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Significance of the Nuclear Gene *RAD54* in Mitochondrial Genome Stability of
Saccharomyces cerevisiae

A Senior Honors Thesis

Submitted in Partial Fulfillment of the Requirements for Graduation in the Honors College

By
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Biology & Psychology Major

The College at Brockport
May 4, 2017

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ABSTRACT

Mitochondria are essential organelles in eukaryotic organisms that synthesize the energy-providing molecule, ATP, through the process of oxidative phosphorylation. As explained by the endosymbiotic theory, mitochondria contain mitochondrial DNA (mtDNA), distinct from nuclear DNA (nDNA). When mitochondrial function is impaired, and mtDNA stability is compromised, detrimental neuromuscular and neurodegenerative disorders such as Mitochondrial Encephalomyopathy, Lactic acidosis and Stroke-like episodes (MELAS) and Leber's Hereditary Optic Neuropathy (LHON) have the potential to occur. The purpose of this study was to determine the role of the nuclear gene *RAD54* in maintaining mtDNA stability in the budding yeast, *Saccharomyces cerevisiae*.

Although the role of Rad54p in maintaining nDNA stability is understood, its impact on mtDNA stability is relatively unknown. *RAD54* is a member of the *RAD52* epistasis group, coding for a protein vital to the initial steps of homologous recombination and double-stranded break (DSB) repair. Given that members of the *RAD52* epistasis group have been shown to contribute to homologous recombination and DSB repair in mtDNA of *S. cerevisiae*, we hypothesized that loss-of-function *RAD54* would decrease the rate at which homologous recombination in mtDNA occurred (Stein, Kalifa & Sia, 2015). A phenotypic respiration loss assay was performed in a *rad54Δ* strain to determine the frequency of spontaneous mutations in mtDNA that blocked the oxidative phosphorylation process. The mutant strain demonstrated a 1.56-fold decrease in spontaneous respiration loss when compared to wild type (p -value = 0.0574). Interestingly, previous research has demonstrated that the nature of these spontaneous mutations is due to large deletions in the mtDNA. To investigate the role of Rad54p in preventing these deletions from occurring, a direct repeat-mediated deletion (DRMD) assay was performed. The DRMD assay demonstrated a significant 3.23-fold increase in nDNA homologous recombination events (p -value = 0.0158) and a statistically insignificant 1.08-fold increase in mtDNA homologous recombination events (p -value = 0.8741) between *rad54Δ* and wild type strains. Given the present findings of this study, it appears the nuclear gene *RAD54* does not play a significant role in maintaining mtDNA stability in respiration loss or DRMD assays.

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Acknowledgments

I would like to formally acknowledge Dr. Rey Sia whose patience and guidance made this research possible.

INTRODUCTION

Saccharomyces cerevisiae

Commonly known as baker's yeast, *Saccharomyces cerevisiae* is a ubiquitous eukaryotic organism that inhabits plants, mammalian gastrointestinal tracts, and aquatic environments (Martini, 1993). The versatile fungus is a facultative anaerobe, capable of surviving in both aerobic and anaerobic conditions by utilizing glucose as its primary carbon source. In the presence of oxygen and diminished glucose levels, *S. cerevisiae* prefers aerobic respiration, efficiently catabolizing one molecule of glucose to sixteen molecules of the energy providing molecule, adenosine-triphosphate (ATP) (Rodrigues, 2006). Conversely, when oxygen is absent and glucose levels are high, *S. cerevisiae* utilizes fermentation, yielding two molecules of ATP from a single glucose molecule. *S. cerevisiae*'s ability to alternate between aerobic and anaerobic pathways is a result of the organism's ability to regulate gene expression. Research has demonstrated that in the presence of excess glucose, nuclear genes associated with fermentation, such as *ADH*, are upregulated, while mitochondrial *COX* genes, associated with aerobic respiration, are repressed (Duenas-Sanchez, 2012). Such findings are significant because they enable researchers to study mitochondrial function by altering carbon availability.

The Mitochondrial Genome of *S. cerevisiae*

Mitochondria are essential organelles responsible for synthesizing ATP through oxidative phosphorylation. According to the endosymbiotic theory, mitochondria are evolutionarily derived from bacterial endosymbionts related to α -proteobacteria (Sanders & Bowman, 2012). Evidence suggests that these bacterial endosymbionts were integrated into larger cells, establishing a permanent mutual relationship between eukaryote and mitochondrion. As explained by this theory, mitochondria contain mitochondrial DNA (mtDNA), distinct from

nuclear DNA (nDNA). Mitochondrial DNA is found throughout the eukaryotic domain, varying in size and composition between species. As shown in Figure 1, the human mitochondrial genome is approximately 16.6 kilobases (kb) long and encodes two ribosomal RNAs (rRNA), 22 transfer RNAs (tRNA), and 13 polypeptides involved in the electron transport chain (Alexeyev, Shokolenko, Wilson, & LeDoux, 2013). In comparison, yeast mtDNA is approximately 85.8 kb long and encodes three subunits of the ATP synthase complex, three subunits of cytochrome *c* oxidase, and a single ribosomal protein (Wolters, Chiu, & Fiumera, 2015). In *S. cerevisiae*, genes encoding various rRNAs are separated by AT-rich intergenic regions. These regions are marked by numerous introns with interspersed palindromic GC-rich regions (Wolters et al., 2015). Interestingly, these GC-rich regions demonstrate preference for intramolecular recombination, and aid in the maintenance of mtDNA structure and stability (Dieckmann & Gandy, 1987).

