Spring 6-24-2016

Mitochondrial DNA Stability and Maintenance in Saccharomyces cerevisiae

Nicole Ashman

The College at Brockport

Follow this and additional works at: http://digitalcommons.brockport.edu/bio_theses

Part of the Biology Commons

Repository Citation

http://digitalcommons.brockport.edu/bio_theses/19

This Thesis is brought to you for free and open access by the Department of Biology at Digital Commons @Brockport. It has been accepted for inclusion in Biology Master’s Theses by an authorized administrator of Digital Commons @Brockport. For more information, please contact kmyers@brockport.edu.
Mitochondrial DNA Stability and Maintenance in *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
Nicole Ashman
June 2016

**Supervisory Committee**
Dr. Rey Sia, Adviser
Dr. Stuart Tsubota
Dr. Michel Pelletier
The College at Brockport
State University of New York
Department of Biology

Thesis Defense

Date: 6/24/16
Name: Nicole Ashman

<table>
<thead>
<tr>
<th>Committee Members</th>
<th>Approved</th>
<th>Not Approved</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prayashi</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stuart Paulhus</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>deHoffman</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Graduate Director: CR

Department Chair: Prayashi
Mitochondrial DNA Stability and Maintenance in *Saccharomyces cerevisiae*

By
Nicole M. Ashman

A thesis submitted to the Department of Biology of the College at Brockport, State University of New York, in partial fulfillment of the requirements for the degree of Master of Science
July 20, 2016
Dedication

This research is dedicated to my mother, Jacqueline Mahar Jaros. This beautiful woman inspired me to pursue education and believe in myself. None of this would be possible without her love and encouragement. Rest in peace.
Acknowledgements

I would like to acknowledge Dr. Sia, Dr. Pelletier, and Dr. Tsubota for guiding me throughout the process or writing and defending my thesis. I would also like to thank Dr. Rich, graduate director. I extend an additional thank you to department chair and mentor, Dr. Sia, and everyone who I worked with in his laboratory.
# Table of Contents

Dedication .......................................................................................................................... i
Acknowledgements ........................................................................................................ ii
Table of Contents ........................................................................................................... iii
Table of Figures and Tables ............................................................................................ iv

Abstract............................................................................................................................ 1

**Background Significance** .............................................................................................. 2
Mitochondria...................................................................................................................... 2
Mitochondrial Morphology and Structure ................................................................. 3
Genes Regulating Mitochondria Morphology ............................................................ 6
Yeast as a Model.................................................................................................................. 9
Aerobic Respiration and Fermentation ........................................................................ 10
Mitochondrial DNA ......................................................................................................... 11
Mutations and Disease..................................................................................................... 12
Direct Repeat-Mediated Deletion and Double Strand Break Repair ..................... 13
Mitochondrial DNA Repair Mechanisms for Point Mutations ............................. 23
Genes of Interest ............................................................................................................... 26

**Materials and Methods** ............................................................................................. 30
Strain List with Genotypes/ Media Used ........................................................................ 30
Respiration Loss............................................................................................................... 31
Respiration Loss with Alternate Carbon Sources .................................................... 33
Direct Repeat-Mediated Deletion ............................................................................... 33
Lea and Coulson Method............................................................................................... 35

**Results** ......................................................................................................................... 36
Respiration Loss............................................................................................................... 36
Respiration Loss with Alternate Carbon Sources .................................................... 41
Direct Repeat-Mediated Deletion ............................................................................... 47

**Discussion** .................................................................................................................... 51
Respiration Loss............................................................................................................... 51
Direct Repeat-Mediated Deletion ............................................................................... 54

**References** ..................................................................................................................... 58
List of Figures

Figure 1.  Fusion and fission ................................................................. 3
Figure 2.  Mitochondrial morphological arrangements .................................. 5
Figure 3.  Fusion, fission, and mitophagy pathway ........................................ 8
Figure 4.  Polymerase slippage .................................................................. 14
Figure 5.  Non-homologous end joining ....................................................... 15
Figure 6.  Non-homologous end joining ........................................................ 17
Figure 7.  Unequal sister chromatid exchange .............................................. 18
Figure 8.  Single-strand annealing ................................................................ 20
Figure 9.  Non-crossing over ....................................................................... 22
Figure 10.  Fission machinery ...................................................................... 27
Figure 11.  Respiration loss ......................................................................... 32
Figure 12.  DRMD ....................................................................................... 35
Figure 13.  Respiration loss ......................................................................... 37
Figure 14.  Average percent respiration loss .................................................. 39
Figure 15.  Average percent respiration loss in double knockout strains ......... 40
Figure 16.  RL for wild-type on various carbon sources ................................... 42
Figure 17.  RL for dnm1Δd on various carbon sources ...................................... 43
Figure 18.  RL for clu1Δ-3 on various carbon sources ...................................... 44
Figure 19.  RL for fis1Δ on various carbon sources .......................................... 45
Figure 20.  RL for 4 strains on dextrose and raffinose ..................................... 46
Figure 21.  DRMD nuclear reporter .............................................................. 48
Figure 22.  DRMD mitochondrial reporter ..................................................... 49
Figure 23.  DRMD nuclear rate .................................................................... 50
Figure 24.  DRMD mitochondrial rate ........................................................... 51

List of Tables

Table 1.  Strain list ....................................................................................... 30
Abstract

This research involved studying mitochondrial DNA stability using *Saccharomyces cerevisiae* as a model organism. Gene knockout strains focused on the genes *FIS1*, *DNM1*, *CLU1* and *RTG1*. Both *FIS1* and *DNM1* are involved in mitochondrial fission. The *CLU1* gene encodes a protein that may associate with the core complex of eukaryotic translation initiation factor 3 (eIF3) in *Saccharomyces cerevisiae*. The eIF3 plays a role in initiation of mRNA translation. The specific function of the Clu1p in this process is undefined. The gene knockout of *CLU1* does not affect growth or translation initiation. The knockout does however cause defects in mitochondrial distribution and organization. The *RTG1* gene encodes a transcription factor (bHLH) involved in interorganellar communication. The protein also contributes to communication between mitochondria, peroxisomes, and the nucleus.

Deletion strains of all four genes were studied for spontaneous respiration loss. This assay reveals if certain nuclear gene products have a role in stabilizing the mitochondrial DNA. The mitochondrial genome encodes proteins that are solely required for respiration. This assay compared the spontaneous respiration loss of a wild type yeast strain to those of the various knockout strains described above. In wild type yeast, a 3.26% spontaneous respiration loss is observed. In the *fis1Δ, dnm1Δ, clu1Δ*, and *rtg1Δ* deletion strains, the percentage of spontaneous respiration loss is 13.29%, 16.36%, ~5.55%, and 8.21%, respectively, using dextrose as a carbon source. When raffinose was used as a carbon source, the spontaneous respiration loss was higher for knockout genes involved in morphology. From this result, we can assume that the nuclear genes above are critical in maintaining mitochondrial DNA stability.

Direct repeat-mediated deletion assays were also performed. These assays were performed for both wild-type and *fis1Δ* strains. Both strains contained nuclear and mitochondrial reporters. Using reporters flanked by homologous repeats we were able to quantify the recombination rates in both nuclear and mitochondrial DNA. The purpose of this assay was to compare nuclear and mitochondrial mutation rates for the wild-type and *fis1Δ* strains. Homologous recombination rates were not significantly different for *fis1Δ* strains compared to wild-type in the nuclear genome. There was an increase in the homologous recombination rate in the mitochondrial genome in *fis1Δ* strains. This result indicates that the *FIS1* nuclear gene plays a role in maintaining mitochondrial DNA stability, for without it, mitochondrial DNA recombination rates increase.
Background and Significance

Mitochondria

The central function of mitochondria is the generation of energy within a cell. Mitochondria generate energy through oxidative phosphorylation. This process is a series of redox reactions, which release energy that gets used in the synthesis of adenosine triphosphate (ATP). Many proteins found in the mitochondria are actually nuclear encoded proteins that are transported to the mitochondria. These proteins enter the mitochondria through selective target signals. Mitochondria are a unique organelle in that they contain their own DNA separate from nuclear DNA. Both nuclear and mitochondrial genes are necessary for mitochondrial dynamics and function (Cooper GM 2000). Mitochondria contain two membranes, and inner mitochondrial membrane and an outer mitochondrial membrane. Within a cell mitochondria appear as a tubular, interconnected network. In addition to energy production, mitochondria also generate heme and phospholipids. Another cellular function of mitochondria is their role in calcium signaling, apoptosis, and cell death (Osellame et al. 2012).

