The Role of *RAD55* in Mitochondrial DNA Stability in *Saccharomyces cerevisiae*

A Senior Honors Thesis

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Abstract

Mitochondria are organelles present in eukaryotic cells. Through the process of cellular respiration mitochondria produce ATP; a vital molecule for the completion of many cellular processes. Mitochondria are unique in that they contain their own DNA separate from the DNA within the nucleus. Mutations in mitochondrial DNA have notable connections to several human pathologies such as various neuromuscular and neurodegenerative disorders. The focus of this study was to determine the role of the nuclear gene *RAD55* in maintaining mitochondrial DNA in budding yeast, *Saccharomyces cerevisiae*. The gene product of *RAD55* cooperates with other proteins to bring about the repair of double-stranded DNA breaks in the nucleus. Specifically, *RAD55* is a member of the *RAD52* epistasis group whose gene product functions as a heterodimer with Rad57p. The Rad55p/57p heterodimer promotes Rad51p filament assembly on single-stranded DNA. Once assembled, Rad51p filaments displace Replication Protein A from single-stranded DNA and its recombinase activity is initiated. To determine the effect loss of Rad55p had on the stability of mitochondrial DNA, two genetic assays were performed. The first assay measured the frequency of spontaneous respiration loss in *rad55*Δ mutants. The lab observed *rad55*Δ mutants did not show a significant increase in spontaneous respiration loss compared to that of the wild type. An additional direct repeat-mediated deletion assay was performed to determine if Rad55p played a role in stabilizing the mitochondrial genome from mutations caused by recombination events. It was discovered that the rate of direct repeat-mediated deletions for *rad55*Δ in the nuclear genome increased 5.8-fold compared to that of the wild type. Surprisingly, the lab found the rate of direct repeat-mediated deletions for *rad55*Δ in the mitochondrial genome decreased by 1.5-fold compared to that of the wild type.

Introduction

Saccharomyces cerevisiae

Yeast are unicellular fungi that, due to their adaptability, are found in terrestrial, aerial, and aquatic environments (Rodrigues 2006). The ubiquitous nature of yeast, specifically the budding yeast *Saccharomyces cerevisiae*, is largely due to their ability to metabolize a number of carbon sources in a respiratory or fermentative fashion. While D-glucose (dextrose) is preferred, *S. cerevisiae* can also utilize alcohols, organic acids, amino acids, or polyols as a source of carbon (Rodrigu es 2006).

As a facultative anaerobe, *S. cerevisiae* will alter its metabolic physiology in response to the sugar composition of the media and the availability of oxygen (Rodrigues 2006). In general, high D-glucose levels in the absence of oxygen favors fermentation while low D-glucose levels in the presence of oxygen favors respiration (Otterstedt 2004). This relationship is logical if the net yield of ATP is taken into consideration. Alcoholic fermentation, a process that occurs in the cytoplasm of *S. cerevisiae*, produces a net of 2 molecules of ATP per molecule of glucose (Rodrigues 2006). The complete respiratory catabolism of a molecule of glucose that occurs mainly in the mitochondria of *S. cerevisiae* produces a net of 16 ATP molecules. Therefore, when *S. cerevisiae* is in a glucose-poor environment, it degrades glucose in a more energetically efficient manner than if it were in a glucose-rich environment (Rodrigues 2006).

The mode of metabolism in *S. cerevisiae* is largely due to the regulation of gene expression. For example, an abundance of D-glucose upregulates genes associated with fermentation such as those of the nuclear ADH family and represses genes associated with respiration such as mitochondrial genes of the COX family (Dueñas-Sánchez 2012). Conversely,

the presence of a non-fermentable carbon source such as glycerol upregulates genes associated with aerobic glycerol catabolism, nuclear GUT1 and GUT2, and represses genes associated with fermentation (Dueñas-Sánchez 2012). The ability of *S. cerevisiae* to utilize alcoholic fermentation or respiration makes it uniquely suited for mitochondrial research. Specifically, because damage to the mitochondrial genome of *S. cerevisiae* will not inhibit its ability to grow, the effects of mitochondrial damage can be easily observed.

Mitochondrial Genome

Mitochondria are organelles present in eukaryotic cells. While mitochondria play a central role in metabolism, apoptosis, and aging, their function is dynamic between and even within an organism; mitochondrial function changes depending on environmental conditions and tissue demands (Rea et al. 2010). The versatility of mitochondria can be partially attributed to mitochondria containing their own genome (Mitochondrial 2011). The presence of multiple genomes within a eukaryotic cell is explained by the endosymbiotic theory. This theory states that mitochondria are no more than bacterial symbionts that evolved over 1.5 billion years into an organelle (Mitochondrial 2011). The evolution of bacterial symbiont to organelle resulted in the disappearance of entire metabolic pathways and the transfer of genetic information necessary for mitochondrial structure and function to the nucleus (Rea et al. 2010). However, the transfer of genetic material did not occur identically between species.

The size and composition of the mitochondrial genome varies between species. For example, the size and composition of human mitochondrial DNA (mtDNA) is different than that of *S. cerevisiae*. Specifically, the human mitochondrial genome is 16.6 kb long (Mitochondrial 2011) whereas the mitochondrial genome of *S. cerevisiae* is 85.8 kb long (Langkjær et al. 2003).

While it might be tempting to infer from their genomic lengths that human mtDNA contains fewer genes than *S. cerevisiae*, this is not the case. As seen in Figure 1, both organisms contain a similar number of genes in their mtDNA; 37 genes in human mtDNA (Mitochondrial 2011) and approximately 35 genes in the mtDNA of *S. cerevisiae* (Langkjær et al. 2003). Additionally, there are a number of orthologs between organisms; genes coding for mitochondrial ribosomes, tRNA, and the enzymes involved in oxidative phosphorylation are similar in both organisms (Mitochondrial 2011). The variation in mtDNA size between species must therefore be attributed to a factor other than gene number.

