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The Role of *CLU1* in Maintaining Mitochondrial Genome Stability and Morphology in *S. cerevisiae*

By

Christine E. Hochmuth

A thesis submitted to the Department of Biological Sciences of the State University of New York College at Brockport in partial fulfillment of the requirements for the degree of

Master of Science

February 8, 2008

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Christine E. Hochmuth

2008

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by Christine E. Hochmuth

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Acknowledgement

I must express sincerest gratitude and utmost respect for my thesis advisor, Rey Sia, PhD., for his guidance during the course of my research at Brockport. Most importantly, I am appreciative of the counsel he offered me in the pursuit of my future educational goals. I would also like to acknowledge my graduate committee members: Dr. Tracey Householder and Dr. Thom Bonner as well as the rest of the faculty of the Department of Biological Sciences for their support. Special thanks to Crystal Allen, Stephanie Carroll, Chad Cornelius, Louis DiDone, and Jonathan Malecki for their assistance and friendship in the laboratory.

Finally, I would like to recognize my parents' instrumental role in my graduate career. Their belief in me provided me with confidence in my abilities. During this process they were there to lend both the support and the words of encouragement that I needed to persevere. I know this same will hold true no matter where science, or life, may take me.

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ABSTRACT

Mitochondrial genome maintenance is essential for the normal function of the cell. Mitochondrial DNA (mtDNA) is located in the matrix, where it is in close proximity to the electron transport chain, which is within the inner mitochondrial membrane. During oxidative phosphorylation, the electron transport chain produces reactive oxygen species (ROS) that may damage the DNA and contribute to mutations within the genome. Mutations in the mitochondrial genome have long been hypothesized as a contributor to diseases, especially those of the neuromuscular system. Mitochondrial mutations have also been linked to some types of cancer, programmed cell death, and aging in humans. The ability to repair this damage is integral for cells to maintain proper function and longevity.

S. cerevisiae is a facultative anaerobe that can grow in the absence of respiration under specific growth conditions, although mitochondria are still required for viability. The lab used a yeast two-hybrid assay with the known mitochondrial protein, Ilv5p, to isolate genes involved in the organization, repair, and recombination of mtDNA. The lab has identified the Clu1p in this screen. Clu1p function was previously found to be required for proper mitochondrial morphology and distribution (1).

My thesis research has focused on creating *clu1*Δ strains and performing fluctuation analysis assays using different reporters that measure specific mitochondrial events. Initial characterization of *CLU1* has shown that loss of Clu1p leads to an increased loss of mitochondrial function which may occur through various

events, such as point mutations, recombinations or deletions, and DNA polymerase slippage. Microscopy has supported previous reports indicating that a *clu1*Δ strain displays a "clustering" phenotype (Fields *et al.*, 1998). This deletion strain exhibits a branched mitochondrial network that is localized to one side within the yeast cell. These data provide evidence that Clu1p plays a central role in mitochondrial genome stability and morphology.

BACKGROUND AND SIGNIFICANCE

Mitochondria.

Mitochondria are the sites of cellular respiration in eukaryotic cells. Their ability to produce adenosine tri-phosphate (ATP), a molecule that stores chemical energy for cellular processes, makes them essential for the viability of the cell. ATP production is accomplished by the catabolism of compounds during glycolysis and in the Kreb's cycle and the transfer of electrons in the electron transport chain leading to the establishment of a chemiosmotic gradient and finally oxidative phosphorylation by the ATP synthase. In addition to their responsibility of producing ATP, mitochondria are also involved in controlling intracellular calcium levels, fatty acid synthesis, heme group synthesis, and apoptosis.

The mitochondrion is a double membrane bound organelle and is approximately 3-4 μm by 1 μm in size. The outer membrane provides a barrier between the internal environment of the mitochondria and the cytosol. Despite this barrier, the outer membrane is not completely impermeable. Necessary proteins can be transported into the mitochondria through the outer membrane. The inner membrane is arranged into folds called cristae. These folds act to increase the surface area of the inner membrane which is the site of cellular respiration. The innermost area of the mitochondria is the matrix. Multiple copies of mitochondrial DNA (mtDNA) are located within the matrix. Also in the matrix are enzymes responsible for the oxidation of various components of the Kreb's Cycle.

There are many mitochondria located in eukaryotic cells. The number of mitochondria present can depend on energy requirements of the cell, the volume of the cell, or how efficiently each mitochondrion produces ATP. The location of mitochondria are also dictated by the energy needs of the cell. Mitochondrial movement and positioning is aided by the intermediate filaments, microtubules, and actin filaments of the cytoskeleton.