Mitochondrial DNA is inherited from an offspring’s mother; hence inheritance of mtDNA mutations does not follow laws of Mendelian genetics. Typically speaking a mtDNA population is heterogeneous. In individuals with a mitochondrial disease, the severity of the phenotype is relative to the accumulation of mtDNA versus wild-type DNA (Osellame et al. 2012). Mitochondrial function and viability is crucial to both cell life as well as cell death.
Mitochondria Morphology and Structure

The exact number and morphology of mitochondria varies from cell to cell and can also change within a cell. Change in mitochondria number and morphology can occur during cell development, cell cycle, or when triggered by environmental factors such as toxins. Mitochondria number and distribution are regulated by fusion and fission, which together maintain homeostasis within the cell. A depiction of fission and fusion is given in Figure 1.

![Mitochondria Diagram](image)

**Figure 1.** Fusion is the joining of mitochondria and is facilitated by various proteins such as Mfn1/2p and Opa1p. Fission is the breaking apart or division of mitochondria and is facilitated by various proteins such as Dnm1p and Fis1p. The ability to regulate fission and fusion of mitochondrial networking is vital to cell stability. (Mandemakers W, Morais VA, De Strooper B (2007) A cell biological perspective on mitochondrial dysfunction in...
Frequent morphology changes as well as motility are characteristic of mitochondria (Karbowski and Youle 2003). Within a cell, mitochondria appear as interconnected, tubular networking (Karbowski and Youle 2003). Rather than isolated organelles as they are often depicted in textbooks, mitochondria are actually assembled as flexible networks within the cell (Vowinckel et al. 2015).

Motility is an important characteristic of mitochondria. Specific molecules facilitate this motility along cytoskeletal elements (Iworima et al. 2016). For example, in neurons, kinesin and dynein molecules are responsible for mitochondria transport along microtubules (Iworima et al. 2016). It is thought that disruption in the motility and morphological arrangement of mitochondria is linked with neurodegenerative disease and aging diseases such as cancer.

The morphology and arrangement of mitochondria is very dynamic and responsive to the environment. For example, when yeast cells shift metabolism from fermentation to respiration, the volume of mitochondria increases (Egner et al. 2002). In response to cell stress such as nutrient deprivation or toxicity, fission is upregulated and mitochondrial networking is condensed into smaller units (Rafelski 2013). Various morphological arrangements of mitochondria are depicted in Figure 2.
Figure 2. Image depicts various mitochondrial morphological arrangements. Mitochondria position can be either a uniform distribution or an asymmetric distribution. The shape may be non-tubular, swollen tubes, or less branching. The size may be a smaller or larger tubular network. The dynamic can be anything from over-fused to over-fragmented. (Rafelski S (2013) Mitochondrial network morphology: building an integrative, geometrical view. BMC Biology 11(1): 1-9)
Another aspect of environmental sensitivity is the ability of mitochondria to induce mitophagy, which is degradation of mitochondria via autophagy. This type of quality control is vital to avoid the accumulation of dysfunctional mitochondria. Several mitophagy pathways have been observed in yeast and mammals, including receptor-dependent and ubiquitination-dependent pathways. The process of receptor-mediated mitophagy acts synergistically with the normal fission/fusion machinery. Typically fragmentation of dysfunctional mitochondria away from the tubular networking of functional mitochondria is a precursor to mitophagy (Wu et al. 2016).

Mitochondrial fusion can be thought of as mitochondria fusing or joining together for complementation. Mitochondrial fission can be thought of as mitochondria dividing or breaking away from each other. Loss of mitochondrial fusion results in an increase in mitochondrial genome mutation rate and deletion (Bertholet et al. 2015). Mitochondrial fission is vital for distribution of mitochondrial nucleoids, and is a necessary precursor for mitophagy (Bertholet et al. 2015). Impairment of these physiological processes is associated with neurodegenerative disease such as Parkinson’s and Alzheimer’s (Bertholet et al. 2015).

**Genes Regulating Mitochondria Morphology**

Several mitochondrial distribution and maintenance (MDM) factors as well as mitochondrial morphology maintenance (MMM) genes have been identified. When these genes are mutated, the result is dysfunctional fission and disrupted mitochondrial transport along the cytoskeleton. The dynamin superclass family is a group of proteins, which play a vital role in mitochondria morphology via fission.
Mutations in genes such as *DNM1*, which is part of the dynamin superclass family, result in a lack of fission. The human homolog for *DNM1* is called dynamin-related protein 1 (*DRP1*). In yeast, genes such as *FIS1* and *MDV1* are also required for proper fission as they interact with *DNM1*. In humans, Drp1p interacts with Fis1p anchored on the outer mitochondrial membrane (De Rose et al. 2015). This process is almost identical in yeast and will be discussed in more detail further on. Both *DNM1* and *FIS1* genes were of interest to us in this study because of their vital roles in mitochondrial morphology.

Several genes are also critical for mitochondrial fusion. The *FZO1* gene and mitofusin genes (*MFN1* & 2 GTPases) are key elements in fusion (Karbowski and Youle 2003). A third large GTPase known as Optic Atrophy 1 (*OPA1*) is also essential for fusion of the outer and inner mitochondrial membranes (De Rosa et al. 2015). There are many genes in addition to those mentioned, which participate in mitochondrial fission and fusion, essential for homeostasis within the cell.

The *PINK1* and *Parkin* genes are essential for sensing mitochondrial damage and signaling mitophagy. *PINK1* accumulates on the outer membrane of damaged mitochondria and recruits *Parkin* and ubiquitin to begin the autophagy signaling process (De Rosa et al. 2015). A representation of the aforementioned proteins and their cellular interactions is given in Figure 3.
Figure 3. This figure shows how Mitofusin 1 and 2 proteins interact with Opa1p to induce mitochondrial fusion. Also depicted is the interaction between Drp1p (Dnm1p) and Fission 1 proteins to facilitate mitochondrial fission. Fis1p is anchored to the mitochondrial membrane and recruits the cytosolic Dnm1p which creates coils that allow for pinching off. Directly below the given fusion and fission diagram is the mitophagy pathway. Pink1p and Parkin interact in this ubiquitination pathway in response to mitochondrial damage. (Powers SK,
Yeast as a Model

*Saccharomyces cerevisiae* or “budding yeast” is a major model organism that has been used in the research of various cellular and molecular pathways in eukaryotes for years (http://www.ncbi.nlm.nih.gov/genome?term=saccharomyces%20cerevisiae). Several characteristics of yeast make it an excellent model organism for eukaryotic research, for example yeast contain mitochondria. They are haploid organisms, which simplifies genetic modification via gene knockouts. The yeast genome is entirely mapped out and it is 12Mb, organized in 16 chromosomes (http://www.ncbi.nlm.nih.gov/genome?term=saccharomyces%20cerevisiae). A smaller, fully mapped out genome is easier to work with. Yeast are facultative aerobes, which can grow via aerobic respiration but prefer fermentation. Much is already known about both respiration pathways, aerobic and anaerobic. Yeast are able to grow at various temperatures including room temperature as well as 30°C. *Saccharomyces cerevisiae* is very easy to culture and maintain in a lab setting (Madigan et al. 2012). An additional benefit to using *Saccharomyces cerevisiae* as a model organism is the abundance of studies and literature that exist. In addition to being a commonly used model organism, *Saccharomyces cerevisiae* is also an important organism for industrial purposes. It is used in the manufacturing of pharmaceuticals, enzymes, beer, wine, and bread.
Due to evolutionary conservation, a simple eukaryote like yeast can be compared to more complex eukaryotes such as humans. Several homologous genes, proteins, and pathways have been identified in yeast and humans.

**Aerobic Respiration and Fermentation**

Aerobic cellular respiration is more efficient for producing adenosine triphosphate (ATP) than fermentation (anaerobic). Aerobic respiration produces 38 molecules of ATP for each molecule of glucose while fermentation only produces 2 molecules of ATP. In aerobic respiration the terminal electron acceptor is $O_2$ and ATP is produced by oxidative phosphorylation. In fermentation an organic compound (not oxygen) serves as the electron donor and acceptor. ATP is produced in fermentation through substrate-level phosphorylation. While the ATP yield is smaller in fermentation, the process is quicker than aerobic respiration (Madigan et al. 2012). *Saccharomyces cerevisiae* or Baker’s yeast has the ability to switch between aerobic and fermentative metabolism. In the presence of a fermentable carbon source such as glucose, yeast prefers fermentation. In the absence of a fermentable carbon source and in the presence of oxygen, yeast with functional mtDNA and nuclear DNA can produce energy through aerobic respiration (Madigan et al. 2012). This characteristic of yeast was critical to quantify respiration loss. Yeast colonies that lost the ability to respire were visible due to a small amount of a fermentable carbon source on plates used. These colonies were termed “petite” because after the carbon source was used up these respiration deficient colonies ceased to grow. Colonies that
retained the ability to aerobically respire appeared larger than the petite colonies as normal growth continues after depletion of the fermentable carbon source.