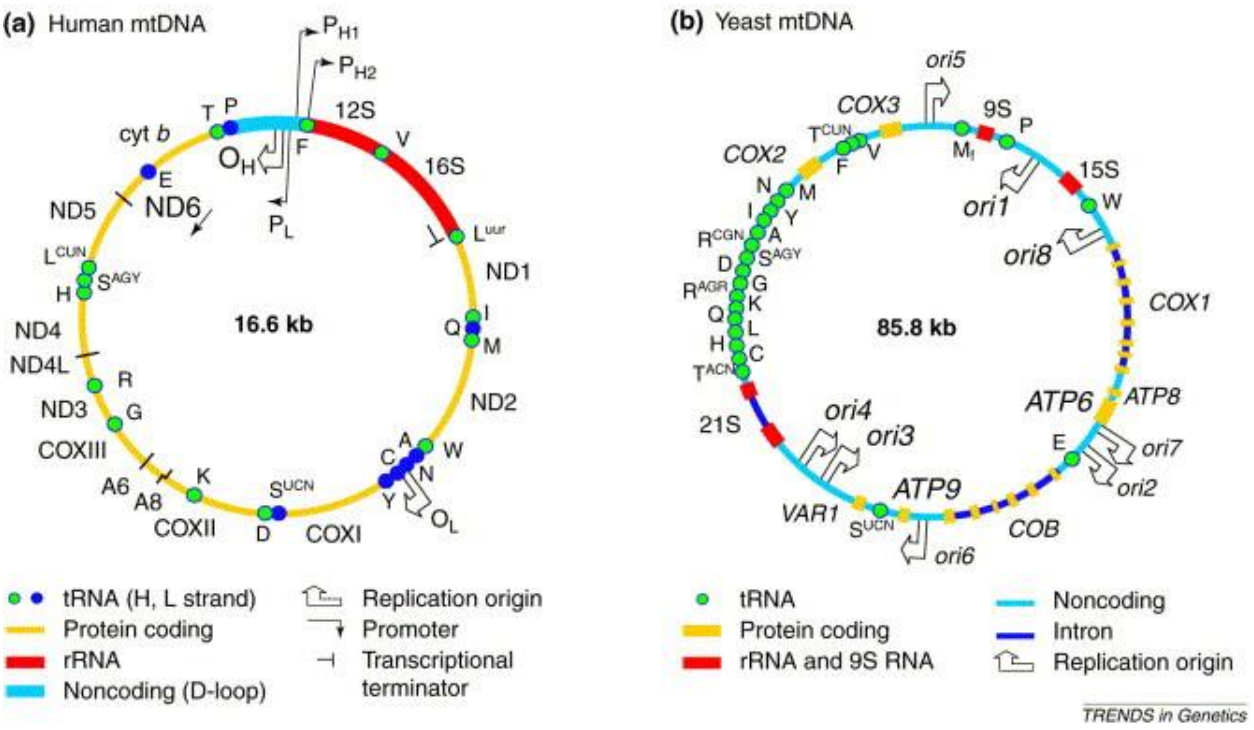

Figure 1. A comparison of the mitochondrial genome of humans and yeast. Compared to the mtDNA of yeast, the mtDNA of humans is smaller, contains significantly less non-coding regions, and does not contain introns. The mtDNA of yeast and humans contains sequences for rRNA, tRNA, and respiratory proteins (Jacobs 2001).

The discrepancy in genomic length between human and yeast mtDNA is attributed to the extremely compact nature of human mtDNA (Jacobs 2001). Compared to *S. cerevisiae*, the mtDNA of humans contains few no non-coding sequences and lacks many of the characteristics typically associated with eukaryotic genes such as trailing sequences and stop codons (Mitochondrial 2011). Researchers use the large mitochondrial genome of *S. cerevisiae* to their advantage when researching mitochondrial functions and disorders.

The mtDNA of *S. cerevisiae* is said to be one of the most thoroughly studied genomes in mitochondrial research (Bernardi 2005). The attractiveness of *S. cerevisiae* mtDNA can be attributed to two factors; an abundance of non-coding sequences and dispensability. The mitochondrial genome of *S. cerevisiae* is approximately five times larger than the mitochondrial genome of animals (Bernardi 2005). As previously stated, the size discrepancy between organisms is due to the large number of non-coding regions in the mtDNA of *S. cerevisiae.* The abundance of non-coding sequences in the mtDNA of *S. cerevisiae* affords an ease in which genes can be knocked-in or knocked-out (Rea et al. 2010). Specifically, genes can be added or removed without interfering with the function or expression of existing mitochondrial genes.

Mitochondrial DNA is referred to as dispensable if an organism can survive without it. As a facultative anaerobe, *S. cerevisiae* can utilize fermentable carbon sources as a means of ATP production even if their mtDNA is massively damaged or lost altogether. This characteristic of *S. cerevisiae* allows researchers to investigate genomic changes in the mitochondria that are normally incompatible with life. The dispensability of *S. cerevisia*e mtDNA has been instrumental in determining the proteins, both nuclear and mitochondrial, that maintain mitochondrial function and stability. The abundance of non-coding regions and dispensability of

S. cerevisiae mtDNA makes it an ideal model organism for the research of a variety of human mitochondrial disorders.

mtDNA of both *S. cerevisiae* and humans lack efficient DNA repair mechanisms and protective histones. A lack of repair and protection coupled with their proximity to reactive oxygen species generated by the mitochondria makes mtDNA highly susceptible to mutations (Mitochondrial 2011). These mutations more drastically affect obligate aerobes such as humans whose mtDNA is not dispensable. For example, human organs such as the heart, brain, and skeletal muscles where aerobic demands are high and regeneration is poor are often the epicenter of mitochondrial disorders (Mitochondrial 2011). Without functioning mitochondria these organs do not generate the ATP needed to maintain homeostasis. Human disorders such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, myoclonic epilepsy with ragged-red fibers, and Leber's Hereditary Optic Neuropathy are all caused by specific mutations in mtDNA (Mitochondrial 2011). Therefore, it is essential to understand the processes involved in the repair of damaged mtDNA. Such processes can be understood using *S. cerevisiae* as a model organism and then applied to human mtDNA. Because 222 of the 601 known yeast mitochondrial proteins have clear human orthologs, the study of yeast mtDNA to better understand human mtDNA is worthwhile.

RAD55

RAD55 is a nuclear gene found in *S. cerevisiae.* It is located on chromosome IV and belongs to the *RAD52* epistasis group (RAD55). This group of genes which includes *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, and *XRS2* is involved in the repair of double-stranded breaks in nuclear DNA (RAD55). The majority of double-stranded DNA (dsDNA)

breaks are a result of ionizing radiation. Consequently, mutants in the *RAD52* epistasis group are known to be extremely sensitive to ionizing radiation (Johnson et al. 1995). For example, *rad55*Δ mutants have been cited to exhibit normal spontaneous mitotic recombination, but an inability to induce recombination in instances of DNA damage caused by ionizing radiation or methyl methanesulfonate (Bashkirov et al. 2000). The importance of *RAD55* in genomic integrity can be better understood by examining the mechanism in which Rad55p repairs nuclear dsDNA breaks.