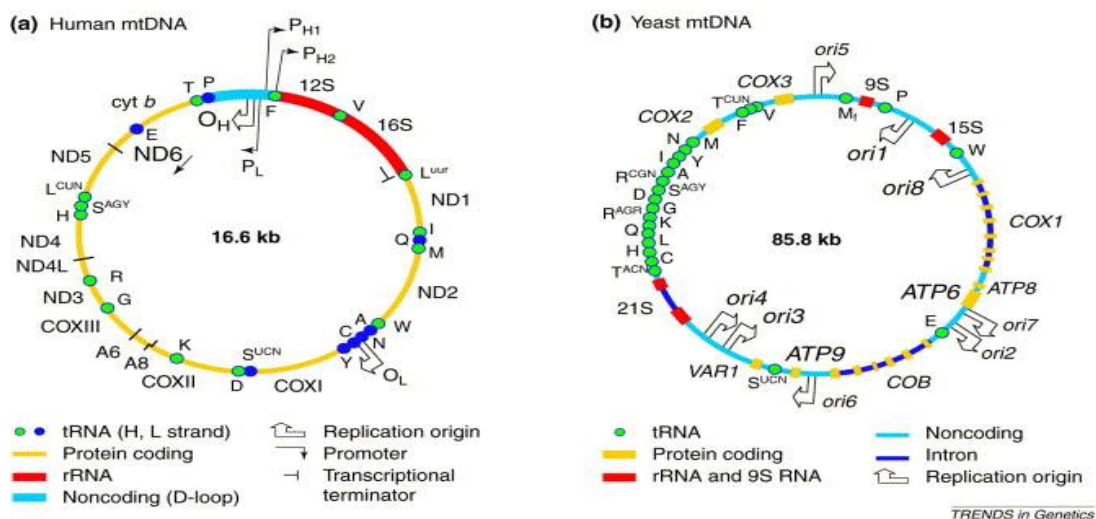


Figure 1. The image above demonstrates the similarities and differences between human and yeast mtDNA. Both yeast and human sequences are circular and lack histone proteins. However, yeast mtDNA contains several origins of replication and possesses noncoding intergenic material in comparison to human mtDNA.

***S. cerevisiae* as a Model Organism in Mitochondrial Research**

Model organisms represent a larger class of organisms sharing related biological processes. Model organisms are useful because they permit ethical experimentation, provide a framework for standardized analysis, and reliably resemble other organisms of interest (Karathia, Vilaprinyo, Sorribas, & Alves, 2011). *S. cerevisiae* has orthologs corresponding to approximately 30% of the genes implicated in human disease, making it an invaluable model organism for studying human biological processes (Karathia et al., 2011). These genetic similarities enable researchers to study various DNA repair mechanisms in genetically damaged *S. cerevisiae* and apply their acquired understanding to DNA repair mechanisms in humans. While nuclear DNA repair mechanisms have been researched extensively, experimentation regarding mitochondrial DNA repair is less prevalent (Bohr, Stevnsner, de Souza-Pinto, 2001). By utilizing *S. cerevisiae* as a model organism in the study of mtDNA repair, understanding the etiology of human mitochondrial dysfunctions such as Mitochondrial Encephalomyopathy, Lactic acidosis and Stroke-like episodes (MELAS) and Leber's Hereditary Optic Neuropathy (LHON) might be augmented.

Possible Mitochondrial DNA Repair Pathways in *S. cerevisiae*

Both nuclear and mitochondrial DNA are vulnerable to endogenous and exogenous damaging agents. Reactive oxidative species (ROS) are formed at high levels as by-products of cellular metabolism, and result in the formation of DNA lesions and oxidized bases (Bohr et al., 2001). Although enzymes in the cytosol such as oxygenases, peroxidases, and oxidases generate small amounts of ROS, more than 95% of ROS superoxide anions ($O_2^{\cdot-}$) are generated by the mitochondria (Bohr et al., 2001). As a result of mtDNA's proximity to ROS anions, mitochondrial DNA is highly susceptible to genetic mutation and damage.

Due to the previous misconception that the mitochondria did not possess DNA repair capabilities, investigation regarding mtDNA repair mechanisms was largely neglected. While controversy regarding repair mechanisms in mtDNA still exists, evidence suggests that mtDNA repair pathways likely include homologous recombination, base excision repair (BER), mismatch repair, and direct reversal (Bohr et al. 2001). Most notably, HR has been indicated in yeast, plant, and mammalian mtDNA; however, specific proteins involved in this process remain largely unknown (Stein, Kalifa, & Sia, 2015).

Homologous Recombination

Homologous recombination (HR) is the exchange of genetic information between homologous DNA sequences, and is critical for maintaining genomic stability by repairing DNA double stranded breaks (DSB). In homologous recombination, the heterotrimeric protein, MRX, binds on either side of the DSB. Sections of DNA around the damaged 5' ends are then resected by the Sae2 protein (Mimitou, Symington, 2008). The resulting 3' overhang invades an intact homologous DNA segment, forming a D-loop, and stimulating either the double-strand break repair (DSBR) pathway or the synthesis-dependent strand annealing (SDSA) pathway.

In the DSBR pathway, the 3' overhang that was not involved in strand invasion forms a cross-stranded Holliday junction with a homologous DNA segment. The resulting Holliday junction is then converted into a recombination product via nicking endonucleases, establishing crossover products. Conversely, in the SDSA pathway the invading 3' overhang strand is extended along the recipient DNA duplex by DNA polymerase. SDSA branch migration forms a Holliday junction between donor and recipient DNA molecules. Through this junction, the synthesized 3' strand anneals to the 3' overhang via complementary base pairing, producing non-crossover recombinants.

Nuclear Gene *RAD54*

The *RAD52* epistasis group plays an essential role in homologous recombination, and consists of genes: *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, and *XRS2* (Symington, 2002). Epistasis group member, *RAD54*, codes for a motor protein involved in double-stranded break repair through synthesis-dependent strand annealing. The DNA-dependent ATPase, Rad54p, is highly conserved in eukaryotic organisms, implicating its functional significance in maintaining genomic stability (Mazin, Mazina, Bugreev, Rossi, 2010). Current studies suggest there is a 66% similarity between *S. cerevisiae* Rad54p and its human homolog, hRAD54 (Mazin et al., 2010).