**Mitochondrial DNA**

The main function of mitochondrial DNA is that it encodes specific proteins, which are vital for synthesis of ATP. Additionally, it encodes proteins required for regular cell functioning and maintenance. Mitochondria are also key players in various intracellular pathways such as apoptosis, calcium signaling, and lipid biosynthesis (Keogh and Chinnery 2015). Although the mitochondrial genome is separate from the nuclear genome, the nuclear genome contains genes that encode for proteins, which are essential for proper mitochondria functioning (Prevost C 2015).

Mitochondrial DNA in *Saccharomyces cerevisiae* is approximately 75 to 85kb in size and each haploid cell contains roughly 25 copies (Langkjaer et al. 2003). Mitochondrial DNA in yeast encodes 8 proteins, which are essential for cellular respiration (Dimmer et al. 2002).

Mitochondrial DNA in humans is approximately 16.6kb of double stranded DNA, which encodes 13 protein products (Mkaouar-Rebai et al. 2016). All 13 protein products are associated with oxidative phosphorylation respiratory complexes. There are also 74 nuclear protein products associated with these respiratory complexes (Mkaouar-Rebai et al. 2016). Most genes are encoded on the heavy strand but some are also on the light strand. The heavy strand contains 12 polypeptide genes, 14 tRNA genes, and 2 rRNA genes. The light strand contains 1 polypeptide gene and 8 tRNA genes. The majority of mtDNA is coding (Mkaouar-Rebai et al. 2016).
Mutations and Disease

Mutations in mitochondrial DNA may occur spontaneously. Accumulated mitochondrial DNA mutations in humans, contributes to aging as well as diseases like cancer, diabetes and heart disease (Kennedy et al. 2012). Impaired mitochondrial function is also associated with neurodegenerative disease such as Alzheimer’s disease (Keogh and Chinnery 2015). In addition to mtDNA mutations leading to impaired proteins in the mitochondria, it can also lead to impaired functions of RNA molecules in the mitochondria (Spangenberg et al. 2016). Mitochondrial diseases are typically the result of disruption in the mitochondrial oxidative phosphorylation processes. Mitochondrial diseases are considered heterogeneous because there can be a wide variation of mitochondrial and nuclear DNA mutations causing the disorder in question (Mkaouar-Rebai et al. 2016). There are several clinical phenotypes to date and the list continues to increase. In the human body, our brains and muscles are the most dependent on ATP generation through oxidative phosphorylation. As a result, mtDNA mutation most often presents phenotypes, which are neurological and neuromuscular (Mkaouar-Rebai et al. 2016). Mitochondrial DNA mutations can be either large-scale rearrangements or point mutations. These point mutations may be in protein-coding genes or in tRNA/rRNA genes (Mkaouar-Rebai et al. 2016). The spontaneous mutation rate of mitochondrial DNA is significantly higher than that of nuclear DNA, by approximately 10 to 17-fold (Tuppen et al. 2010). Additional diseases associated with mitochondrial DNA mutation include: Leigh syndrome, Kearns-Sayre syndrome, Pearson syndrome, Mitochondrial encephalopathy, lactic
acidosis, and stroke-like episodes (MELAS), Chronic progressive external ophthalmoplegia (CPEO), Neuropathy, ataxia, and retinitis pigmentosa (NARP), Leber's hereditary optic neuropathy, and Myoclonic epilepsy and ragged red fibers (MERRF) (Tuppen et al. 2010).

**Direct Repeat-Mediated Deletion and Double Strand Break Repair**

Several proteins have been identified that are responsible for deletion rates and mtDNA repair. Without these proteins, mtDNA stability is greatly compromised. Mitochondrial DNA mutations can also be inherited (Spangenberg et al. 2016). The deletion of mitochondrial DNA at locations of direct repeat sequences is common. Roughly two-thirds of reported mtDNA deletions in humans are flanked by direct repeats (Phadnis et al. 2005). These homologous sequence regions can be referred to as deletion hotspots.

Two pathways that cause direct repeat-mediated deletion (DRMD) have been identified in *Saccharomyces cerevisiae* nuclear DNA. Double-strand breaks (DSBs) initiate both of these identified pathways. These DRMD pathways are called unequal sister chromatid exchange and single-strand annealing, both of which occur during homologous recombination.

In humans, the exact mechanism of these direct repeat-mediated deletions is uncertain, but proposed methods of DRMD include: polymerase slippage, non-homologous end joining, and homologous recombination. All three of these pathways can cause direct repeat-mediated deletion.

**DNA Polymerase Slippage**
One proposed mean of direct repeat-mediated deletion in human mtDNA is the DNA polymerase slippage model. This model involves the template strand and coding strand dissociating during DNA replication. When the strands re-associate, they are misaligned as the first homologous repeat sequence on the coding strand anneals to the second homologous repeat sequence on the template strand. This results in a deletion of one of the repeat sequences from each strand and deletion of the intervening sequence (Phadnis et al. 2005). Figure 4 is a model representation of polymerase slippage deletion.

![Figure 4](image)

**Figure 4.** Model A shows polymerase slippage resulting in a deletion of a homologous repeat and the intervening sequence. (Lovett ST (2004) Encoded errors: mutations and rearrangements mediated by misalignment at repetitive DNA sequences. Molecular Microbiology 52(5): 1243-53)

**Non-Homologous End Joining**
The NHEJ pathway is activated in response to double-strand breaks. This model is a second proposed means of DRMD in human mtDNA. After a double-strand break, the NHEJ complex localizes to the DNA ends at the site of the break. The NHEJ complex with all of its subunits is shown in Figure 5.

![Figure 5](image)

After the Ku70/80 heterodimer is bound to the DSB site, the nuclease complex (Artemis:DNA-PKcs), DNA polymerases (Pol λ and Pol μ), and the ligase complex (XLF:XRCC4:DNA ligase IV) are recruited (Lieber 2010). The ends are modified by the nuclease (deletion) and polymerases (re-synthesis) in order to become compatible and allow for ligation. The final step is ligation of the broken
strands. During end modification, deletions and/or additions of nucleotides often occurs (Gu et al. 2008). DRMD may occur through end modification or two DSBs near each other. The overall process of Non-Homologous End Joining is given in Figure 6.
Unlike HR, NHEJ does not require a homologous template for DSB repair. End processing in this repair pathway may lead to deletion of damaged or mismatched nucleotides. (Lieber MR (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end joining pathway. Annual Review of Biochemistry 79:181-211)

Homologous Recombination
Homologous recombination contains two sub-categories: Unequal sister chromatid exchange and single-strand annealing. Homologous recombination is the third proposed means of direct repeat-mediated deletion in human mtDNA.

- **Unequal Sister Chromatid Exchange**

  Unequal sister chromatid exchange occurs during homologous recombination after a double-strand break in a repeat sequence. The ends created after a double-strand break can invade a sister chromatid homologous sequence in a misaligned matter leading to a deletion (Phadnis et al. 2005). A figure of unequal sister chromatid exchange is given in Figure 7.

![Unequal sister chromatid exchange](https://sciknowledge.wordpress.com/2013/01/06/genome-evolution/)

**Figure 7.** Unequal sister chromatid exchange occurs with homologous sequences on sister chromatids misaligning and a deletion often results. In this diagram one sister chromatid ends up with 3 repeat sequences and 2 intervening sequences while the other sister chromatid only has one repeat sequence.