While the repair of double-stranded DNA breaks can occur in a variety of ways, homologous recombination is the pathway in which *RAD55* is involved. In homologous recombination the broken ends of a chromosome utilize a sister or homologous chromosome as a template for DNA repair (Sugawara 2003). In order for this to occur, single-stranded DNA (ssDNA) must find its complementary sequence within a double-stranded DNA molecule. This process is facilitated by DNA strand exchange proteins that complex with ssDNA and aid in the interaction with homologous dsDNA. The gene product of *RAD55* plays an essential role in the interaction between DNA strand exchange proteins and ssDNA.

Rad55p is the gene product of *RAD55*. Rad55p contains 406 amino acids and has a molecular weight of 46.3 kDa (RAD55). Rad55p has been cited to form a heterodimer with Rad57p (Sung 1997). The Rad55p-Rad57p heterodimer is thought to function by enhancing the activity of Rad51p (Sung 1997). Rad51p is a DNA strand exchange protein that is capable of binding 3bp of ssDNA per monomer (Sugawara 2003). Rad51p can only bind ssDNA in the presence of ssDNA binding proteins. ssDNA binding proteins, such as eukaryotic replication protein A (RPA), function to prevent ssDNA from forming secondary structures. However,

incubation of RPA with ssDNA before the addition of Rad51p has been cited to drastically reduce the loading of Rad51p onto ssDNA (Sugawara 2003). Eukaryotic cells overcome the reduction in Rad51p binding affinity by utilizing the Rad55p-Rad57p heterodimer as a cofactor (Sung 1997). The interaction between the Rad55p-Rad57p heterodimer and Rad51p is enough overcome the rate-limiting step of Rad51p binding to RPA bound ssDNA (Sung 1997). Consequently, loss of Rad55p has been cited to impair the normal activity of Rad51p and therefore the homologous recombination of dsDNA breaks in the nucleus (Sung 1997). The interactions between ssDNA, RPA, Rad51p, and the Rad55p-Rad57p heterodimer are summarized in Figure 2.

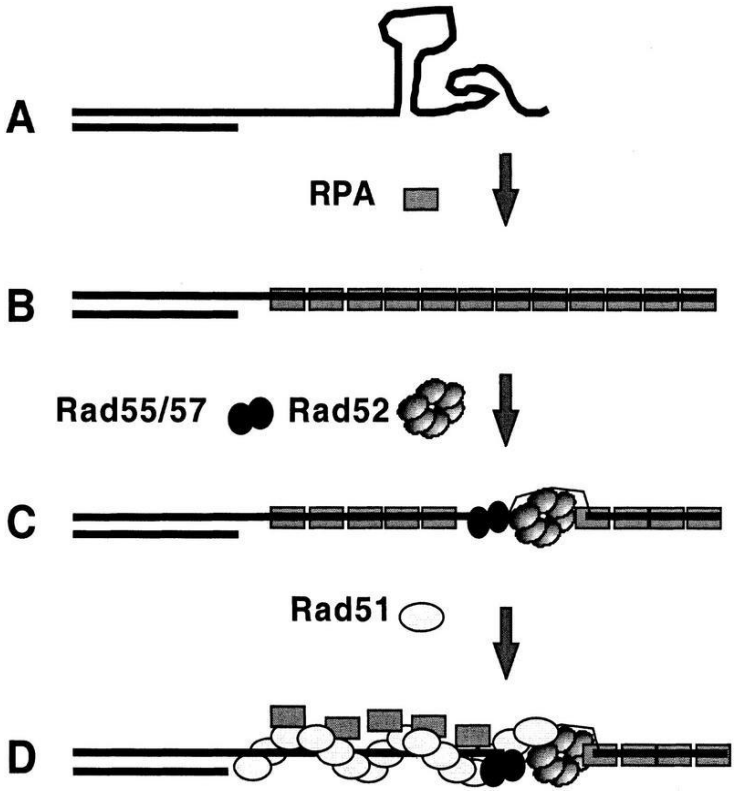

Figure 2. Model for assembly of RPA, Rad52p, and Rad51p. (A) Resection of DSB produces 3' single-stranded tails. (B) RPA assembles onto resected DSBs removing secondary structures. (C) Rad52p and the Rad55p/57p heterodimer bind the ssDNA-RPA filament generating a nucleation site for Rad51p filament assembly. (D) Rad51p filaments are assembled in a cooperative manner and displace RPA from binding sites on ssDNA (Gasior et al. 1998).

Research Focus

While research on the role of *RAD55* in the homologous repair of dsDNA breaks in the nucleus has been conducted, there is not currently literature on its effects in the repair of mtDNA. Therefore, the focus of this study was to begin to characterize the role of nuclear *RAD55* in maintaining mtDNA in budding yeast, *S. cerevisiae*. To accomplish this goal, two assays were performed; a respiration loss assay and a direct repeat-mediated deletion (DRMD) assay. The respiration loss assay is a phenotypic assay used to determine the frequency of spontaneous mutations that impact mitochondrial function. The DRDM assay is used to determine if a particular gene functions in stabilizing the mitochondrial genome from mutations caused by recombination events. The results of these assays better characterized the function of *RAD55* in the mitochondrial stability of *S. cerevisiae*.

Materials and Methods

Yeast Strains

Strain Name	Genotype
DFS188	*MATa ura3-52 leu2-3,112 lys2 his3 arg8::hisG*
NRY112	*MATa rad55Δ::KAN^{R} ura3-52 leu2-3,112 lys2 his3 arg8::hisG*
LKY196	*MATa Rep96::ARG8^{m}::cox2 Rep96::URA3::trp1 ura3-52 leu2-3,112 lys2 his3 arg8::hisG*
LKY953	*MATa rad55Δ::KAN^{R} Rep96::ARG8^{m}::cox2 Rep96::URA3::trp1 ura3-52 leu2-3,112 lys2 his3 arg8::hisG*

Figure 3. Strain names and corresponding genotypes. DFS188 and NRY112 were the wild type and mutant (*rad55Δ*) strains utilized in the respiration loss assay. LKY196 and LKY953 were the wild type and mutant (*rad55Δ*) strains utilized in the DRMD assay. All strains were graciously provided by the lab of Dr. Rey A. Sia at The College at Brockport.