Rad54p is a member of Superfamily (SF) 2 and possesses seven signature motifs: I, Ia, II, III, IV, V, and VI which constitute the “motor” responsible for Rad54p translocation abilities (Mazin et al., 2010). Within SF2, Rad54p belongs to the SWI2/SNF2 protein family; specifically, this family is involved in chromatin remodeling, DNA topology alterations, and protein displacement (Mazin et al., 2010). Interestingly, overexpression of *RAD52* epistasis group members *RAD51* and *RAD54* results in augmented frequencies of gene repair (Liu, Cheng, van Brabant, & Kmiec, 2002). Such findings indicate that loss of Rad54p function would have significant consequences in DNA repair.

Nuclear Gene *RAD54* in Homologous Recombination

Rad54p is a multifunctional and versatile protein involved in nearly every step of nuclear homologous recombination. Expression of Rad54p is critical for effective displacement loop (D-loop) formation, Holliday junction binding, and D-loop dissociation by branch migration (Mazin et al., 2010). Interestingly, loss of Rad54p function does not inhibit the success of other DNA

repair mechanisms such as single-strand annealing, break-induced replication, and on-homologous end joining (Dudas & Chovanec, 2004).

By interacting both physically and functionally with Rad51p, Rad54p stimulates D-loop formation and aids in the successful creation of heteroduplex DNA (hDNA). While the exact mechanism remains unknown, it is believed Rad54p stimulates D-loop formation by acting as a heteroduplex DNA “pump” (Wright & Heyer, 2014). Research suggests Rad54p supports D-loop formation through the following mechanisms: first, Rad54p associates with, and stabilizes, Rad51p, single-stranded DNA (ssDNA), and chromatin; this process enables Rad54p to translocate along heteroduplex DNA via ATPase activity and remodel nucleosomes (Mazin et al., 2010). This translocation induces conformational change through the generation of positive and negative supercoiled domains, enabling DNA polymerase to access the invading 3’ hydroxyl end upon the dissociation of Rad51p from hDNA (Wright & Heyer, 2014).

Upon the formation of Holliday junctions, Rad54p binds DNA to form a multimeric active complex (Mazin et al., 2010). Rad54p subsequently drives branch migration at the Holliday junction by interacting with the structure-specific endonuclease Mus81-Eme1(Mms4) (Mazin et al., 2010). The interactions between these two proteins stimulates DNA cleavage activity, resulting in D-loop dissociation and homologous recombination termination (Mazin et al., 2010). Given the variety of functional roles Rad54p plays in nDNA homologous recombination, understanding its function in mtDNA is of interest.

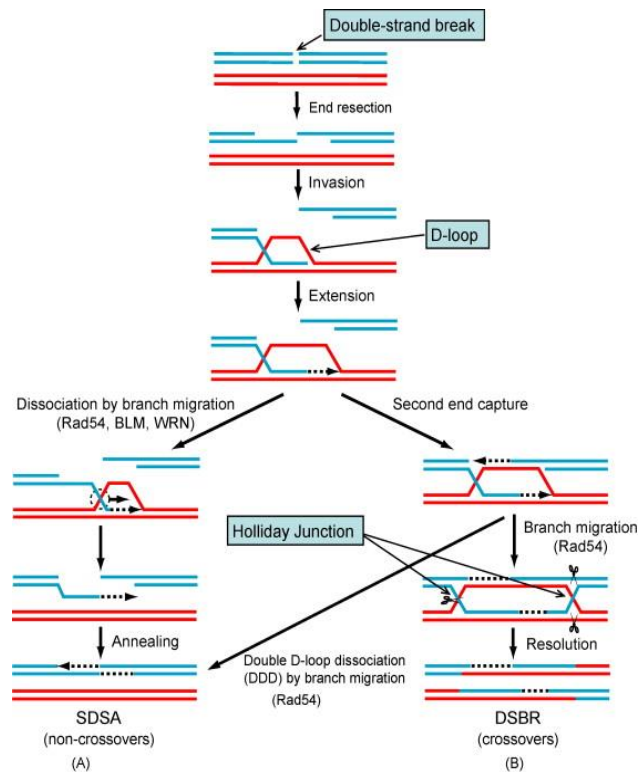


Figure 2. Rad54p is involved in homologous recombination through double-stranded break (DSB) repair and synthesis-dependent strand annealing (SDSA). Initial steps of homologous recombination result in the formation of joint molecules, or D-loops, that are extended by DNA polymerase and processed through one of two major pathways: SDSA or DSB repair. SDSA repair results in non-crossover recombinants, whereas DSB repair results in crossover recombinants. *This image was obtained from Mazin et al., 2010.*

The Role of *RAD54* in Mitochondrial DNA Repair in *S. cerevisiae*

The role of *RAD54* in maintaining nuclear DNA stability is understood; however, its impact on mitochondrial DNA integrity remains relatively unknown. The purpose of this experiment was to determine if the nuclear gene *RAD54* played a role in maintaining mtDNA stability in *S. cerevisiae*. By replacing functional *RAD54* with heterologous DNA coding for the Kanamycin resistance gene, production of the wild type protein, Rad54p, was eliminated, creating a recessive loss-of-function allele, *rad54Δ*. Quantification of mtDNA homologous recombination rates in wild type and mutant strains provided an augmented understanding of the role of *RAD54* in maintaining mtDNA integrity. In order to accomplish this experiment, respiration loss assays and direct repeat-mediated deletion assays were performed. The respiration loss assay phenotypically determined the frequency of spontaneous mutations that detrimentally impacted mitochondrial function, while the direct repeat-mediated deletion assay

determined the functional role of *RAD54* in stabilizing the mitochondrial genome following mutation. Given members of *RAD52* epistasis group contribute to mitochondrial homologous recombination and DSB repair in *S. cerevisiae*, it was hypothesized that loss-of-function *RAD54* would decrease the rate at which homologous recombination in mtDNA occurred (Stein, Kalifa & Sia, 2015).

