crossing over, synthesis-dependent strand annealing (SDSA), or single-strand annealing with direct repeats. Non-homologous end joining anneals double-strand breaks directly. The overhangs may be perfectly compatible and repair accurately or may be non-compatible and cause loss of nucleotides. Single-strand annealing occurs when a double strand break is detected in or near regions of homology. The regions of homology anneal, flaps of nonhomology are created and excised by a flap endonuclease. Single-strand annealing can commonly occur during DRMD due to the regions of homology in the direct repeats. Crossing over may not lead to a DRMD event if they do not occur at or within the direct repeats. Crossing over will cause a DRMD if the break occurs in the direct repeat and recombines at the other repeat. Synthesis-dependent strand annealing does not cause a DRMD when it repairs a double-strand break; strand invasion occurs and repair occurs without recombination. This assay can measure more accurately if wildtype strains have significantly different rates of mutations than rad1Δ and rad10Δ strains. If a significant difference is observed it can indicate that cells without RAD1 and RAD10 are unable to repair mutations in the mitochondria using one or more of the aforementioned repair pathways. Nuclear recombination events are also measured as a control as the genes tested are known to encode nuclear DNA repair proteins.

By plating on selective media, serial dilutions can be used to obtain recombination rates for both nuclear and mitochondrial DNA. The nuclear and
mitochondrial reporters used in this assay both contain genes located 99 bp downstream of the ATG start codon for another gene. The nuclear reporter used contains the *URA3* gene flanked by two regions of 96 bp repeats fused within the *TRP1* gene following the ATG start codon, see Figure 14. Cells possessing this reporter are phenotypically *Ura*⁺ and *Trp*⁻. Spontaneous DRMD events occurring between the 96 bp repeats causes the *URA3* gene to be excised. Once the *URA3* gene has been excised, a functional *TRP1* gene is present and cells become phenotypically *Ura*⁻ and *Trp*⁺. A similar construct is present in the mitochondrial reporter involving the *ARG8m* and the *COX2* genes. The *ARG8m* gene is inserted within the *COX2* gene rendering it respiration deficient, see Figure 14. When the *ARG8m* gene is removed by recombination within the repeated sequences flanking it, the *COX2* gene becomes functional and cells are able to grow on nonfermentable carbon sources such as glycerol.
Figure 14. **Nuclear and Mitochondrial Reporters.** The top image illustrates the nuclear reporter including the **URA3** and **TRP1** genes along with regions of 96 bp repeated sequences shown by the striped box regions. The **URA3** gene is inserted 99 bp into the **TRP1** gene rendering it inactive. When recombination occurs between the 96 bp repeated sequences the **URA3** gene is excised leaving a functional **TRP1** gene behind. A similar construct is present in the mitochondrial reporter shown in the bottom image. The **ARG8** gene is located 99 bp into the **COX2** gene and flanked by two regions of 96 bp repeats. The **COX2** gene may become active if recombination occurs removing the **ARG8** gene.

The YPG plates in the assay contain a non-fermentable carbon source and measure mitochondrial recombination. Without a fully functioning mitochondrial genome, cells are unable to utilize glycerol and therefore do not grow. The **ARG8** gene is removed through recombination from the mitochondrial reporter to allow a
functional COX2 gene. When the COX2 gene is functional, cells may utilize respiration and grow on YPG plates. The SD-TRP plates in the assay measure nuclear recombination. When recombination occurs in the URA3 gene of the nuclear reporter, the TRP1 gene becomes functional and the cells are able to grow on SD-TRP.