Yeast Growth Media

YPG medium consisted of 10 g/L yeast extract, 20 g/L peptone, 25 g/L agar, and 2% glycerol added after autoclaving. YPG + 0.2% dextrose medium contained the same concentration of ingredients as YPG medium with an additional 2 g/L dextrose. YPD medium consisted of 10 g/L yeast extract, 20 g/L peptone, 25 g/L agar, and 20 g/L dextrose. SD-Ura-Arg medium contained 0.72 g/L CSM-Ura-Arg, 1.7 g/L yeast nitrogen base, 5.0 g/L ammonium sulfate, 20 g/L dextrose, and 25 g/L agar. SD-Trp medium contained 0.74 g/L CSM-Trp, 1.7 g/L yeast nitrogen base, 5.0 g/L ammonium sulfate, 20 g/L dextrose, and 25 g/L agar.

Respiration Loss Assay

Assays were performed with wild type (DFS188) and *rad55Δ* (NRY112) yeast strains. Each strain was suspended in 250 µL of a 20% glycerol solution and frozen at -72 °C. As illustrated in Figure 4, strains were first patched on YPG medium. Patched yeast cells were

incubated for two days at 30 °C. Cells from the incubated YPG plate were streaked for singles on plates containing YPD medium. Streaked yeast cells were incubated for three days at 30 °C.

Individual yeast colonies from streaked YPD plates were transferred to 15 different microcentrifuge tubes such that each microcentrifuge tube contained one yeast colony. Each yeast colony was serially diluted in sterile dH_2O to a final dilution factor of 10^{-5}. For DFS188, 50 µL of the serial dilution was plated on plates containing YPG + 0.2% dextrose medium. For NRY112, 100 µL of the serial dilution was plated on YPG + 0.2% dextrose. Plated cells were incubated for four days at 30 °C.

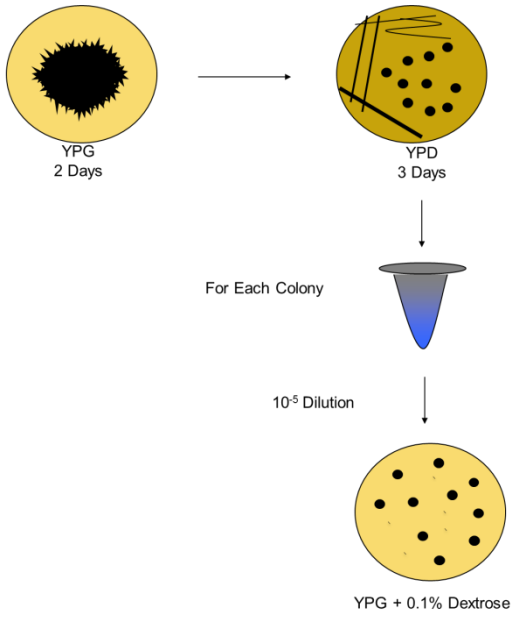

Figure 4. A pictorial representation of the respiration loss assay. Strains were patched on YPG medium and incubated for 2 days. Next, patched cells were streaked for singles on YPD medium and incubated for 3 days. Individual yeast colonies were then transferred to microcentrifuge tubes and diluted by a dilution factor of 10^{-5}. Finally, diluted cell colonies were plated on YPG + 0.2% dextrose medium and incubated for 4 days.

Colonies were counted based on phenotype. Non-respiring (rho^-) colonies appeared petite while respiring (rho^+) colonies appeared large.

The percent respiration loss was calculated by dividing the number of petite colonies by the total number of colonies on each plate. The fraction of petite colonies was multiplied by 100 to obtain the percent petite for each plate. Once the percent petite for each plate was determined, the median percent petite was calculated for all plates in the assay. At least three assay repetitions were performed for mutant and wild type strains. The average of the median percent petites was calculated to give the overall frequency of respiration loss for each strain. Standard deviations and p-values were also calculated.

Direct Repeat-Mediated Deletion Assay

Assays were performed with wild type (LKY196) and *rad55Δ* (LKY953) yeast strains. Each strain was suspended in 250 µL of a 20% glycerol solution and frozen at -72 °C. As outlined in Figure 5, strains were first patched on SD-Ura-Arg medium. Patched yeast cells were incubated for 2 days at 30 °C. Cells from the incubated SD-Ura-Arg plate were streaked for singles on plates containing YPD medium. Streaked yeast cells were incubated for three days at 30 °C. Individual yeast colonies from streaked YPD plates were transferred to fifteen microcentrifuge tubes labeled A_1 through O_1 such that each microcentrifuge tube contained one yeast colony. Each yeast colony was suspended in 100 µL of sterile dH_2O. Five microliters of yeast cell suspension, from tubes A_1 through O_1, was transferred to fifteen corresponding microcentrifuge tubes containing 500 µL sterile dH_2O (a dilution factor of 100) labeled A_2 through O_2. Five microliters of cell suspension from microcentrifuge tubes labeled A_2 through O_2 was transferred to corresponding microcentrifuge tube labeled A_3 through O_3 containing 500 µL sterile dH_2O (a dilution factor of 20,000). Ninety five microliters of yeast cell suspension from microcentrifuge tubes A_1 through O_1 was plated on SD-Trp medium. One hundred microliters of

yeast cell suspension from microcentrifuge tubes labeled A_2 through O_2 was plated on YPG medium. Fifty microliters of yeast cell suspension from microcentrifuge tubes A_3 through O_3 was plated on YPD medium along with 50 µL sterile dH_2O.

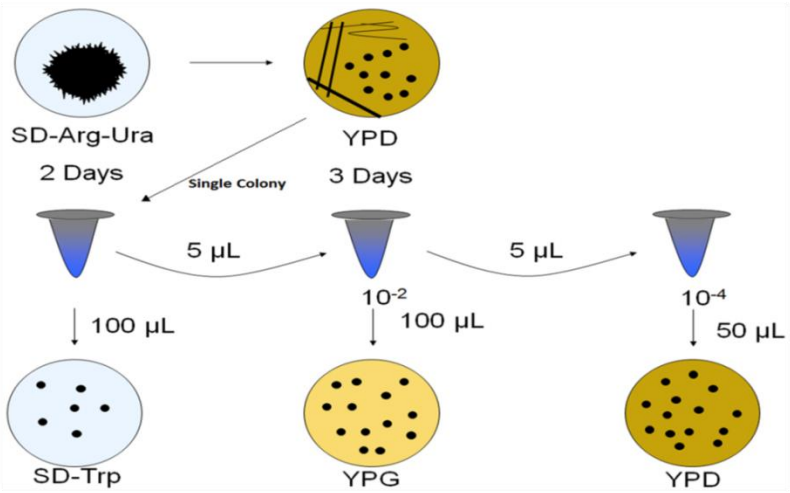

Figure 5. A pictorial representation of the steps involved in the direct repeat-mediated deletion assay. Each strain was patched on SD-Arg-Ura medium and incubated for 2 days. Patched strains were streaked for singles on YPD medium and incubated for 3 days. A single colony was transferred from the streaked YPD plate to three serially diluted microcentrifuge tubes. Diluted portions of each yeast colony were transferred to SD-Trp, YPG, or YPD media which measure the nuclear recombination rate, mitochondrial recombination rate, and total cell count respectively.