(https://sciknowledge.wordpress.com/2013/01/06/genome-evolution/)
• Single-Strand Annealing

Single-strand annealing occurs when the double-strand break is located in the sequence between homologous repeats. In the single-strand annealing (SSA) pathway, the double-strand break generates 3’ single-strand ends. The now exposed homologous repeat sequences anneal to each other. The resulting non-homologous flaps are cleaved and the ends undergo ligation. SSA results in one of the flanking repeat sequences and the intervening sequence being deleted (Phadnis et al. 2005). A diagram of single-strand annealing is given in Figure 8.
It is important to note that the last two proposed means of direct repeat-mediated deletion, NHEJ and homologous recombination, are both examples of double-strand break repair. Double-strand break repair does not always result in DRMD. There are DSBR pathways that do not cause DRMD, such as CO (crossing over) and SDSA.
(synthesis-dependent strand annealing). Both of these pathways fall under the heading of meiotic recombination. Double-strand breaks are intentionally induced in meiosis and are repaired by meiotic recombination. This process is highly regulated by the cell. Crossing over occurs between segments of non-sister chromatids during meiosis. This pathway occurs when chromosome arms on opposing sides of the DSB swap with each other. This recombination event is responsible for the genetic variance of gametes. Synthesis-dependent strand annealing is another form of meiotic recombination and it is often called non-crossing over. This repair mechanism is more common than crossing over. In this pathway, the starting chromosomal arm configuration is preserved (Bernstein et al. 2011). A diagram of these pathways is given in Figure 9.
Figure 9. Non-crossing over via synthesis-dependent strand annealing is shown on the left side of the diagram. Crossing over (in this instance double crossing over) is shown on the right. Both DSBR mechanisms occur during meiotic recombination. (Bernstein H, Bernstein C, Michod RE (2011) Meiosis as an Evolutionary Adaptation for DNA Repair. DNA Repair, Dr. Inna Krumian (Ed.), InTech)
Mitochondrial DNA Repair Mechanisms for Point Mutations

Mitochondrial DNA repair mechanisms are critical for repairing oxidative damage done to mtDNA. Accumulation of mutated mitochondrial DNA and increase in respiration loss are correlated with loss of such repair mechanisms. One example is BER or base excision repair. This repair pathway replaces incorrect and damaged bases. Base modifications are one of the most common forms of mutations as far as endogenous DNA mutations. Examples of base modifications include alkylation, oxidation, abasic sites, DNA strand breakage, and incorrectly incorporated nucleotides (Kim and Wilson 2012). In aerobic species, the leading cause of base modification is ROS or reactive oxygen species. For example, purines are often modified when their ring atoms become oxidized by ROS produced during cellular metabolism. While there are variations of the BER pathway, the overview of steps remains the same. Initially, a DNA glycosylase removes the damaged or incorrect base, leaving a temporary abasic site. Next, an apurinic/apyrimidinic endonuclease or lyase creates an incision in the abasic site. After this step, a lyase or phosphodiesterase removes the sugar fragment that remains at the incision site. Finally, a DNA polymerase fills in the gap left from the base removal and the incision in sealed by a DNA ligase (Kim and Wilson 2012). Oxidative damage in mitochondrial DNA is five to ten times higher than it is in nuclear DNA. Studies have shown that in neurodegenerative diseases such as Alzheimer’s, a significant loss of base excision repair is observed (Canugovi et al. 2015). Pertaining to our study, an
increase in percent respiration loss may also correlate with loss of base excision repair.

Direct reversal is another critical repair mechanism for maintaining genomic stability. This repair mechanism has been reportedly observed in yeast mitochondria, but its existence in higher eukaryotes is still unclear (Alexeyev et al. 2013). Only a couple of types of DNA damage can be repaired by direct reversal. Some types of DNA mutations repaired by direct reversal are pyrimidine dimers and guanine alkylation. Pyrimidine dimers are usually caused by UV exposure and guanine alkylation is usually caused by a methyl or ethyl addition on the purine ring.

Pyrimidine dimers are formed when two adjacent pyrimidines on the same strand of DNA dimerize via a cyclobutane ring. This happens when the double bonds between carbons 5 and 6 become saturated. A structural DNA mutation like this inhibits transcription and replication, so the repair of pyrimidine dimers is crucial (Cooper GM 2000). Alkylation occurs when alkylating agents transfer methyl or ethyl groups to a DNA base. A common example of this is guanine methylation. When the O\textsuperscript{6} position of a guanine base becomes methylated, guanine then pairs with thymine rather than cytosine (Cooper GM 2000).

Direct reversal repair of pyrimidine dimers is accomplished through a process termed photoreactivation. This process uses visible light as the energy source to break the bonds of the cyclobutane ring. After these bonds are broken the pyrimidines are no longer dimerized and transcription/replication can proceed. Direct reversal of alkylation is accomplished through the action of specific enzymes. For
example when an alkylating agent methylates guanine, O6-methylguanine methyltransferase enzyme transfers the methyl to the active site of a cysteine residue (Cooper GM 2000). Due to the increased levels of mutation in mitochondrial DNA, all of these repair mechanisms are crucial to genome stability. There is still a lack of evidence in the literature as to all of the mtDNA repair mechanisms and a need for further investigation.

Mismatch repair (MMR) is another critical repair mechanism in the mitochondrial genome. Mismatch repair targets base-to-base mismatches as well as mismatches resulting from insertions and deletions, which may occur during replication or recombination. MMR acts during replication to inhibit the transfer of DNA mutations that would otherwise occur during cell division. The MMR pathway has been fully defined in *E. coli* as a model and several homologous proteins have been identified in human MMR by comparison. Proteins involved in human MMR include homologs for MutS, MutL, EXO1, ssDNA-binding protein RPA, PCNA, DNApolα, and DNAlig I (Li G-M 2008). First, MutS is formed as a heterodimer ATPase and can be either hMutSα or hMutSβ. The hMutSα heterodimer recognizes smaller scale mismatches while the hMutSβ heterodimer recognizes larger scale mismatches. Upon recognition, the ATPase initiates MMR. hMutLα also possesses ATPase activity and is responsible for regulating the termination of MMR excision. Next, PCNA interacts with subunits of MutS and MutL. This interaction contributes to initiation and DNA re-synthesis. EXO1 is an exonuclease responsible for cleaving mismatched nucleotides. It also interacts with subunits of MutS and MutL. 5’
mismatch excision requires the presence of MutS and RPA. 3’ mismatch excision requires the presence of MutLα endonuclease. MutLα endonuclease requires PCNA and RFC for activation. After the mismatch is detected and excision is complete, DNApolδ is responsible for DNA re-synthesis and DNAlig I ligates the nick in the strand (Li G-M 2008). A MutS homolog has been identified in yeast. This homolog (MSH1) localizes to mitochondrial DNA. A knockout of this MMR protein in yeast results in severe mtDNA instability (De Souza-Pinto NC et al. 2009).

RER or ribonucleotide excision repair is another repair pathway, which may be present in mitochondria. Ribonucleotide mutations occur when DNA polymerases incorporate ribonucleotides into DNA during replication. During this repair pathway RNase H2 incises the inappropriately incorporated ribonucleotide. Excision is further carried out by FEN1 or flap endonuclease. DNA polymerase δ, a PCNA clamp, and the clamp loader RFC are all responsible for strand displacement re-synthesis. DNA ligase I ligates the nick created by the excision process (Sparks et al. 2012). While the process is not entirely understood, loss of RNase H2 function results in genomic instability.

Genes of Interest

FIS1

The FIS1 gene is 468bp in length, the Fis1p protein is 155aa long, and the molecular weight of the Fis1p protein is 17,738.7Da. The Fis1p protein is a positive regulator of mitochondrial fission located on the outer membrane surface of mitochondria (http://www.uniprot.org/uniprot/P40515). Fis1p is involved in
mitochondrial fission and peroxisome abundance. Peroxisomes contain enzymes vital to various metabolic processes. The balance of fission and fusion rates is what controls mitochondria size and their ability to form closed networks within a cell. Various factors regulate fission and fusion rates, including metabolic and pathogenic conditions internally as well as the external cellular environment. Fission and fusion are important for mitochondrial growth, for redistribution, and for maintaining a functional interconnected network. Fission and fusion mechanisms also play vital roles processes such as apoptosis and autophagy (Van der Bliek et al. 2013).

Proper mitochondrial membrane fission in yeast requires Fis1p, Mdv1p and Dnm1p. Fis1p recruits Mdv1p to the mitochondria surface. Next, Dnm1p is also recruited and this protein complex initiates mitochondrial division (Bradshaw et al. 2012). The entire assembled fission complex is given in Figure 10 with Fis1p anchored to the mitochondrial membrane, Mdv1 anchored to Fis1p, and Dnm1p anchored to Mdv1.

**Figure 10.** Westermann B (2010) Mitochondrial fusion and fission in cell life and death. *Nature Reviews Molecular Cell Biology* 11: 872-884
In humans, Fis1p is also present on the mitochondrial membrane. Rather than Mdv1p or Caf4p, three proteins Mff (mitochondrial fission factor), MiD49 and MiD51 (mitochondrial dynamics proteins) are responsible for recruiting the Dnm1 homolog, Drp1 (dynamin-related protein). Overexpression of FIS1 results in mitochondria fragmentation from increased fission. The knockout of FIS1 results in elongated mitochondria from the absence of fission (Losón et al. 2013).