The mitochondrial recombination rates are not significantly different between wildtype LKY196 strains, rad10Δ strains CSY001 and CSY002, and the rad1Δ strain CSY012. The average rate of recombination was found using the Lea Coulson method of the mean. From three trials the average rate of recombination in wildtype strains was found to be 4040x10^-7 with a standard deviation of 215x10^-7. The average rate of recombination in rad10Δ strains was calculated to be 4330x10^-7 with a standard deviation of 1240x10^-7, see Figure 15. The difference in recombination rates was not statistically significant with a p value of a two-tailed t-test equaling 0.73 for three trials. The average rate of recombination events in rad1Δ strains was calculated to be 4750x10^-7 with a standard deviation of 1270x10^-7, see Figure 15. This 1.2 fold increase was found to be insignificant using a two-tailed t test with a p value of 0.44. The insignificance indicates that RAD10 loss is not vital for repairing DRMD events in the mitochondria. Loss of RAD1 was also insignificant and did not affect the cell's ability to repair DRMD events in the mitochondria.
Nuclear recombination is measured by growth on SD-TRP plates. If homologous recombination occurs in the URA3 gene of the nuclear reporter, the TRP1 gene becomes functional and allows colonies to grow on media lacking tryptophan. The nuclear recombination rate in rad1Δ strains was found to be decreased 2.3 fold in comparison to the wildtype among three trials of DRMD.

Figure 15. Average rate of mitochondrial mutations per cell division in wild-type, rad1Δ, and rad10Δ strains. There is no significant difference between the rate of mutations found in the mitochondria of wildtype (LKY196) and rad10Δ strains (CSY001 and CSY002). There is also no significant difference between the rate of mutations found in the mitochondria of wildtype and rad1Δ strains (CSY012) The p value of a two-tailed t-test was calculated to be 0.732 for CSY001 and CSY002. The p value of a two-tailed t-test was calculated to be 0.436 for CSY012.
LKY196 had an average recombination rate of $13.1 \times 10^{-7}$ with a standard deviation of $0.70 \times 10^{-7}$. CSY001 and CSY002 had an average recombination rate of $5.65 \times 10^{-7}$ and a standard deviation of $0.47 \times 10^{-7}$, see Figure 16. The two fold decrease in mutant recombination compared to wildtype was found significant using a two-tailed t-test with a p value of 0.00026. The nuclear recombination rate in rad1Δ strains was decreased 1.8 fold compared to wildtype strains. The calculated rate for three trials of CSY012 was $7.43 \times 10^{-7}$ with a standard deviation of $0.58 \times 10^{-7}$, see Figure 16. This 1.8 fold decrease was found to be significant using a two-tailed t test with a p value of 0.0005. The decrease in recombination indicates that RAD10 and RAD1 are needed in order for nuclear direct repeat-mediated deletion to occur compared to wildtype.
Induced Direct Repeat-Mediated Deletion Assay

The Induced Direct Repeat-Mediated Deletion assay is used to determine the frequency of recombination events occurring in the mitochondria following a double-strand DNA break. This separates itself from the DRMD by limiting the events measured to those following the double-strand break such as single-strand annealing, crossing over, and non-homologous end joining. The spontaneous DRMD...
assay does not necessarily involve a double-strand break. The induced DRMD assay does not measure replication dependent events such as DNA polymerase slippage or template switching measured in the spontaneous DRMD assay. The events measured are replication independent unlike some which occur in the DRMD. The strains used in this assay have lost the nuclear reporter. This loss of reporter is essential to ensure transformants have taken up the plasmids E234 and E240. Both reporter and plasmids mentioned contain the *URA3* gene (see Figures 6 and 10), without losing the reporter first it is unclear if strains are URA⁺ due to successful uptake of plasmid DNA.

The strains used for the induced DRMD assay have been transformed to include the plasmids E234 and E240; these plasmids contain an intein which inactivates the protein function of Kpn1. E240 contains a nonfunctional intein which does not excise at low temperatures. The KPN1 endonuclease is controlled by a GAL1 promoter which is activated in the presence of galactose. These features are identified in Figure 17 along with other important aspects.

The mitochondrial reporter is maintained and a temperature sensitive intein is utilized to block the Kpn1 endonuclease. When the intein excises, a double-strand break in is created the *ARG8ʷ* gene of the mitochondrial reporter, see Figure 14. If this double-strand break causes a DMRD event the *ARG8ʷ* gene is excised and a functional *COX2* gene allows cells to grow on non-fermentable carbon sources such
as glycerol in YPG plates. YPG plates measure mitochondrial recombination events following the double-strand breaks, YPD plates give a cell count, and SD-ARG plates measure other events as a control. Cells which are YPG^ARG^- may have experienced a complete degradation of mitochondrial DNA, lost the mitochondrial reporter, or not had a recombination event following the double-strand break. This control allows isolation of mitochondrial DNA recombination events following the double strand DNA break. The double-strand breaks may be repaired by non-homologous end joining, crossing over, or single strand annealing with direct repeats.