MATERIALS AND METHODS

***S. cerevisiae* Growth Media**

For this experiment several types of growth media were used. YPG media agar consisted of: 10.0 g/L yeast extract, 20.0 g/L peptone, 25.0 g/L agar, and 2% glycerol. YPD media agar was composed of: 10.0 g/L yeast extract, 20.0 g/L peptone, 25.0 g/L agar, and 20.0 g/L dextrose. YPD + 0.2% dextrose consisted of: 10.0 g/L yeast extract, 20.0 g/L peptone, 25.0 g/L agar, 2% glycerol, and 2 g/L dextrose. The synthetic agar media SD-Ura-Arg contained: 0.72 g/L CSM-Ura-Arg, 1.7 g/L yeast nitrogen base, 5.0 g/L ammonium sulfate, 20.0 g/L dextrose, and 25.0 g/L agar. Finally, SD-Trp agar media consisted of: 0.74 g/L CSM-Trp, 1.7 g/L yeast nitrogen base, 5.0 g/L ammonium sulfate, 20.0 g/L dextrose, and 25.0 g/L agar.

***S. cerevisiae* Strains**

As illustrated in Table 1, four different *S. cerevisiae* strains were involved in the completion of this experiment. The wild type strains, DFS188 and LKY196, were used in the respiration loss and DRMD assays, respectively. The *rad54*Δ mutant strains, NRY155 and LKY950, were utilized in the respiration loss and DRMD assays, respectively. All strains were suspended in 250 μL of 20% glycerol and stored at -72°C.

Table 1. *S. cerevisiae* Strains and Their Corresponding Genotypes

The table below provides the *S. cerevisiae* strains used in this experiment and their corresponding genotypes. The *S. cerevisiae* strains used were provided by the laboratory of Dr. Rey Sia at The College at Brockport.

Strain	Genotype
DFS188	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG</i>
NR117	DFS188 with <i>rad54Δ::KanMX</i>
LKY196	<i>MATa Rep96::ARG8^m::cox2 Rep96::URA3::trp1 ura3-52 leu2-3, 112 lys2 his3 arg8::hisG</i>
LKY950	LKY196 with <i>rad54Δ::KanMX</i>

Respiration Loss Assay

Both the wild type, DFS188, and *rad54Δ* mutant, NR117, were patched on YPG agar media and incubated for approximately 24 hours at 30°C. Cells from the incubated YPG plate were then streaked for individual colonies on YPD media agar and incubated for three days at 30°C.

A serial dilution was performed by transferring individual colonies from the YPD agar media to distinct Eppendorf tubes containing 100 μL sterile water. The solutions were vortexed, and 5 μL of each suspension was transferred to a corresponding Eppendorf tube containing 500 μL sterile water. 5 μL from the resulting solution was then vortexed and transferred to a third Eppendorf tube containing 500 μL sterile water. 100 μL of the resulting 10⁻⁵ dilution was plated on YPD + 0.2% dextrose and incubated at 30°C for three days. Figure 4 illustrates this procedure.

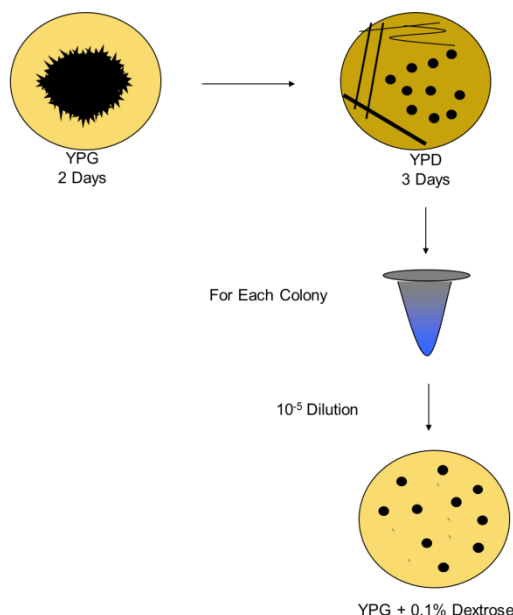


Figure 4. The image to the left illustrates the respiration loss assay procedure. *S. cerevisiae* was patched onto YPG media and incubated at 30°C for 24 hours. Cells were then streaked and incubated on YPD at 30°C for three days. A 10^{-5} serial dilution was performed and cells were plated onto YPG + 0.2% dextrose. The YPG + 0.2% dextrose plates were incubated at 30°C for three days.

The resulting colonies on YPG + 0.2% dextrose were differentiated based on phenotype. Non-respiring, ρ^- colonies had a petite appearance, while respiring ρ^+ colonies had a larger appearance. Percent respiration loss was calculated by dividing petite colony formation by the total cell count. In addition, median respiration loss frequencies for the wild type and mutant strains were calculated.

Direct Repeat Mediated Deletion Assay

Both the wild type, LKY196, and *rad54* Δ mutant, LKY950, were patched onto SD-Ura-Arg agar media and incubated at 30°C for 24 hours. Cells from the incubated SD-Ura-Arg plate were then streaked onto YPD for individual colonies and incubated at 30°C for three days.

A serial dilution was performed by transferring individual colonies from the YPD agar media to distinct Eppendorf tubes containing 100 μ L sterile water. The solution was vortexed, and 5 μ L of the suspension was transferred to corresponding Eppendorf tubes containing 500 μ L sterile water. 5 μ L of the resulting solution was then vortexed and transferred to a third Eppendorf tube containing 500 μ L sterile water. 95 μ L of the original solution was plated on SD-

Trp agar media, 100 μL of the 10^{-2} dilution was plated on YPG media, and 50 μL of the final 10^{-4} dilution was plated on YPD with 50 μL sterile water. SD-Trp, YPG, and YPD plates were incubated at 30°C for three days. Figure 5 illustrates this procedure.