Yeast colonies plated on YPG medium were incubated for 3 days at 30°C. Only larger yeast colonies were counted on YPG plates. Colonies on YPG medium also contained a significant amount of background that was not counted. Yeast colonies plated on SD-Trp and YPD media were incubated for 4 days at 30°C. All yeast colonies on YPD and SD-Trp media were counted.

An initial mean was determined from the number of colonies that grew on each of the fifteen YPD plates. The initial mean was then multiplied and divided by 2 to give upper and lower limits. If the number of colonies that grew on a YPD plate did not fall within the range of

the upper and lower limit, the data associated with those plates was thrown out. For example, if the number of colonies on plate A_3 was less than the lower limit of the initial mean, all the data associated with A was thrown out. A new mean was determined for the data that did fall within the upper and lower limits of the initial mean. A total colony count was determined by multiplying the new mean from the YPD plates by its dilution factor (20,000). Using the edited data (excluding the data from plates that did not fall within the upper and lower limit of the initial mean) the median number of colonies that grew on SD-Trp and YPG media was determined. From this point, the Lea-Coulson method of the median was utilized to determine the rate of mutation in nuclear and mitochondrial DNA of LKY196 and LKY953 strains. Because the colonies plated on SD-Trp medium were not diluted, the median number of colonies that grew on SD-Trp represented the revertant frequency (r_o) for nuclear mutations. To determine the revertant frequency for mitochondrial mutations, the median number of colonies that grew on YPG medium was first multiplied by its dilution factor (100) to give r_o. The value of r_o/m could then be interpolated from the Lea-Coulson table (Sia Lab Modified Lea-Coulson Table). If the calculated revertant frequency did not match the values of r_o from the table, the value of r_o/m was extrapolated as shown on the Lea-Coulson table. The mutation frequency (m) was found by dividing r_o and r_o/m. Finally, the rate of mutation was determined by dividing the mutation frequency by the total colony count.

At least three assays were performed for LKY196 and LKY953 yeast strains. The average nuclear and mitochondrial mutation rate was calculated along with corresponding standard deviations and p-values.

Results

Respiration Loss Assay

The respiration loss assay is a phenotypic assay used to determine the rate of spontaneous mutations in yeast mtDNA. Yeast cells that are mitochondria deficient have lost the ability to respire, but can still implement fermentation as a means of energy production. Utilizing the knowledge that glycerol is a non-fermentable carbon source and dextrose is a fermentable carbon source the rate of spontaneous mutation can be determined. As depicted in Figure 6, yeast cells patched on medium containing 2% glycerol (YPG) as the sole source of carbon ensures only respiring cells (rho+) grow. Cells streaked on medium containing 20 g/L dextrose (YPD) allows respiring and non-respiring cells to grow enabling spontaneous mutations to occur without detrimental effects on colony growth. Yeast colonies plated on medium containing 2% glycerol and 0.1% dextrose (YPG + 0.1% dextrose) allows respiring and non-respiring cells to grow, but non-respiring cells arrest growth after the limited supply of dextrose is fermented. Thus, while these colonies do not grow as large as respiring colonies, they still appear petite. Comparing the number of petite colonies to the total number of colonies provides a method to quantitatively determine differences in spontaneous mutation rates between wild type and mutant (*rad55*Δ) strains of *S. cerevisiae*. Therefore, this assay was used to determine what effect, if any, *RAD55* has on the frequency of spontaneous mutations within mtDNA.

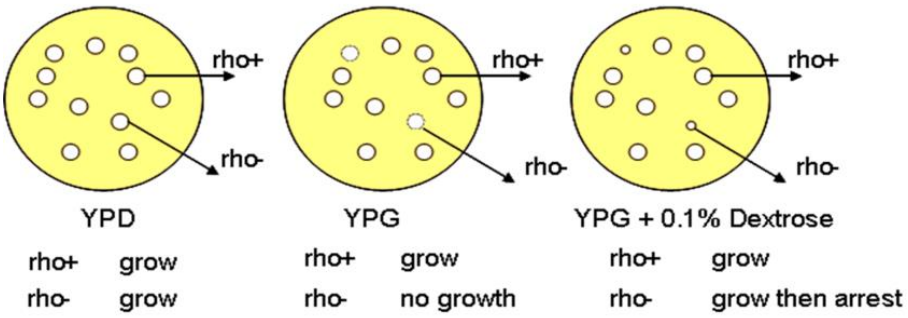

Figure 6. A pictorial representation of respiring (rho^+) and non-respiring (rho^-) colonies' ability to grow on YPD, YPG, and YPG + 0.1% dextrose media. Both respiring and non-respiring colonies grow on YPD media. Only respiring colonies grow on YPG media. Respiring and non-respiring colonies grow on YPG + 0.1% dextrose, but non-respiring colonies arrest growth and remain petite in size.

The rate of spontaneous mutations in mtDNA was compared in wild type (DFS188) and *rad55Δ* (NRY112) strains of *S. cerevisiae*. Three respiration loss assays were performed for each strain. The results of these assays are illustrated in Figure 7. It was determined that the average frequency of respiration loss for DFS188 was 2.84% with a standard deviation of 0.275%. Additionally, the average frequency of respiration loss for the three assays performed with NRY112 was 2.87% with a standard deviation of 0.161%. The average frequency of respiration loss for DFS188 and NRY112 strains were compared and T-test analysis produced a p-value of .858. Therefore, a significant difference did not exist in the average frequency of respiration loss between wild type and *rad55Δ* strains of *S. cerevisiae*.