The assay is performed with two strains in parallel, one containing E234 and on containing E240. The E240 strain serves as a control with a nonfunctional intein to measure spontaneous recombination both pre and post induction. Induction occurs when the strains are in the presence of galactose at 19°C, see Figures 17 and 18.
Figure 17. **E234 and E240 Plasmids.** The pYES2.1 plasmid backbone is shown along with the inserted Kpn1 gene and VMA1 intein present in E234 and E240. The KPN1 gene encodes a restriction endonuclease that induces a break in ARG gene of the mitochondrial reporter. The VMA1 intein inactivates KPN1, when VMA1 is removed KPN1 function is restored. VMA1 is temperature sensitive, when exposed to temperatures above 20°C, it removes itself from the plasmid. The plasmids also contain a mitochondrial localization sequence (MTLS) which allows the Kpn1 gene product to localize to the mitochondria.

Adapted from http://www.snapgene.com/resources/plasmid_files/yeast_plasmids/pYES2.1-E/
The rate of recombination events in the mitochondria for rad1Δ and rad10Δ strains was not significantly different from wildtype strains following induction of Kpn1. Wildtype strains had a calculated rate of 63.81% recombination with a standard deviation of 0.066. This rate was 1.25 times lower than that of rad1Δ strains and 1.20 times lower than that of rad10Δ strains. The calculated rate for rad1Δ strains was 76.55% with a standard deviation of 0.028. This was found to be
insignificant using a two-tailed t test with a p value of 0.063. The calculated rate for \textit{rad10Δ} strains was 79.69\% with a standard deviation of 0.0071, see Figure 19. The 1.25 fold increase in recombination in \textit{rad10Δ} strains was found to be insignificant using a two-tailed t test with a p value of 0.052.

The mild increase in induced DRMD events in \textit{rad1Δ} and \textit{rad10Δ} strains was found to be insignificant. This insignificant increase is not disadvantageous to the cells.

![Induced Direct Repeat Mediated Deletion in Wild-type, \textit{rad1Δ}, and \textit{rad10Δ} Strains](image)

Figure 19. \textbf{Average frequency of mutations per cell division in wild-type, \textit{rad1Δ}, and \textit{rad10Δ} strains.} The average rate of mutations per cell division in \textit{rad1Δ} strains was found to be increased 1.20 times from wildtype. The average rate of mutations per cell division in \textit{rad10Δ} strains was found to be increased 1.29 times from wildtype. Both increases in mutations were found to be insignificant using a two-tailed t-test, p values 0.0632 and 0.0522 respectively.
**Discussion**

The role of *RAD1* and *RAD10* in the mitochondria is not as well understood as in the nucleus. In the nucleus Rad1p and Rad10p are involved in repairing double strand breaks through single strand annealing and in nucleotide excision repair. The roles of *RAD1* and *RAD10* in mitochondrial stability is the main focus of this research as previous research has not focused on *RAD1* and *RAD10* in mitochondrial stability but only nuclear. The results of the respiration loss assay along with the DRMD assay do not provide a complete picture but only a fraction of insight. The induced DRMD assay provides a more comprehensive understanding of their role in mitochondrial stability. Based on the preliminary results of this research, *RAD1* and *RAD10* do not appear to have a major role in repair of mitochondrial DNA damage. At present it has been determined that *RAD1* and RAD10 do not significantly contribute to spontaneous DRMD events in the mitochondria but do contribute to spontaneous DRMD events in the nucleus. Based on these conclusions, spontaneous DRMD events in the mitochondria are repaired by a mechanism other than SSA.