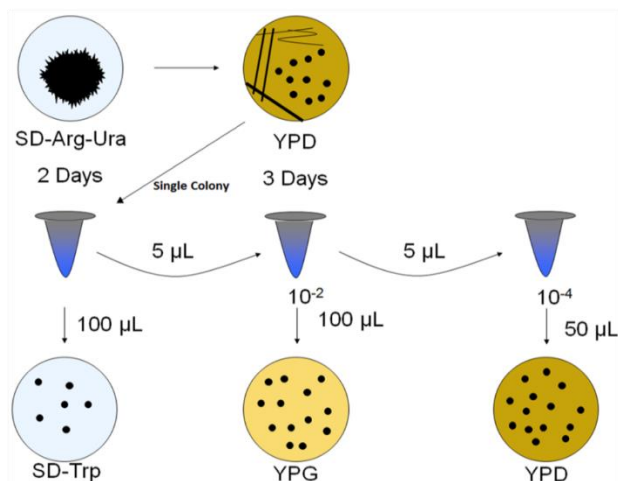


Figure 5. The figure to the left illustrates the DRMD procedure. *S. cerevisiae* was incubated at 30°C for 24 hours on SD-Ura-Arg media. Resulting cells were streaked and incubated on YPD at 30°C for three days. A serial dilution was performed, and cells were plated and incubated on SD-Trp, YPD, and YPD for three days at 30°C .

The resulting colonies were quantified based on the media used. Colonies on SD-Trp and YPD were counted regardless of size, whereas colonies formed on YPG plates were counted based on an individually determined size threshold. In order to determine spontaneous direct repeat mediated deletion events, an initial mean was calculated from the YPD plates. This initial mean was multiplied and divided by two to give upper and lower limits, respectively. Data falling outside of the upper and lower limits was omitted from the remainder of the analysis, and a new mean was calculated accordingly. The new mean was multiplied by the dilution factor, 20,000, to approximate the number of cell divisions on YPD. The medians for SD-Trp and YPG were also calculated based on the upper and lower limit parameters.

Using the SD-Trp and YPG medians, the Lea-Coulson method was applied to determine the rate of mutation in nuclear and mitochondrial DNA (Lea & Coulson, 1949). The nuclear revertant frequency (r_0) was determined using the median number of colonies on SD-Trp; the

mitochondrial revertant frequency was determined by multiplying the median number of colonies on YPG by the dilution factor, 100. Using the Lea-Coulson Table, individual r_0 range values were determined, and the smaller range value was subtracted from the experimentally determined r_0 . The resulting r_0 value was multiplied by the difference between r_0/m , 0.1, and added to the corresponding r_0/m value provided by the Lea-Coulson Table to determine the experimental r_0/m value. In order to determine the number of mutations (m) that occurred, the determined r_0 value was divided by r_0/m . The number of mutations per cell division was then found by dividing m by the total cell count of the given agar plate.

RESULTS

In order to verify the hypothesis that loss-of-function *RAD54* would decrease the rate at which homologous recombination in mtDNA occurred, respiration loss assays and DRMD assays were conducted.

Respiration Loss Assay

By quantifying petite colony formation on YPG + 0.2% dextrose, the rate of spontaneous mutations in *S. cerevisiae* mtDNA was determined. As shown in Figure 6, YPD plates contain the fermentable carbon source dextrose, while YPG and YPG + 0.2% dextrose plates contain the non-fermentable carbon source, glycerol. YPD plates ensure only respiring cells grow, while YPG and YPG + 0.2% dextrose plates permit the growth of both respiring and non-respiring cells. Because dextrose is fermentable, YPD plates enable spontaneous mutations to occur in both ρ^+ and ρ^- cells without largely impacting colony growth. YPG + 0.2% dextrose media permits growth of both ρ^+ and ρ^- cells, however, it arrests ρ^- cell growth once dextrose is depleted, creating petite ρ^- colonies. YPG permits only the growth of ρ^+ cells, which are capable of metabolizing glycerol through oxidative phosphorylation.

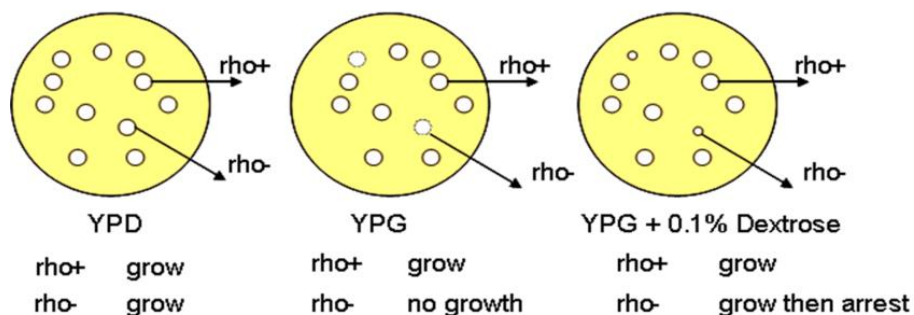


Figure 6. The image above represents the quantitative determination of spontaneous respiration loss in *S. cerevisiae*. YPD plates permit growth of ρ^+ and ρ^- colonies, while YPG plates permit only ρ^+ growth. YPG + 0.2% dextrose permits ρ^+ and ρ^- colonies, arresting ρ^- growth upon depletion of dextrose. By quantifying cell growth on YPG + 0.2% dextrose media a total cell count was obtained. The percentage of petite colonies represent the frequency of spontaneous loss of mtDNA stability.