**CLU1**

The CLU1 gene is 3,834bp long, the Clu1p protein is 1277aa in length, and the molecular weight of the Clu1p protein is 145147.6Da (http://www.yeastgenome.org/). The Clu1p is a protein that associates with the core complex of eukaryotic translation initiator factor 3 (eIF3) in *Saccharomyces cerevisiae*. The eIF3 is present in mammals, plants, and yeast, but is comprised of different subunits. The eIF3 plays a role in initiation of mRNA translation (Browning et al. 2001). The specific function of the Clu1p in this process if undefined. The gene knockout of CLU1 does not effect growth or translation initiation. The knockout does cause defects in mitochondrial distribution and organization. The phenotype for CLU1 inhibition is clustered mitochondria rather than a spread out interconnected network (http://www.yeastgenome.org/).

**RTG1**

The RTG1 gene encodes a transcription factor (bHLH) involved in interorganelle communication. The protein also contributes to communication between mitochondria, peroxisomes, and the nucleus. RTG1 is a main component of
the retrograde signaling pathway. This pathway facilitates communication between the nucleus and mitochondrion. *RTG1* regulates expression of various nuclear genes and allows the cell to respond appropriately to mitochondrial damage (Torelli et al. 2015). *RTG1* also plays a part in antioxidant and stress defense. Knocking out *RTG1* inactivates retrograde signaling, even though there are also *Rtg2* and *Rtg3* proteins. In order for *Rtg1p* to act as a functional transcription factor, it needs to heterodimerize with *Rtg3p* (Torelli et al. 2015). Inhibiting the *RTG1* gene inhibits mitophagy through the retrograde pathway and thus results in an increase of mutant mitochondria.

The main objectives of this project are to determine if various nuclear genes play a role in stabilizing the mitochondrial genome, to compare respiration loss using different carbon sources, and to compare mitochondrial and nuclear DRMD mutation rates between wild-type strain and knockout strain, *fis1Δ*. 
**Materials and Methods**

**Strain List with Genotypes/ Media Used**

The yeast strains, which were used in this study, are given in table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFS188</td>
<td>\textit{MATa ura3-52 leu2-3, 112 ly2 his3 arg8::hisG}</td>
<td>Sia, et al. (2000)</td>
</tr>
<tr>
<td>rtg1Δ</td>
<td>DFS 188 \textit{rtg1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>dnm1Δ</td>
<td>DFS 188 \textit{dnm1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>dnm1Δ rtg1Δ</td>
<td>DFS 188 \textit{dnm1Δrtg1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>dnm1Δ rtg1Δ5b</td>
<td>DFS 188 \textit{dnm1Δrtg1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>clu1Δ-1</td>
<td>DFS 188 \textit{clu1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>clu1Δ-3</td>
<td>DFS 188 \textit{clu1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>clu1Δ-5</td>
<td>DFS 188 \textit{clu1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>CPY127</td>
<td>DFS 188 \textit{fis1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
<tr>
<td>LKY196</td>
<td>DFS 188 \textit{Rep96::ARG8M::cox2 Rep96::URA3::trp1}</td>
<td>Kalifa, et al. (2009)</td>
</tr>
<tr>
<td>CPY139</td>
<td>LKY196 \textit{fis1Δ::kanMX}</td>
<td>R. Sia lab, SUNY Brockport</td>
</tr>
</tbody>
</table>

*Table 1.* All strains of \textit{Saccharomyces cerevisiae} used in this study along with the corresponding genotype and source given.

Growth media used included YPG, YPD, YPG + 0.2% dextrose, YPGal, YPFruc, YPRaff, SD-Trp, and SD-Ura-Arg. Yeast peptone glycerol, or YPG, included the following ingredients: 10 grams of yeast extract, 20 grams of bacto peptone, 25 grams of agar, 900 milliliters of deionized (DI) water, and 100 milliliters of 20% glycerol. Yeast peptone dextrose, YPD, was made using 10 grams of yeast extract, 20 grams of bacto peptone, 25 grams of agar, 1000 milliliters of DI water,
and 20 gram of dextrose. YPG plus 0.2% dextrose plates were made with the same ingredients as the YPG plates, however, 2 grams of dextrose was also added. Yeast peptone galactose, YPGal, was made using 10 grams of yeast extract, 20 grams of bacto peptone, 25 grams of agar, 1000 milliliters of DI water, and 20 gram of galactose. Yeast peptone fructose, YPFruc, was made using 10 grams of yeast extract, 20 grams of bacto peptone, 25 grams of agar, 1000 milliliters of DI water, and 20 gram of fructose. Yeast peptone raffinose, YPRaff, was made using 10 grams of yeast extract, 20 grams of bacto peptone, 25 grams of agar, 1000 milliliters of DI water, and 20 gram of raffinose. All of these types of media were used in respiration loss assays.

Another medium, known as synthetic dextrose (SD), was used in direct repeat-mediated deletion assays. Specifically, SD minus tryptophan (SD-Trp) and SD minus uracil and arginine (SD-Ura-Arg) were made, and YPG and YPD were used again. SD-Trp contained 0.74 grams of Complete Supplement Mixture (CSM)-Trp, 1.7 grams of yeast nitrogen base, 5 grams of ammonium sulfate, 20 grams of dextrose, 25 grams of agar, and 1000 milliliters of DI water. The SD-Ura-Arg and SD-Trp plates contained the same ingredients, however, instead of using 0.74 grams of CSM-Trp, 0.74 grams of CSM-Ura-Arg were used.

**Respiration Loss**

Yeast strains, both wild-type and various gene knockouts, were initially patched on YPG media and incubated overnight at 30°C. The YPG media consisted of yeast extract, peptone, and 2% glycerol to permit general growth. Growth on YPG
indicated functioning mitochondria because respiration is used to metabolize glycerol. After the 24-hour incubation period, patches were streaked for isolation on YPD plates. The YPD plates consisted of yeast extract, peptone, and 2% dextrose. This media allows for spontaneous respiration loss, as both respiration + (rho+) and – (rho-) colonies can utilize dextrose via respiration or fermentation. YPD plates were incubated at 30°C for 3 days (72 hours). From these streak plates, 15 individual colonies of roughly the same size were selected for serial dilution. Serial dilutions were conducted using sterile deionized water to a final dilution of 10^-5. The 15 dilutions (per assay) were plated on YPG+0.2% dextrose media. A diagram of this assay is shown in Figure 11.

![Diagram of assay](image)

**Figure 11.** This figure shows initial patching of yeast on YPG media, followed by streaking for isolated colonies on YPD media. From YPD plates, isolated colonies were diluted and solutions were plated on YPG+0.2% dextrose media to screen for percent respiration loss.

On YPG+0.2% dextrose plates, colonies that retained the ability to respire and utilize glycerol grew to normal size. Colonies that had lost the ability to respire could only utilize the 0.2% dextrose via fermentation and then stopped growing when the dextrose was used up. These rho- (mutant mtDNA) colonies were easily
distinguishable due to their significantly smaller size compared to rho+ (wild-type mtDNA) colonies. To calculate the percent respiration loss, the number of rho- colonies was divided by the total number of colonies and then multiplied by 100 to obtain a percentage. The median percentage for all 15 plates was used as the percent respiration loss for each assay. Sometimes plates became contaminated and could not be counted. At least ten out of fifteen plates had to be counted in each assay in order for the median values to be deemed statistically relevant. Ideally, the assay should be performed at least three times for each strain, but due to time constraints and the amount of strains this was not always possible. This assay was performed once for dnm1Δ, twice for dnm1Δrtg1Δ, twice for dnm1Δrtg1Δ5b, three times for rtg1Δ, three times for clu1Δ3, once for clu1Δ1 and clu1Δ5 and three times for fis1Δ.

**Respiration Loss with Alternate Carbon Sources**

This assay was also modified to observe the effects of using different carbon sources in place of YPDextrose plates for streaking. Other carbon sources included fructose, galactose, and raffinose. The respiration loss assay with fructose was performed once for clu1Δ3 and three times for fis1Δ. The respiration loss assay with galactose was performed once for clu1Δ3 and three times for fis1Δ. The respiration loss assay with raffinose was performed once for dnm1Δrtg1Δ, once for dnm1Δrtg1Δ5b, once for rtg1Δ, twice for clu1Δ3, twice for clu1Δ5, and three times for fis1Δ.