*Rad10Δ and Rad1Δ strains show increased rate of respiration loss compared to wildtype*

From three trials of a respiration loss assay, the average of the medians for *rad1Δ* strains were found to have 1.2 times increased rates of respiration loss.
Rad10Δ strains were found to have an average median of respiration loss 1.5 times increased compared to wildtype strains. Respiration loss measures the rate of mtDNA mutations affecting respiration. MtDNA mutations may occur in which the entire genome is degraded (rho0) or just sections are altered through deletions or point mutations (rho−). The respiration loss assay does not discern between mutations involving the loss of the mitochondrial genome and point mutations or between mutations which would be repaired through differing mechanisms. The significant increase in respiration loss between wildtype, rad1Δ, and rad10Δ strains indicates significant mtDNA mutations affecting respiration. These mutations occur randomly and may indicate that these genes play a key role in mitochondrial stability in Saccharomyces cerevisiae. The non-discriminative nature of the assay makes it difficult to pinpoint the exact cause of the significant increases in respiration loss of those strains.

Due to the increased number of copies of mtDNA compared to nuclear DNA, mtDNA can be mutated and a cell may still be viable because of the redundancy. When respiration loss occurs it is not clear whether the entire mitochondrial genome has been degraded or point mutations occurred thereby preventing respiration. From the increased respiration loss it can be determined that RAD1 and RAD10 are potentially involved in repairing mitochondrial mutations that prevent respiration. Mutations measured may also be due to nuclear proteins that localize to the mitochondria for use in respiration, but that is much less common. The
increased respiration loss present in \textit{rad}10\text{Δ} and \textit{rad}1\text{Δ} strains reveals a decrease in mtDNA stability compared to wildtype. To provide a larger scale of how \textit{RAD}1 and \textit{RAD10} functions in mtDNA stability, DRMD assays were performed to measure recombination due to spontaneous direct repeat-mediated deletions.

\textbf{Rad1\text{Δ} and Rad10\text{Δ} strains show no significant difference in mitochondrial DRMD events}

Compared to wildtype strains, \textit{rad}1\text{Δ} and \textit{rad}10\text{Δ} strains show no significant difference in the rate of spontaneous mitochondrial DRMD events. The DRMD assay measures recombination events in both the nucleus and mitochondria that are associated with direct repeat-mediated deletions. Cells able to grow on YPG plates have undergone recombination removing the \textit{ARG} gene in the mitochondrial reporter. When the \textit{ARG}8\text{m} gene is removed, the \textit{COX}2 gene becomes functional allowing respiration and cells to grow on YPG. The recombination must occur so that the entire \textit{ARG} gene is removed in order for proper transcription and translation of \textit{COX}2. The large amount of background noise on YPG plates of DRMD prevents the results from being as accurate as nuclear recombination results.

From the insignificant change in mitochondrial recombination rates between wildtype, \textit{rad}1\text{Δ}, and \textit{rad}10\text{Δ} strains it can be inferred that \textit{RAD}1 and \textit{RAD10} do not play a major role in spontaneous DRMD events in the mitochondria. If the proteins do not play a major role in spontaneous DRMD events in the mitochondria, single-
strand annealing is likely not a major repair pathway used during those events. Single-strand annealing is the only DNA repair pathway measured in the spontaneous DRMD which RAD1 and RAD10 have previously been documented to work in. Other pathways such as non-homologous end joining, crossing over, and DNA polymerase slippage are also tested in the spontaneous DMRD assay. Although the mitochondrial recombination rates show no significant difference between rad1Δ, rad10Δ, and wildtype strains that is not the case for nuclear recombination rates.

**Rad1Δ and RAD10Δ strains show a significant decrease in nuclear DRMD events**

Compared to wildtype strains, rad1Δ and rad10Δ strains show a 1.8 fold and 2.3 fold decrease respectively in recombination during spontaneous nuclear DRMD events. Nuclear recombination is measured in the DRMD assay by plating on selective media lacking tryptophan. The URA3 gene in the nuclear reporter must be removed by recombination to leave a functional TRP1 gene behind. This removal could occur through single strand annealing, crossing over, non-homologous end joining, or DNA polymerase slippage. The significant decrease in nuclear recombination during spontaneous DRMD events indicates that RAD1 and RAD10 are involved in the spontaneous DRMD events of the nucleus. The endonuclease complex formed by RAD1 and RAD10 has previously been established to work in single-strand annealing. Since the spontaneous DRMD assay tests the ability of a cell
to use this repair pathway which has been established in the nucleus, the significant
decrease in nuclear recombination that was observed is as expected. Cells lacking
these genes are less capable of repairing double-strand breaks occurring in the
nucleus, this prevents recombination which would be evident in this assay.