By comparing the number of petite colonies to the total cell count on YPG + 0.2% dextrose, the effect loss-of-function *RAD54* had on the frequency of spontaneous mutations in mtDNA was calculated. As shown in Figure 7, the rate of spontaneous mutations in mtDNA was compared between wild type, DFS188, and *rad54* Δ mutant, NRY117, strains. The *rad54* Δ mutants had a 1.95% ($SD = 0.492$) average spontaneous respiration loss frequency, while the wild type demonstrated a respiration loss frequency of 3.04% ($SD = 0.883$). Overall, *rad54* Δ resulted in a 1.56-fold decrease in respiration loss. A t-Test Paired Two Sample for Means was performed producing a statistically insignificant p -value of 0.0574.

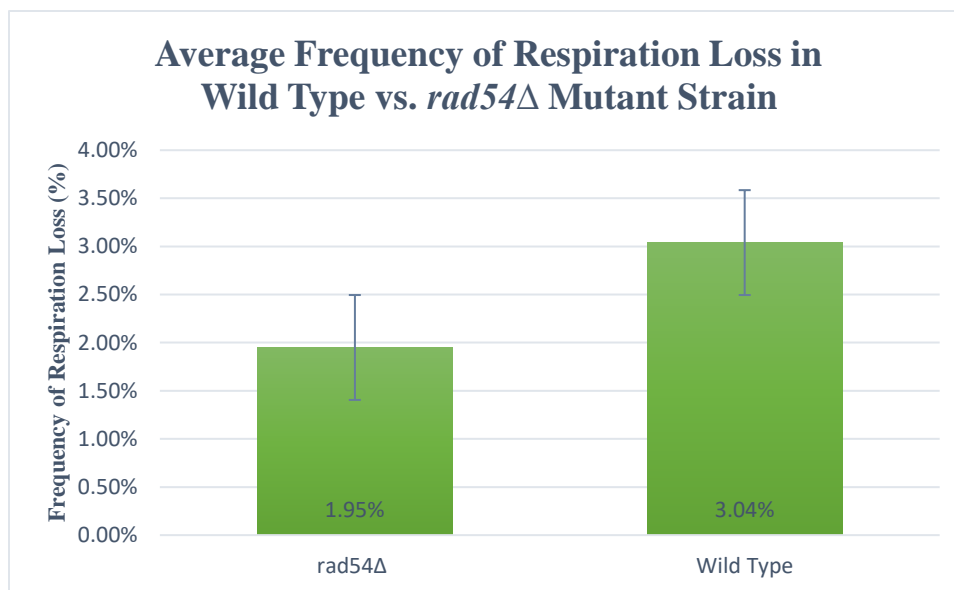


Figure 7. The graph above depicts the average frequency of respiration loss for *rad54Δ* and the wild type. The mutant strain demonstrated a 1.56-fold decrease to 1.95% in spontaneous respiration loss compared to the 3.04% observed in wild type strains. A t-Test Paired Two Sample for Means was performed producing a statistically insignificant *p*-value of 0.0574.

Direct Repeat Mediated Deletion Assay

The direct repeat mediated deletion (DRMD) assay was used to measure the rate of homologous recombination in *S. cerevisiae*. Specific nuclear and mitochondrial reporters were used to quantify the rate at which DRMD events occurred in both mtDNA and nDNA. The rate of deletion events in nDNA was determined by using the nuclear gene, *TRP1*, interrupted with *URA3*. By interrupting the expression of *TRP1*, tryptophan synthesis was inhibited and uracil production was permitted through expression of *URA3*. By patching on SD-Arg-Ura media, only cells with a functional *URA3* reporter gene were able to successfully grow in the absence of uracil. As shown in Figure 8, *URA3* is flanked by direct repeat regions of DNA, prone to nearly two-thirds of mtDNA direct repeat mediated deletions (Phadnis, Sia & Sia, 2005). When these

deletions occur, *URA3* is excised and *TRP1* function is restored, permitting the synthesis of tryptophan. Cells plated on SD-Trp media enabled the determination of the rate at which the interrupting *URA3* gene was deleted in the nuclear genome restoring *TRP1* expression.

Nuclear DRMD Reporter

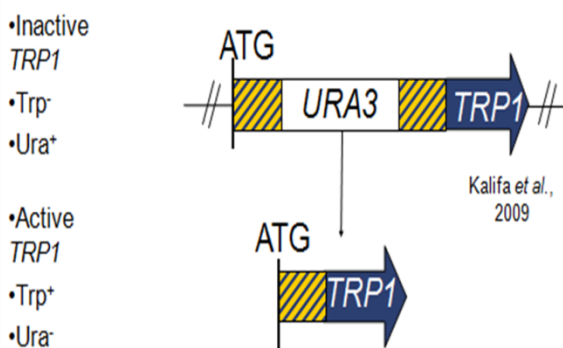


Figure 8. The figure to the left illustrates the nuclear DRMD reporter. When *URA3* is present, *TRP1* is inactive and tryptophan is not synthesized. When recombination occurs, *URA3* is lost and *TRP1* function is restored. This phenomenon is observed by plating on selective media lacking tryptophan.

Through the nuclear DRMD reporter the average number of nuclear direct repeat mediated deletions events were determined for *rad54Δ* and the wild type. As shown in Figure 9 the loss of *RAD54* function resulted in a 3.23-fold increase in nuclear DRMD events. The average rate of nuclear deletion events per cell division was found to be 4.01×10^{-6} ($SD = 1.88 \times 10^{-6}$) for the *rad54Δ* mutant and 1.24×10^{-6} ($SD = 1.44 \times 10^{-7}$) for the wild type. A t-Test Two Sample Assuming Unequal Variances was performed producing a statistically significant *p*-value of 0.0158.