**Direct Repeat-Mediated Deletion**
In order to determine mutation rates in individual genomes, strains of wildtype yeast as well as *fis1Δ* strains with nuclear and mitochondrial reporters were implemented. Initially cells were patched on SD-Ura-Arg plates. This media lacks uracil nucleotides as well as the amino acid arginine. These plates were incubated at 30°C for approximately 24 hours to select for cells containing the reporter genes. From cell patches, cells were streaked onto YPD plates. YPD plates were incubated at 30°C for approximately 72 hours. This incubation period allowed for spontaneous direct repeat-mediated deletion to occur in both the mitochondrial and nuclear genomes. After the incubation, fifteen colonies were added to 100ul of deionized water. A serial dilution was performed to create $10^{-2}$ and $10^{-4}$ dilutions. From the undiluted samples, 95ul were plated onto SD-Trp plates (lacking tryptophan). 100ul of the $10^{-2}$ diluted samples were plated on YPG plates. 50ul of the $10^{-4}$ diluted samples (and 50ul H2O) were plated on YPD plates. All three sets of plates were incubated at 30°C for approximately 72 hours. A diagram of this assay is given in Figure 12.
Figure 12. The DRMD assay involved initially patching on SD-Arg-Ura media with a 2 day incubation. From the patch, YPD media was streaked for isolated colonies. From an isolated colony, various dilutions were performed. From different dilutions as given, solutions were plated on SD-Trp (nuclear genome), YPG (mitochondrial genome), and YPD (total cells) media.

Colonies were counted on the SD-Ura-Arg, YPG, and YPD plates. These numbers were used along with the Lea and Coulson method to determine mutation rates in the mitochondrial as well as nuclear genome. Four assays were performed for the wildtype strain and four assays were performed for the \( fis1\Delta \) strain.

**Lea and Coulson Method**

The data collected from the direct repeat-mediated deletion assays was used to determine mutation rates. First the mean number of colonies on YPD plates was calculated. Then the upper limit was determined by multiplying the mean by two. The lower limit was calculated by dividing the mean by two. Any data from YPD
plates that fell outside of these limits was discarded along with the corresponding data for YPG and SD-Trp plates. The nuclear median was found from the SD-Trp data and the mitochondrial median was found from the YPG data. Next, the YPD mean was multiplied by 20,000 to yield the total number of cells. The $r_0$ of mitochondrial data was calculated by multiplying the YPG median by 100. The $r_0$ of nuclear data was calculated by multiplying the SD-Trp median by 1. After calculating these $r_0$ values, the Lea and Coulson chart was used. The determined $r_0$ value fell in between two chart values in the $r_0$ column. The determined $r_0$ value was subtracted from the higher of the two chart $r_0$ values it fell in between. This number was divided by the difference of the two chart $r_0$ values, and was then multiplied by 0.1. The $r_0/m$ value was calculated by adding this number to the $r_0/m$ chart value corresponding with the lower chart $r_0$ number. The determined $r_0$ was then divided by the calculated $r_0/m$ value to yield variable $m$. Variable $m$ was divided by total number of cells to give the mutation per cell division rate. The Lea and Coulson method was used to determine mutation rates for both mitochondrial and nuclear data (Lea and Coulson 1949).

**Results**

**Respiration Loss**

The respiration loss assay was used to determine the roles of various genes in maintaining mitochondrial DNA stability. As previously mentioned, the mitochondrial genome contains genes solely required for respiration. To examine whether or not certain nuclear genes were involved in maintaining mitochondrial DNA stability we performed various gene knockouts. The rate of spontaneous
respiration loss was compared between wild-type strains and knockout strains. If the knockout strains had higher incidence of respiration loss it was determined that these genes must be critical in mitochondrial genome maintenance and repair. For the standard assay, dextrose was used as a carbon source as seen in Figure 13.

**Respiration Loss Assay**

![Figure 13](image)

Figure 13. A respiration loss assay monitors the rate at which cells lose the ability to respire by quantitating petite colony formation on media containing 2% glycerol and 0.2% dextrose. Glycerol is a non-fermentable carbon source. Cells must have functional mitochondria (rho+) in order to grow on media containing glycerol as the sole carbon source. If cells are lacking functional mitochondria (rho-), they can ferment dextrose through an anaerobic metabolic pathway to remain viable.

Yeast cells can use either fermentation or oxidative phosphorylation for their metabolism. When a carbon source such as dextrose is present, yeast will utilize fermentation to generate ATP. This fermentation process is independent of mitochondrion and occurs in the cell’s cytoplasm. When a fermentable carbon source is absent, yeast cells generate ATP through the oxidative phosphorylation pathway. This pathway is dependent on functional mitochondrion. When the yeast cells are plated on YPG + 0.2% dextrose, cells with functional mitochondria can continue to grow via oxidative phosphorylation once the fermentable carbon source it used up.
Petite colonies on these plates were representative of cells that had lost the ability to respire through oxidative phosphorylation and could only utilize the 0.2% dextrose before growth was arrested. These petite (rho-) colonies were easily distinguishable from the normal-sized (rho+) colonies. The percent respiration loss was calculated by dividing the number of petite colonies by the total number of colonies on the YPG + 0.2% dextrose media. The respiration loss assay was performed a minimum of three times for each strain, with the exception of $clu1\Delta-5$ and $clu1\Delta-1$. Averages from these multiple assays were calculated. Various nuclear genes were knocked out to determine the possibility of their role in stabilizing mitochondrial DNA. Along with wild-type, the incidence of respiration loss was observed for $fis1\Delta$, $clu1\Delta$, $rtg1\Delta$ and $dnm1\Delta$ knockout strains. Figure 14 shows the average respiration loss values from the assays performed.
Figure 14. Respiration loss assays were performed for wild-type yeast along with \textit{dnm1\Delta d}, \textit{rtg1\Delta}, \textit{fis1\Delta}, and three different \textit{clu1\Delta} knockouts (\textit{clu1\Delta-1}, \textit{3}, and \textit{5}). A higher incidence of respiration loss was observed for all four gene knockouts. The highest percent respiration loss was observed with \textit{dnm1\Delta d} and \textit{fis1\Delta} knockouts.

Spontaneous respiration loss in wild-type yeast occurs at approximately 3.26%. If the nuclear genes tested had no role in stabilizing mitochondria and mitochondrial function then we would have expected the percent respiration loss to remain the same. For \textit{clu1\Delta} the percent respiration loss was doubled. With our \textit{rtg1\Delta} knockout the percent respiration loss increased by 4.95%. The greatest increase of respiration loss was seen with \textit{dnm1\Delta d} and \textit{fis1\Delta} knockouts. With \textit{dnm1\Delta d} we
observed a 5-fold increase in respiration loss. With fis1Δ we observed a 10.03% increase in respiration loss.

Respiration loss assays were also performed for two double knockout strains. The objective was to compare the percent respiration loss for each individual knockout with the percent respiration loss of a double knockout. The results of the double knockouts are seen in Figure 15.

![Average Percent Respiration Loss in Double Knockout Strains](image)

**Figure 15.** Respiration loss incidence was compared between wild-type, single knockout, and double knockout strains. Respiration loss was higher in single and double knockout strains than it was in wild-type yeast. The P value and standard deviation for dnm1Δrtg1Δ is greater because the assay was only performed twice and the two values differed from each other by roughly 6%.
Again the average percent respiration loss for wild-type yeast is only 3.26%. The \textit{dnm1\textDelta rtg1\textDelta} double knockouts had an increase in respiration loss when compared to the single \textit{rtg1\textDelta} knockout. The \textit{dnm1\textDelta rtg1\textDelta \textDelta5b} average respiration loss was 3.97% higher than \textit{rtg1\textDelta}. The \textit{dnm1\textDelta rtg1\textDelta} average respiration loss was 4.23% higher than \textit{rtg1\textDelta}. Odd results were observed when comparing these double knockouts to the single \textit{dnm1\textDelta d} knockout. The percent respiration loss actually decreased for double knockouts. The \textit{dnm1\textDelta rtg1\textDelta \textDelta5b} average respiration loss was 4.18% less than \textit{dnm1\textDelta d}. The \textit{dnm1\textDelta rtg1\textDelta} average respiration loss was 3.92% less than \textit{dnm1\textDelta d}.

**Respiration Loss with Alternate Carbon Sources**

Another aspect of this assay was to observe if the results varied when a different carbon source was used. The standard assay uses dextrose as a fermentable carbon source. For comparison this was substituted with carbon sources such as galactose, fructose, and raffinose. We were especially interested to see if this made a difference in respiration loss with gene knockouts involved in morphology. The results are given below in Figures 16-20.
Figure 16. Figure 16 gives the average percent respiration loss for the wild-type strain on four different fermentable carbon sources, dextrose, fructose, galactose, and raffinose.

For wild-type cells, the percent respiration loss was slightly increased (by 0.49%) when fructose was the carbon source, compared to dextrose as the carbon source. The percent respiration loss decreased when the carbon source was galactose or raffinose by 2.23% and 2.55% respectively. Figure 16 illustrates that alternate carbon sources galactose and raffinose cause significant decrease in the percent of spontaneous respiration loss for wild-type yeast.
Figure 17. Figure 17 gives the average percent respiration loss for the $dnm1Δd$ knockout strain on four different fermentable carbon sources, dextrose, fructose, galactose, and raffinose.