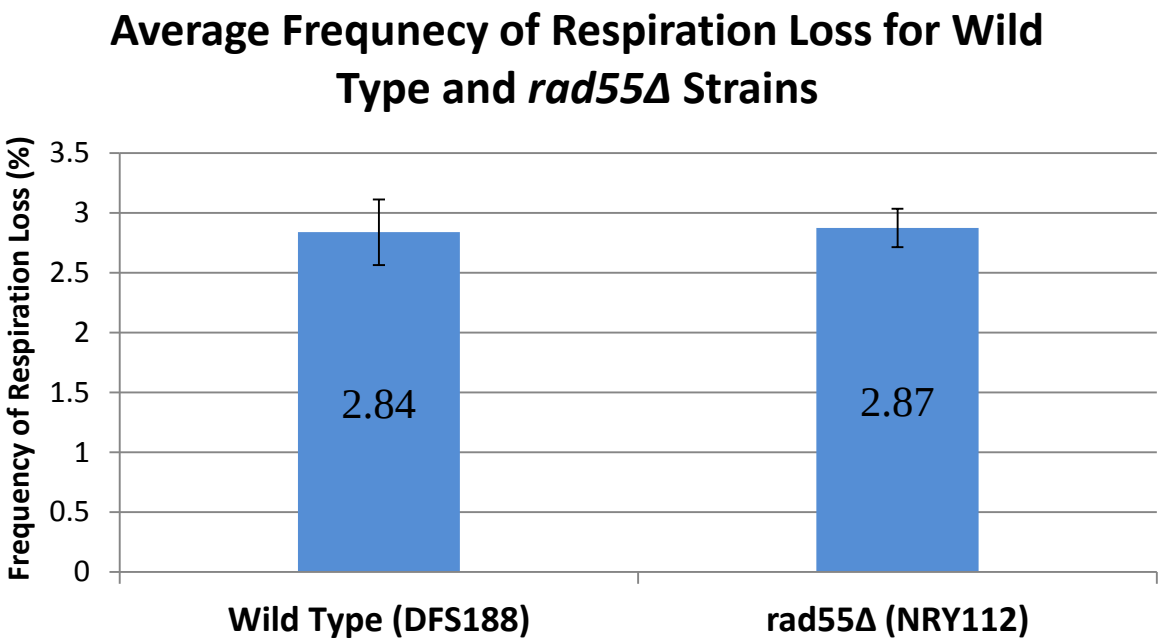

Figure 7. The average frequency of respiration loss for wild type (DFS188) and *rad55Δ* (NRY112) strains of *S. cerevisiae*. The average frequency of respiration loss for DFS188 was 2.84%. The average frequency of respiration loss for NRY112 was 2.87%. No significant difference (p=.859) was seen in the frequency of respiration loss between DFS188 and NRY112 strains.

Direct Repeat-Mediated Deletion Assay

Direct repeats flank two-thirds of reported mtDNA deletions (Phadnis 2005). While the mechanisms by which these deletions arise remains elusive, direct repeat-mediated deletions involving polymerase slippage, homologous recombination, and non-homologous end joining have been proposed (Phadnis 2005). The DRMD assay utilizes existing knowledge on direct repeat-mediated deletions and specific reporters to quantify the rate of direct repeat-mediated deletions in the mitochondrial and nuclear genome. The average number of nuclear and mitochondrial direct repeat-mediated deletion events were determined for wild type (LKY196) and *rad55Δ* (LKY953) strains of *S.* cerevisiae. At least three iterations of the DRMD assay were performed for each strain.

The nuclear DRMD reporter utilized in this experiment consisted of the nuclear gene *TRP1* interrupted by the *URA3* gene. When not interrupted, *TRP1* is responsible for the production of the amino acid tryptophan and *URA3* is responsible for the production of the amino acid uracil. Patching cells on media lacking uracil ensures that only cells containing the functional *URA3* reporter gene are utilized in the assay. Because *URA3* is flanked by direct repeats, it is prone to direct repeat-mediated deletions. When these deletions occur, *URA3* is removed and the gene function of *TRP1* is restored. By plating on media lacking tryptophan, the rate of direct repeat-mediated deletion events in the nuclear genome can be determined. The nuclear DRMD reporter is illustrated in Figure 8.

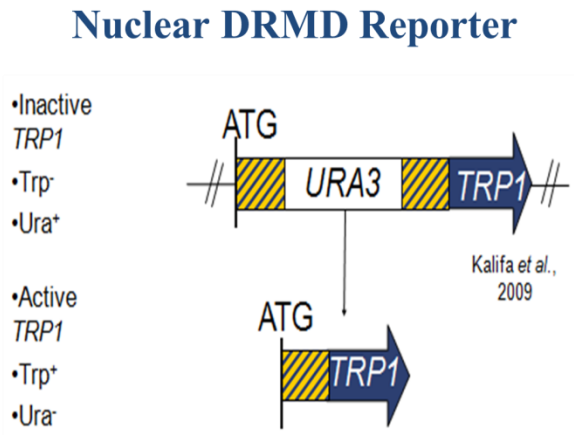

Figure 8. An illustration of the DNA construct created to determine if a direct repeat-mediated deletion occurred in the nucleus. The *TRP1* gene is interrupted by the *URA3* gene. When recombination occurs, the cells' ability to synthesize tryptophan is restored. This can be detected by plating on SD-Trp medium.

The loss of RAD55 gene function resulted in a 5.8-fold increase in nuclear DRMD events. These results are summarized in Figure 9. Specifically, the average number of deletion events per cell division in the nucleus of LKY196 was found to be 16.2×10^{-7} with a standard deviation of 5.73×10^{-7} and the average number of deletion events per cell division seen in

LKY953 was 94.6×10^{-7} with a standard deviation of 1.82×10^{-6}. The average nuclear DRMD rate for the wild type and *rad55Δ* strains were compared and T-test analysis produced a p-value of .00127. It was concluded that a significant difference in nuclear DRMD rates existed between wild type and *rad55Δ* strains of S. cerevisiae.