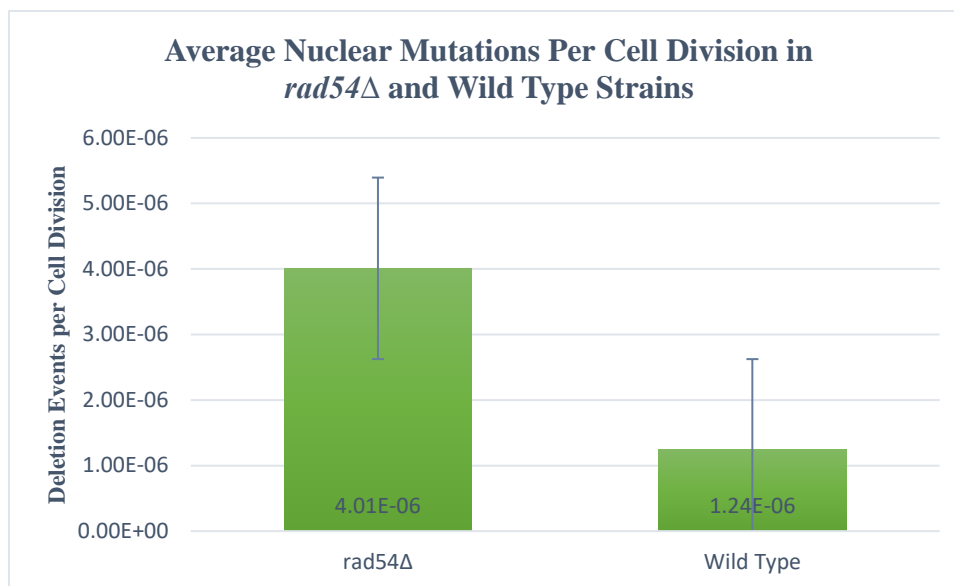


Figure 9. The graph above depicts the average number of nuclear deletion events for *rad54Δ* and the wild type. The mutant strain demonstrated a 3.23-fold increase to 4.01×10^{-6} in homologous recombination events compared to the observed 1.24×10^{-6} in wild type strains. A t-Test Two Sample Assuming Unequal Variances was performed producing a statistically significant *p*-value of 0.0158.

In order to determine the rate of deletion events in the mitochondria, a DRMD mitochondrial reporter was used. As illustrated in Figure 10, the reporter consisted of the mitochondrial respiratory *COX2* gene interrupted by the gene responsible for synthesizing arginine, *ARG8^m*. By patching on SD-Arg-Ura media, only cells containing a functional *ARG8^m* reporter gene were able to successfully grow in the absence of arginine. As shown in Figure 10, *ARG8^m* is flanked by direct repeat regions of DNA, prone to mtDNA direct repeat mediated deletions. When these deletions occur, *ARG8^m* is excised and *COX2* function is no longer interrupted, permitting cellular respiration. Cells with restored *COX2* function were able to successfully grow on YPG media, enabling the determination of the rate at which the interrupting *ARG8^m* gene was deleted in the mitochondrial genome.

Mitochondrial DRMD Reporter

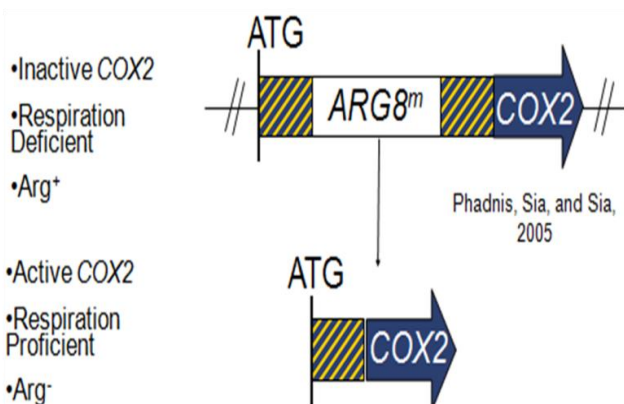


Figure 10. The figure to the left illustrates the mitochondrial DRMD reporter. When *ARG8^m* is present, *COX2* is inactive and cellular respiration is inhibited. When recombination occurs, *ARG8^m* is lost and *COX2* function is restored. This is observed by plating on selective media lacking a fermentable carbon source such as glycerol.

Through the mitochondrial DRMD reporter, the average number of mitochondrial direct repeat mediated deletions events were determined for *rad54Δ* and the wild type. As shown in Figure 11, the loss of *RAD54* function resulted in a 1.08 fold increase in mitochondrial DRMD events. The average rate of mitochondrial deletion events per cell division was found to be 2.57×10^{-4} ($SD = 1.39 \times 10^{-4}$) for the *rad54Δ* mutant and 2.39×10^{-4} ($SD = 1.47 \times 10^{-4}$) for the wild type. A t-Test Two Sample Assuming Unequal Variances was performed producing a statistically insignificant *p*-value of 0.8741.