For $dnm1Δd$, the percent respiration loss on dextrose was 16.36%. This frequency increased by 0.63% with fructose. There was a more dramatic increase in percent respiration loss when the carbon sources were galactose or raffinose. Percent respiration loss with galactose increased by 20.62% and with raffinose it increased by 41.92%. Figure 17 indicates that fructose, as an alternate carbon source, does not drastically increase the percent spontaneous respiration loss. A significant increase in
spontaneous respiration loss is observed when the alternate carbon source is galactose or raffinose for the *dnm1Δ* strain.

**Figure 18.** Figure 18 gives the average percent respiration loss for the *clu1Δ-3* knockout strain on four different fermentable carbon sources, dextrose, fructose, galactose, and raffinose. The P value and standard deviation for percent respiration loss on Raffinose is high because the assay was only performed twice and the two figures obtained differed from each other by almost 8%.

For *clu1Δ-3*, the percent respiration loss on dextrose was 5.55%. This frequency remained relatively the same when the carbon source was fructose or galactose. With fructose there was a minor increase by 0.24%. With galactose there was a minor decrease by 0.9%. A large increase in respiration loss was observed
when raffinose was the fermentable carbon source. With raffinose the percent respiration loss increased by 7.52% but this increase is not significant. Figure 18 highlights that with the clu1Δ-3 strain, alternate carbon sources do not make a significant impact on percent respiration loss.

Figure 19. Figure 19 gives the average percent respiration loss for the fis1Δ knockout strain on four different fermentable carbon sources, dextrose, fructose, galactose, and raffinose.

For the fis1Δ strain, percent respiration loss was greater on fructose, galactose, and raffinose in comparison with dextrose. The percent respiration loss increased by 5.84% with fructose, by 8.29% with galactose, and by 27.87% with raffinose. Figure 19 shows observations that alternate carbon sources increase the percent spontaneous
respiration loss for the *fis1A* strain. The increase is insignificant on fructose, but is significant on galactose and raffinose.

The *clu1Δ-5* strain had 7.05% respiration loss with dextrose as the carbon source and 18% respiration loss with raffinose as the carbon source. The *rtg1Δ* strain had 8.21% respiration loss with dextrose as the carbon source and 0.94% respiration loss with raffinose as the carbon source. The *dnm1Δrtg1Δ* strain had 12.44% respiration loss with dextrose as the carbon source and 60.95% respiration loss with
raffinose as the carbon source. The clu1Δ-5 strain had 7.05% respiration loss with dextrose as the carbon source and 18% respiration loss with raffinose as the carbon source. The dnm1rtg1Δ5b strain had 12.18% respiration loss with dextrose as the carbon source and 56.53% respiration loss with raffinose as the carbon source. Figure 20 illustrates a 10.95% increase in respiration loss on raffinose for the clu1Δ-5 strain. A significant decrease in respiration loss was observed on raffinose for the rtg1Δ strain. Significant increases in respiration loss were observed on raffinose for the dnm1rtg1Δ and dnm1rtg1Δ5b strains.

**Direct Repeat-Mediated Deletion**

This assay was used to determine the rate of homologous recombination events in the nuclear and mitochondrial genomes. Direct repeat-mediated deletions occur spontaneously in both genomes. We compared the frequency of these homologous recombination events in wild-type cells with the frequency of recombination in the fis1Δ strain. In order to observe these recombination events, both nuclear and mitochondrial reporters were created. The nuclear reporter was a TRP1 gene, which was silenced by a URA3 gene. An ATG start codon was located upstream of the URA3 gene, and the URA3 gene was flanked on either side by 96bp homologous sequences. In the event of homologous recombination, this URA3 gene was deleted and the TRP1 gene was activated. This phenotype was quantifiable through our assay. The layout of this reporter construction is given in Figure 21.
Figure 21. Figure 21 illustrates the nuclear reporter created to detect homologous recombination. Upstream is the ATG start codon followed by a *URA3* gene, flanked on either side by a 96bp homologous sequence. The *URA3* gene silences a *TRP1* gene. In the event of homologous recombination, the *URA3* gene is deleted (Ura-) and the ability of the cell to synthesize tryptophan is restored (Trp+). This phenotype is detectable on SD-Trp media, which lacks tryptophan.

The mitochondrial reporter was a *COX2* gene, which was silenced by an *ARG8m* gene. An ATG start codon was located upstream of the *ARG8m* gene, and the *ARG8m* gene was flanked on either side by 96bp homologous sequences. In the event of homologous recombination, this *ARG8m* gene was deleted and the *COX2* gene was activated. This phenotype was quantifiable through our assay. The layout of this reporter construction is given in Figure 22.
Figure 22. Figure 22 illustrates the mitochondrial reporter created to detect homologous recombination. Upstream is the ATG start codon followed by an ARG8\textsuperscript{m} gene, flanked on either side by a 96bp homologous sequence. The ARG8\textsuperscript{m} gene silences a COX2 gene. In the event of homologous recombination, the ARG8\textsuperscript{m} gene is deleted (Arg-) and the ability of the cell to respire (rho+) is restored. This phenotype is detectable on YPG media, which lacks a fermentable carbon source and contains glycerol instead.

Four assays were performed for each strain, wild-type and fis1\textDelta. The direct repeat-mediated deletion (DRMD) rates from all four assays (per strain) were averaged. The nuclear DRMD rates are given in Figure 23. The mitochondrial DRMD rates are given in Figure 24. Using reporters allowed us to screen for nuclear and mitochondrial recombination events. When comparing nuclear recombination events for wild-type and fis1\textDelta strains, no significant difference was observed as seen in Figure 23. However, when comparing mitochondrial recombination events, a significant increase was observed in the fis1\textDelta strain (Figure 24). This indicates that
*FIS1* plays a role in stabilizing mitochondrial DNA. Without the *FIS1* activity, mutation rates increased as mitochondrial genomic stability was compromised.

**Figure 23.** This figure gives the nuclear mutation rates ($10^{-7}$) for both wild-type and *fis1Δ* strains from the DRMD assays. Four assays were performed for each strain and the mutation rates were averaged. Error bars are based on standard deviation. For *fis1Δ* $P=0.4118$. 
Figure 24. This figure gives the mitochondrial mutation rates \(10^{-7}\) for both wild-type and fis1Δ strains from the DRMD assays. Four assays were performed for each strain and the mutation rates were averaged. Error bars are based on standard deviation. For fis1Δ \(P=0.0458\).

Discussion

Respiration Loss

Results from the respiration loss data indicate that DNM1, FIS1, CLU1, and RTG1 genes all play a critical role in maintaining mitochondrial stability. With wild-type yeast cells spontaneous respiration loss occurs at a low rate (3.26%). Genes in mitochondrial DNA encode proteins solely required for respiration. Respiration loss is therefore typically the result of a mutation or damage to the mtDNA. The genes we
knocked out in this experiment were nuclear genes. Several nuclear genes products have been identified that are transported to the mitochondria. Many of these nuclear proteins are responsible for mtDNA repair and general maintenance of the mitochondrial genome and mitochondrial function.

To determine if these nuclear genes, *DNM1*, *FIS1*, *CLU1*, and *RTG1*, had a role in maintaining mitochondrial DNA stability, we knocked them out and compared the resulting respiration loss with that of wild-type cells. With the *dnm1Δ* knockout strain, respiration loss increased 5-fold. The rate increased from 3.26% in wild-type to 16.26% in *dnm1Δ*. It is evident that the nuclear Dnm1p is significant in maintaining the integrity of mtDNA. Without functional Dnm1p, mitochondrial fission is disrupted.

As for the *fis1Δ* knockout strain, another gene involved in morphology, a 4-fold increase in respiration loss was observed. This is again highly indicative of a crucial role in maintaining mtDNA stability. The percent respiration loss increased from 3.26% in wild-type to 13.29% in *fis1Δ*. Without functional Fis1p, mitochondrial fission is disrupted.

With the *clu1Δ* knockout strain, respiration loss was approximately doubled. The percent respiration loss increased from 3.26% in wild-type to 5.55% in *clu1Δ-3*. Respiration loss was also performed for two other *clu1Δ* strains, but was not repeated three times for the values to be statistically relevant. With *clu1Δ-5* respiration loss was 7.05% and with *clu1Δ-1* respiration loss was 6.08%. For these two strains, assays were only performed once each.
Percent respiration loss for \( rtg1^{\Delta} \) increased from 3.26% in wild-type to 8.21% with the knockout. As with the three genes already mentioned, \( RTG1 \) is indicated as a nuclear gene important for maintaining mtDNA stability. Without Rtg1p functioning, the retrograde signaling pathway is disrupted.