The indication that RAD1 and RAD10 function in nuclear DRMD events also
suggests that single strand annealing is involved and therefore double strand breaks
may be present. Single-strand annealing may be present in nuclear spontaneous
DRMD events but not mitochondrial. However, this does not provide a complete
picture of how RAD10 affects mtDNA stability. The spontaneous DRMD assay does
not discriminate in repair methods tested. RAD1 and RAD10 have only ever been
documented to work in single-strand annealing and nucleotide excision repair.
None of the assays performed test the nucleotide excision repair pathway. The
spontaneous DRMD assay tests crossing over, non-homologous end joining, DNA
polymerase slippage, and single-strand annealing. From the insignificant increase in
mitochondrial recombination rates in rad1Δ and rad10Δ strains compared to
wildtype it has been concluded that these genes are not involved in repairing
spontaneous DRMDs in the mitochondria. To further determine the role of RAD1
and RAD10 in mitochondrial stability an induced DRMD assay will be performed.
Rad1Δ and Rad10Δ strains show no significant difference in induced DRMD events

The induced DRMD assay creates a double-strand break in mtDNA through the use of a plasmid containing an intein. The plasmids E234 and E240 contain a YES2.1 backbone along with the *KPN1* gene, the *VMA1* intein, and a mitochondrial localization sequence (shown in Figure 17). The difference between E234 and E240 is that E240 contains a point mutation which prevents the intein from splicing out. Without the intein removed the *KPN1* gene is rendered nonfunctional, this acts as a control in this assay. The *VMA1* intein is temperature sensitive, when exposed to temperatures above 19°C it splices itself and leaves behind a functional *KPN1* gene (see Figure 18). Kpn1p is a restriction endonuclease which induces a double strand break in the *ARG8m* gene of the mitochondrial reporter. When the break is induced, either recombination occurs repairing it or the DNA is degraded. If no recombination occurs, the cells will grow on SD-ARG because the reporter is still intact. When recombination occurs a functional *COX2* gene is present and growth on YPG is observed. The assay uses replica plating in order to track growth of the same colony on YPG, YPD, and SD-ARG. If a colony does not grow on both SD-ARG and YPG the mitochondrial DNA may have degraded, the reporter may have been lost, or no recombination occurred following the incision made by Kpn1p. Such colonies are adjusted for in the final results to exclude them. The final results are also adjusted for pre-induced spontaneous respiration loss.
The induced DRMD assay involves a double-strand break. These breaks are repaired through single-strand annealing, non-homologous end joining, and crossing over. The double-strand break repair pathway RAD1 and RAD10 work in is single-strand annealing. By limiting the number of repair pathways which could be employed in the assay, the induced DRMD aims to narrow down which pathways could be utilized in the mitochondria and determine if specific genes employ any of the possible pathways. This assay does not account for results caused by degradation of nuclear genes which are unable to be repaired. Both rad1Δ and rad10Δ strains showed a small increase in recombination rates compared to wildtype rates. The 1.2 fold increase in rad1Δ recombination and the 1.25 fold increase in rad10Δ recombination were found to be insignificant using a two-tailed t test. This insignificance in recombination rate differences implies that RAD1 and RAD10 are not involved in mitochondrial stability during DRMD events whether spontaneous or caused by an induced double strand break. The genes currently have been determined to not be involved in single-strand annealing in the mitochondria.

Although the genes have been determined to be uninvolved in mitochondrial stability of the pathways tested, it is possible the mitochondria use other pathways as yet unknown. It is also possible that the results of the assay were affected by the instability of the nuclear genome caused by the loss of the genes. To address this possible confounding factor, future research could be performed in which RAD1 and
RAD10 are prevented from localizing to the mitochondria rather than rendering them nonfunctional. The results of the assays may also have been affected by only rendering one gene nonfunctional per assay. Although the Rad1p/Rad10p endonuclease complex has only been shown to work with both units present in the nucleus it is possible another protein interacts in complex in the mitochondria. The possible presence of another protein in the complex in the mitochondria could leave cells with a semi-functional complex and therefore skewing the results. Repeating the assays with the aforementioned alterations may indicate RAD1 and RAD10 as having a role in mitochondrial stability.
References