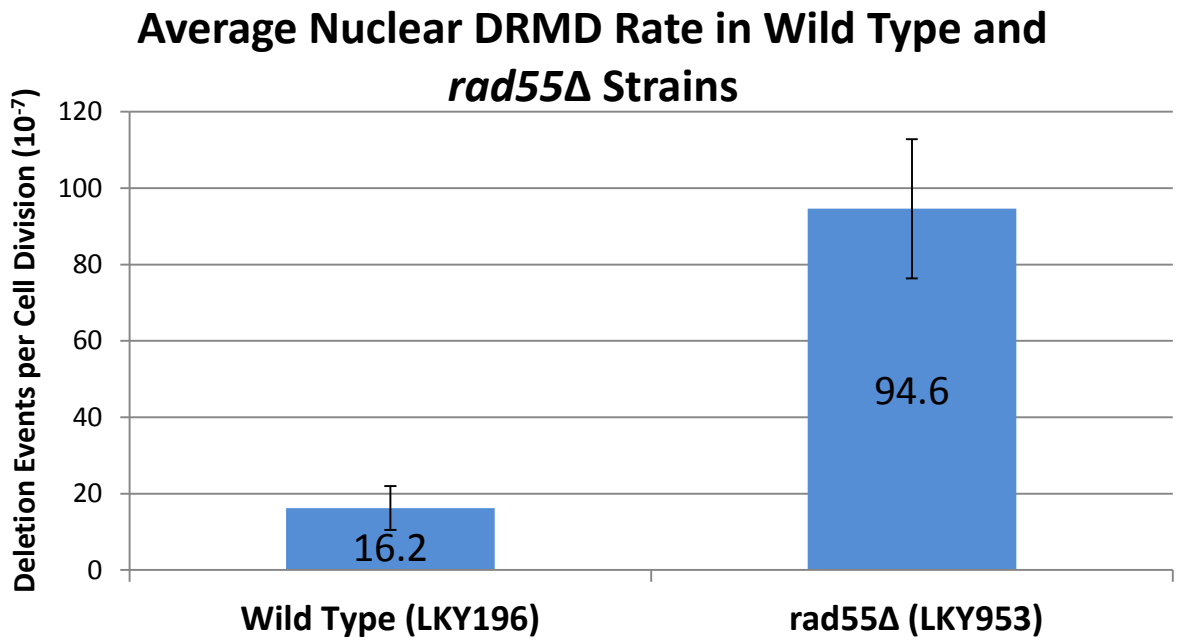

Figure 9. The average nuclear DRMD rate for wild type (LKY196) and *rad55Δ* (LKY953) strains was 16.2×10^{-7} and 94.6×10^{-7} deletion events per cell division respectively. The 5.8-fold increase in deletion events between LKY953 and LKY196 was determined to be significant (p=.0013).

An additional reporter was used to determine the rate of direct repeat-mediated deletions in the mitochondria. The mitochondrial DRMD reporter, depicted in Figure 10, consisted of the essential mitochondrial respiratory gene *COX2* interrupted by a gene producing arginine, *ARG8*^{m}. To ensure the reporter was present in all of the initial cells, cells were patched on media lacking arginine. *ARG8*^{m}, which is flanked by direct repeats, is prone to direct repeat-mediated deletions. When these deletions occur, the activity of *COX2* is restored and cells

regain their ability to respire. The restoration of respiration is measured by plating yeast colonies on media containing an exclusively non-fermentable carbon source such as glycerol.

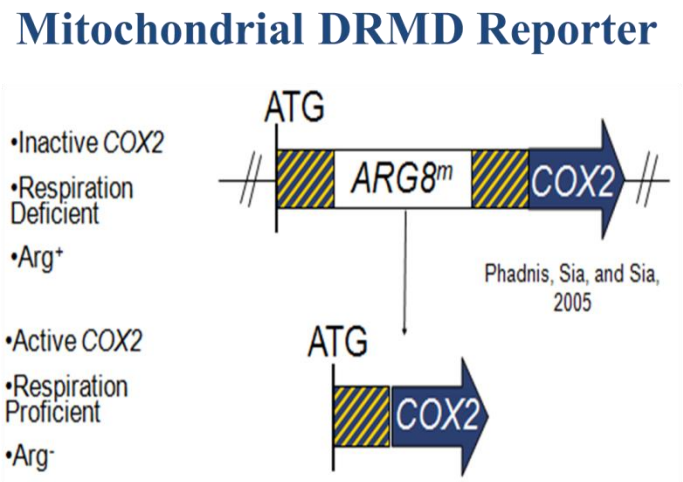

Figure 10. An illustration of the DNA construct created to determine if a direct repeat-mediated deletion occurred in the mitochondria. The *COX2* gene is interrupted by the *ARG8*^{m} gene. When recombination occurs, the cells' ability to synthesize Cox2p is restored enabling the cells to undergo cellular respiration. This can be detected by plating on selective media.

Interestingly, the loss of *RAD55* produced a 1.5-fold decrease in mitochondrial DRMD events. As depicted in Figure 11, the wild type strain underwent an average of 2558.7×10^{-7} deletion events per cell division and the *rad55Δ* strain underwent an average of 1719.1×10^{-7} deletion events per cell division. Additionally, the wild type strain had a standard deviation of 3.82×10^{-5} and the *rad55Δ* strain had a standard deviation of 2.42×10^{-5}. Statistical analysis of these results produced a p-value of .0444 suggesting a significant difference in DRMD events existed between wild type and *rad55Δ* strains.