The increase in percent respiration loss was higher for \( dnm1^{\Delta} \) and \( fis1^{\Delta} \) and both of these proteins are involved in mitochondrial morphology. Although the increase in respiration loss was significant in \( rtg1^{\Delta} \) and \( clu1^{\Delta} \), the increase was not as great as genes involved in morphology. Both \( RTG1 \) and \( CLU1 \) are not morphology-related genes. A knockout of \( CLU1 \) does however result in a collapsed mitochondria network phenotype.

Another observation was the effect of double knockouts on respiration loss percentage. When we knocked out \( DNM1 \) and \( RTG1 \) simultaneously, percent respiration loss was \(~12.31\%\). This can be considered and increase in respiration loss when comparing to the single \( RTG1 \) knockout (8.21%). Respiration loss decreased when comparing to the single \( DNM1 \) knockout (16.26%) and reasoning for this result is inconclusive.

It was previously observed that respiration loss is also increased on different carbon sources. This only seems to be a trend for gene knockouts involved in morphology. To study this phenomenon we repeated respiration loss assays on raffinose, fructose, and galactose. For \( dnm1^{\Delta} \), respiration loss was 16.36% with dextrose, 16.99% on fructose, 36.98% on galactose, and 58.28% on raffinose. Similar results were observed for \( fis1^{\Delta} \). With \( fis1^{\Delta} \), percent respiration loss was
13.29% with dextrose, 19.13% on fructose, 21.58% on galactose, and 41.16% on raffinose. An explanation for why a different carbon source for fermentation causes an increase in percent respiration loss is still unclear. This phenomenon is not observed in wild-type strains or mutant strains not involved in mitochondrial morphology. For both dnm1Δ and fis1Δ, the highest percent respiration loss is observed when the fermentable carbon source is raffinose. While a possible explanation could be that raffinose requires the most energy for modification before the sugar can enter glycolysis, this is not observed for wild-type yeast. In fact, spontaneous respiration loss for wild-type strains decreases on raffinose when compared with dextrose as the fermentable carbon source.

With the wild-type strain, respiration loss was 3.26% with dextrose, 3.75% on fructose, 1.03% on galactose, and 0.71% on raffinose. In this instance, increase in sugar complexity results in a minor decrease in respiration loss. Respiration loss for clu1Δ-3 was 13.07% on raffinose, 4.65% on galactose, and 5.79% on fructose. These percent respiration loss values are compared with 5.55% on dextrose as the fermentable carbon source. Other than a minor increase in respiration loss on raffinose, there is no significant difference in respiration loss among carbon sources. Respiration loss for clu1Δ-5 was 18% on raffinose compared to 7.05% on dextrose. This increase is still not comparable to that observed in dnm1Δ and fis1Δ. This varying percent respiration loss on different fermentable carbon sources proposes an interesting area of study for further research.

**Direct Repeat-Mediated Deletion**
The rate of spontaneous homologous recombination events in the nuclear genome is not significantly different in \textit{fis1Δ} strains when compared to wild-type strains. The rate decreases from $14.8 \times 10^{-7}$ in wild-type to $12.5 \times 10^{-7}$ in \textit{fis1Δ} strains and the statistical P-value is 0.4118. A high P-value like this indicates that the difference observed is not statistically relevant and could be due to chance. The rate of spontaneous homologous recombination events in the mitochondrial genome is significantly different in \textit{fis1Δ} strains when compared to wild-type strains. The rate increases from $2982.6 \times 10^{-7}$ in wild-type to $5294.225 \times 10^{-7}$ in \textit{fis1Δ} strains and the statistical P-value is 0.0458. A P-value of ~0.05 is significant and not due to chance.

The direct repeat-mediated deletion assays were performed for both wild-type and \textit{fis1Δ} strains. Mitochondrial and nuclear reporters were used to measure the rate of recombination. In this assay, direct repeats or homologous sequences served as “hot spots” for deletion. Double-strand breaks occur and through various pathways of recombination, homologous sequences get deleted. The reporters used allowed us to compare homologous recombination events in both strains and in both the mitochondrial and nuclear genomes. Four assays were performed for wild-type strain \textit{Saccharomyces cerevisiae}. An additional four assays were performed for \textit{fis1Δ} knockout strain yeast. A single assay measures the mitochondrial and nuclear recombination rates simultaneously.

An increase in mitochondrial recombination rates for \textit{fis1Δ} strains can be linked to the critical role of \textit{FIS1} in mitochondrial fission. The absence of \textit{FIS1} leads to a lack of mitochondrial fission. Mitochondrial fission is upregulated to break
dysfunctional mitochondria away from functional mitochondrial networking, and is typically a precursor to mitophagy. In the absence of FIS1, accumulation of dysfunctional mitochondria occurs. This could explain the increase in direct repeat-mediated deletions. Another explanation would be that without this FIS1 gene, involved in mtDNA repair and stability, other genes with similar roles are affected. This could lead to an increase in homologous recombination events in the mitochondrial DNA.

As previously mentioned, homologous recombination events in the mitochondrial and nuclear DNA were quantified using reporters. The nuclear reporter contained a start codon, a 96bp homologous sequence, a URAT gene, another 96bp homologous sequence, and a TRP1 gene. In the presence of the URAT gene, TRP1, which allows for tryptophan synthesis, was silenced. In the event of homologous recombination, URAT would be deleted and TRP1 would become expressed. The mitochondrial reporter consisted of a start codon, a 96bp homologous sequence, a ARG8m gene, another 96bp homologous sequence, and a COX2 gene. In the presence of the ARG8m gene, COX2, which allows for aerobic respiration, was silenced. In the event of homologous recombination, ARG8m would be deleted and COX2 would become expressed.

To select for yeast colonies containing our reporters, S. cerevisiae was initially patched on synthetic SD-Arg-Ura media. This media lacks arginine and uracil. Next, patches were streaked on YPDextrose plates to allow for direct repeat-mediated deletions to occur. Lastly, homologous recombination events were detected
by plating on SD-Trp and YPG plates. Colonies that grew on SD-Trp media, which lacks tryptophan allowed us to quantify nuclear recombination. Colonies that grew on YPG which has glycerol instead of a fermentable carbon source, allowed us to quantify mitochondrial recombination (rho+).

A possible source of error could be miscounting colonies on YPG plates. Colonies were rather small and difficult to see. In addition to these small yeast colonies, there were even smaller colonies present, which were not to be counted as DRMD yeast. The media was also translucent and this added to the difficulty in accurate counting. A uniform minimal size was used for counting in all assays, and P-values indicated statistical relevance for mitochondrial recombination rate differences (P=0.0458). The same difficulty was not encountered with counting colonies on SD-Trp plates. Colonies on these plates were a larger size, visible, and appeared in much lower numbers. Despite margin for error, P-values indicate a significant increase in mitochondrial recombination with fis1Δ strains. This is further evidence of the vital role of FIS1 in maintaining mitochondrial genome stability. Without fission machinery operating successfully, accumulation of damaged mtDNA occurs, and an increase in homologous recombination events is observed. As previously stated, mitochondrial fission is vital for distribution of mitochondrial nucleoids, and is a necessary precursor for mitophagy (Bertholet et al. 2015). Without functional Fis1p, mitophagy is not initiated and a build up of dysfunctional mtDNA results.
References

Journal Articles:


Bradshaw E, Yoshida M, Ling F (2012) Mitochondrial fission proteins Fis1 and Mdv1, but not Dnm1, play a role in maintenance of heteroplasmy in budding yeast. FEBS Letters 586: 1245-1251, ISSN 0014-5793


Iworima DG, Pasqualotto BA, Rintoul GL (2016) Kif5 regulates mitochondrial movement, morphology, function and neuronal survival. Molecular and Cellular Neuroscience 72: 22-33


Losón OC, Song Z, Chen H, Chan DC (2013) Fis1, Mff, MiD49, and MiD51 mediate
Drp1 recruitment in mitochondrial fission. Molecular Biology of the Cell 24(5): 659-667


**Textbooks:**

Bernstein H, Bernstein C, Michod RE (2011) Meiosis as an Evolutionary Adaptation for DNA Repair. *DNA Repair, Dr. Inna Kruman (Ed.), InTech*


**Websites:**


[https://sciknowledge.wordpress.com/2013/01/06/genome-evolution/](https://sciknowledge.wordpress.com/2013/01/06/genome-evolution/)

[http://www.uniprot.org/uniprot/P40515](http://www.uniprot.org/uniprot/P40515)

[http://www.yeastgenome.org](http://www.yeastgenome.org)