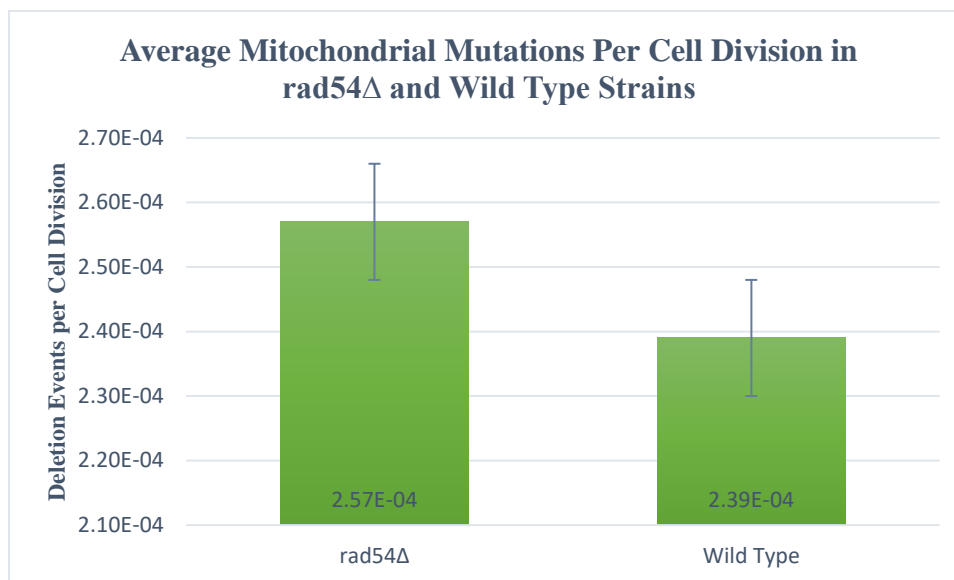


Figure 11. The graph above depicts the average number of mitochondrial deletion events for *rad54Δ* and the wild type. The mutant strain demonstrated a 1.08-fold increase to 2.57×10^{-4} in homologous recombination events compared to the observed 2.39×10^{-4} in wild type strains. A t-Test Two Sample Assuming Unequal Variances was performed producing a statistically insignificant *p*-value of 0.8741.

DISCUSSION

Extensive nuclear research suggests Rad54p plays a versatile role in nuclear homologous recombination, aiding in D-loop formation, binding Holliday junctions, and promoting branch migration. However, the functional role *RAD54* plays in maintaining mitochondrial DNA stability remains relatively unknown. Data from the respiration loss and DRMD assays indicates that the hypothesis loss-of-function *RAD54* would decrease the rate at which homologous recombination in mtDNA occurred should be rejected.

Respiration Loss Assay

***rad54*Δ strains did not demonstrate a significant difference in respiration loss**

Spontaneous respiration loss occurs when mutations eliminate *S. cerevisiae*'s ability to perform oxidative phosphorylation. The purpose of the respiration loss assay was to phenotypically determine the rate at which these spontaneous mutations occurred in *S. cerevisiae* mtDNA. The findings of this study indicate that *rad54*Δ strains did not play a statistically significant role in altering the frequency of mtDNA spontaneous mutations (p -value = 0.0574). While the respiration loss assay indicated diminished mtDNA stability in *rad54*Δ strains, it did not indicate specific mutations responsible for mitochondrial dysfunction. A possible reason for these statistically insignificant findings is that a loss of Rad54p may have been caused by a subtle mutation not detectable in such a general mitochondrial function loss assay. Consequently, further investigation into the role of *RAD54* in maintaining mtDNA stability was warranted.

Direct Repeat Mediated Deletion Assay

***rad54*Δ strains demonstrated a significant increase in nuclear DRMD events**

The nuclear DRMD assay measured the rate at which *URA3* was excised, restoring transcription of *TRP1* in *S. cerevisiae*. The findings of this study indicate that *rad54*Δ strains resulted in a statistically significant increase in nuclear DRMD events (p -value = 0.0158). Interestingly, the absence of functional Rad54p lead to a 3.23-fold increase in nuclear hyper-recombination events. Given Rad54p is involved in nuclear DSB repair through synthesis-dependent strand annealing, it was expected that *rad54*Δ strains would demonstrate decreased nuclear DRMD events. However, current data suggests that loss of Rad54p function might actually augment nuclear recombination events (Schmuckli-Maurer et al., 2003). In a study conducted by Schmuckli-Maurer and colleagues, chromosome loss rates in haploid *S. cerevisiae*

colonies were phenotypically analyzed using the nuclear reporter *ade2-1* interrupted by *SUP11* (Schmuckli-Maurer et al., 2013). Similar to the findings of this study, Schmuckli-Maurer et al. found that wild type strains exhibited a 5.9×10^{-7} mutation rate compared to an increased mutation rate of 13.3×10^{-7} in *rad54* cells (Schmuckli-Maurer et al., 2013). While findings of Schmuckli-Maurer and colleagues were statistically insignificant, they do provide insight into the role Rad54p plays in nuclear recombination events.

One plausible explanation for an increase in nuclear recombination events is that homologous recombination was initiated but not properly terminated in the absence of functional Rad54p (Schmuckli-Maurer et al., 2003). Conversely, genomic stability in loss of function *rad54*Δ mutants may not be caused by inadequate termination of homologous recombination, but might instead be caused by misrepair, resulting in augmented mutation rates (Schmuckli-Maurer, et al., 2003). Given the present findings of this study, it can be concluded that loss of Rad54p function results in increased nuclear mutation rates. Such findings suggest that *RAD54* exerts a negative regulatory control on recombination, and that intergenic recombination is not hindered by loss of Rad54p.

***rad54*Δ strains did not demonstrate a significant difference in mitochondrial DRMD events**

The mitochondrial DRMD assay measured the rate at which *ARG8^m* was excised, restoring *COX2* function. While extensive research has been conducted regarding the role Rad54p plays in nuclear homologous recombination, very little has been conducted investigating the gene's role in mitochondrial genome stability (Bohr et al. 2001). The findings of this study indicate that *rad54*Δ strains did not result in a statistically significant difference in mitochondrial mutation events (p -value = 0.8741). This finding could be explained by the presence of the mitochondrial endonuclease, Cce1, or by the notion that homologous recombination is not used

as a specific repair pathway for double stranded mtDNA breaks (Larsen, Rasmussen & Rasmussen, 2005). Analogous to the function of Rad54p in the nucleus, Cce1 is believed to play a supporting role in maintaining mtDNA stability by resolving Holiday junctions in DSB repair (Larsen et al., 2005). In the presence of the mitochondrial endonuclease Cce1 suggests mtDNA is repaired through HR; however, it is possible that HR is not utilized as a specific repair pathway for double-stranded mtDNA breaks. Further verification for either of these findings would aid in explaining the statistically insignificant difference in *rad54*Δ mitochondrial DRMD events.

Conclusion

Based on the respiration loss and DRMD assay findings, it can be concluded the nuclear gene *RAD54* does not play a significant role in maintaining mtDNA stability. However, further study is needed to definitively support or refute these findings.

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