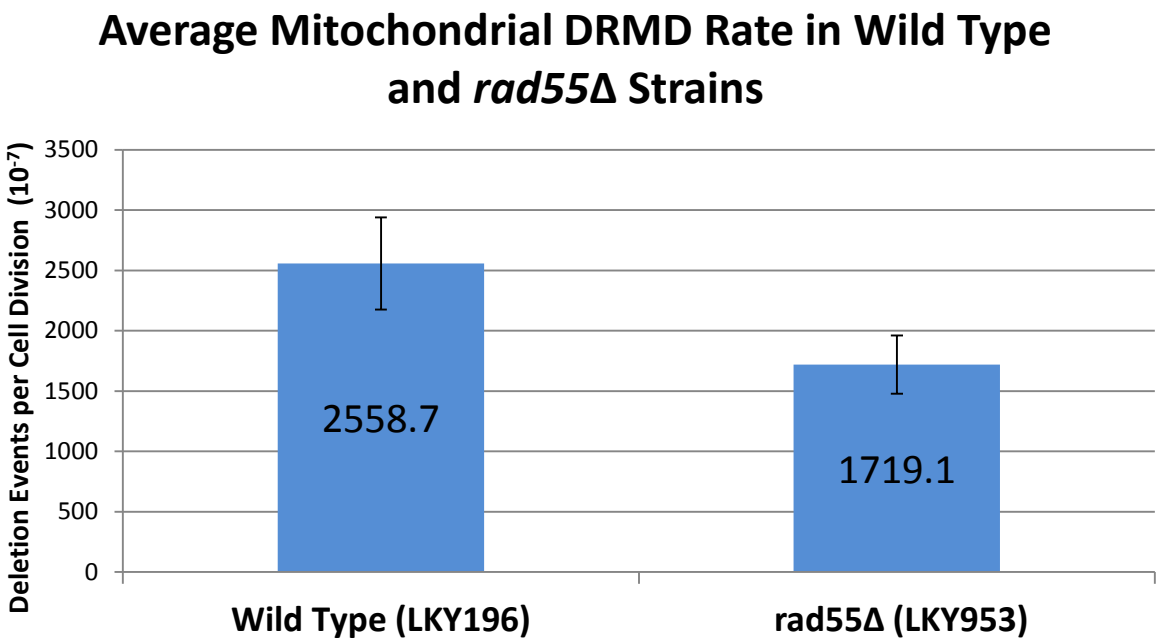

Figure 11. The average mitochondrial DRMD rate in wild type (LKY196) and *rad55Δ* (LKY953) strains was 2558.7×10^{-7} and 1719.1×10^{-7} deletion events per cell division respectively. The 1.5-fold decrease in deletion events between LKY953 and LKY196 was determined to be significant (p=.044).

Discussion

The role of *RAD55* in mitochondria is not as well documented as its role in the nucleus of *S. cerevisiae*. In the nucleus, Rad55p is involved in facilitating strand exchange during homologous recombination of double-stranded DNA breaks. Consequently, loss of *RAD55* has been cited to impair the normal activity of homologous recombination of dsDNA breaks in the nucleus. Limited research has been conducted to investigate the role of *RAD55* in the maintenance and stability of the mitochondrial genome of *S. cerevisiae*. Therefore, the focus of this study was to determine the role *RAD55* plays within the mitochondria.

rad55Δ Strains do not Show a Significant Difference in Respiration Loss

Three iterations of the respiration loss assay for wild type and *rad55Δ* strains resulted in a frequency of spontaneous mutation of 2.84% and 2.87% respectively. These frequencies produced a p-value of .859. Therefore, it was concluded that *RAD55* does not significantly impact the rate of spontaneous mutations within mtDNA.

While the respiration loss assay can be used to determine if respiration is occurring, it has its limitations. Specifically, the respiration loss assay does not discern the specific cause of respiration loss or give an accurate depiction of the number of mutations occurring in mtDNA. For example, a single point mutation that renders the mitochondria non-functional will give the same result, the formation of petite colonies, as a deletion of the entire mitochondrial genome. Additionally, the respiration loss assay only reports mutations that result in a loss of mitochondrial function. This would be beneficial if any and every mutation within the mtDNA of *S. cerevisiae* guaranteed a loss of mitochondrial function, but this is not the case. Mutations in non-coding regions or introns found within the 85.8 kb of *S. cerevisiae* mtDNA do not necessarily result in loss of mitochondrial function. Therefore, while the respiration loss assay can provide some insight into the role of *RAD55* in mitochondrial stability, it is far from the full story. Consequently, the seemingly absent role of *RAD55* in the mitochondrial genome according to the respiration loss assay was not sufficient to squelch further investigation. DRMD assays were conducted to more fully characterize the function of *RAD55* in the stability of the mitochondrial genome. These assays measured the rate of recombination due to spontaneous direct repeat-mediated deletions.

***rad55Δ* Strains Show a Significant Increase in Nuclear DRMD Events**

At least three repetitions of the DRMD assay were performed for wild type and *rad55Δ* strains of *S. cerevisiae*. The nuclear rate of deletion events per cell division for wild type and *rad55Δ* strains was found to be $16.2 \times 10^{-7}$and 94.6×10^{-7} respectively. Statistical analysis of the 5.8-fold increase between wild type and *rad55Δ* strains produced a p-value of .0013 indicating a significant difference.

In the absence of *RAD55* hyper-recombination was observed. Specifically, a 5.8-fold increase in DRMD events was seen. The drastic increase in DRMD events in the absence of *RAD55* contrasts the expected function of *RAD55*. Loss of *RAD55* has been established to impair normal homologous recombination of dSDNA breaks in the nucleus (Sung 1997). Homologous recombination has been proposed as a mechanism in which direct repeat-mediated deletions arise (Phadnis 2005). Consequently, a decrease in homologous recombination, the anticipated effect of losing *RAD55*, would be expected to decrease the frequency of direct repeat-mediated deletions. Therefore, the 5.8-fold increase in DRMD events that accompanied the loss of *RAD55* was unexpected.

Evidently the overall function of Rad55p is to suppress the frequency of recombination within the nucleus. Interestingly, this suggests that in addition to Rad55p facilitating the binding of Rad51p to ssDNA for subsequent strand exchange and homologous recombination, Rad55p also exerts negative regulatory control over recombination. This finding indicates that Rad55p may have additional downstream effects that are distinct from its role in promoting Rad51p function. Future research can be conducted to determine additional functions of *RAD55* in the nucleus.

rad55Δ Strains Show a Significant Decrease in Mitochondrial DRMD Events

Until now there has been little research on the role of Rad55p in the stability of the mitochondrial genome. The results of the mitochondrial DRMD assay indicate a significant 1.5-fold decrease in deletion events following the loss of *RAD55*. The average mitochondrial DRMD rate decreased from 2558.7×10^{-7} deletion events per cell division in the wild type strain to 1719.1×10^{-7} deletion events per cell division in the mutant strain.

The 1.5-fold decrease in deletion events is a novel finding that suggests Rad55p functions differently in the mitochondria than in the nucleus. In the mitochondria Rad55p functions to promote recombination while in the nucleus Rad55p functions primarily to inhibit recombination. Perhaps Rad55p only acts to promote strand exchange within the mitochondria, thus promoting homologous recombination and related direct repeat-mediated deletions, and does not act as a negative regulator of homologous recombination as seen in the nucleus. While this proposed mechanism of action is largely speculative, further research can be conducted to determine the true nature of Rad55p in the stability of the mitochondrial genome.

Future Research

The repair of double-stranded DNA breaks can occur via non-homologous end joining, homologous recombination, or single-strand annealing. One of the pitfalls of the standard DRMD assay is that it does not discern between these modes of DNA repair. The future of this research involves performing an assay that induces double-stranded breaks in mtDNA through the use of a plasmid containing an intein. The induced DRMD assay is a more specific assay in

that it only measures the rate of homologous recombination. This assay will more accurately determine if the 1.5-fold decrease in deletion events per cell division seen in the mitochondria is a result of homologous recombination or some other form of dsDNA break repair.

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