In dividing cells mitochondria must be evenly dispersed to the newly forming daughter cells. This is accomplished through a fission mechanism closely resembling the process of binary fission seen in bacteria. However just as in nuclear division, the mtDNA must also undergo replication.

Mitochondrial Metabolism.

The first stages in cellular metabolism, glycolysis, do not occur within the mitochondria, but rather in the cytosol (Figure 1). Glycolysis is the conversion of glucose to pyruvate requiring two equivalents of ATP. The process of glycolysis produces a net of two equivalents of ATP and two equivalents of NADH. Under anaerobic conditions, pyruvate is converted to lactate by lactate dehydrogenase. During aerobic conditions, pyruvate can enter the mitochondria and go through the Tricarboxylic Acid (TCA) or Krebs cycle (Reviewed in Campbell and Reece 6th ed., 2005).

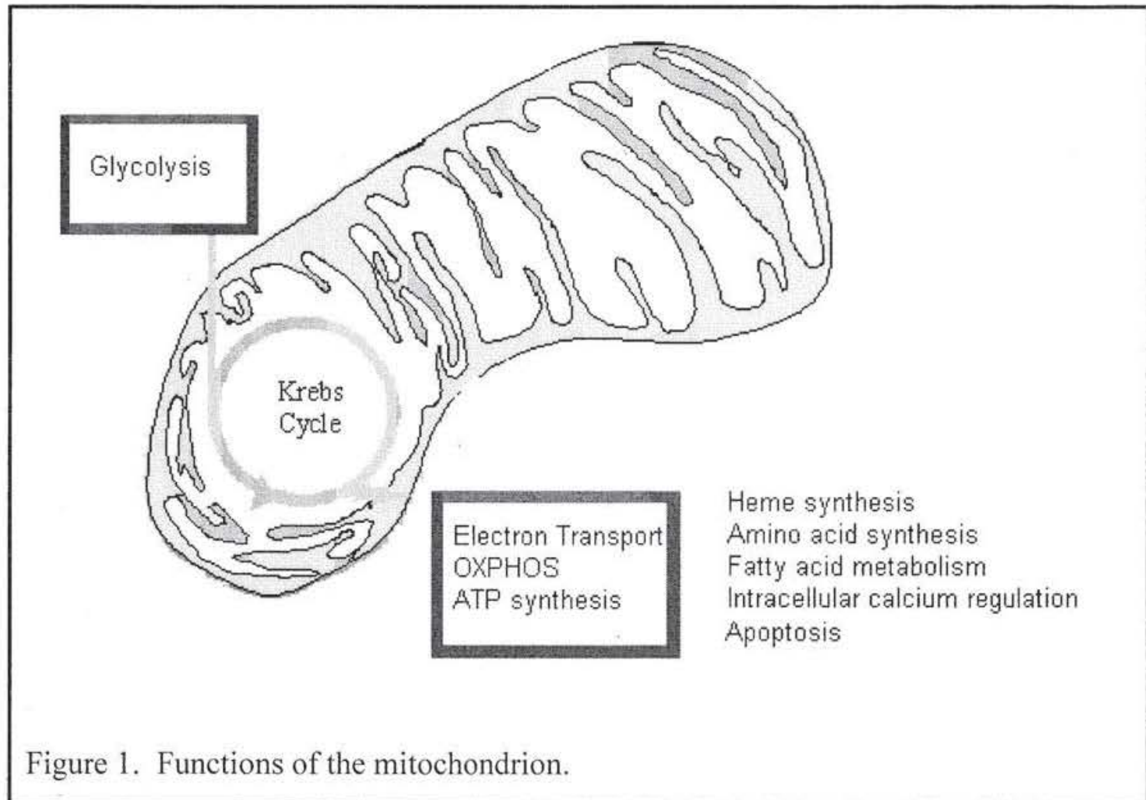

Figure 1. Functions of the mitochondrion.

In the initial stage of the TCA cycle, pyruvate is converted to acetyl CoA by the enzyme pyruvate dehydrogenase. Acetyl CoA then enters the TCA cycle where four oxidation-reduction reactions occur to produce three molecules of nicotinamide adenine dinucleotide (NADH) and one molecule of flavin adenine dinucleotide ($FADH_2$). NADH and $FADH_2$ donate the electrons that are eventually transferred to oxygen through four inner-membrane proteins of the electron transport chain (Figure 2). With this passage of electrons, protons are released into the intermembrane space creating an electrochemical gradient. This membrane potential drives the production of ATP through the ATP synthase complex (Fernie et al., 2004).

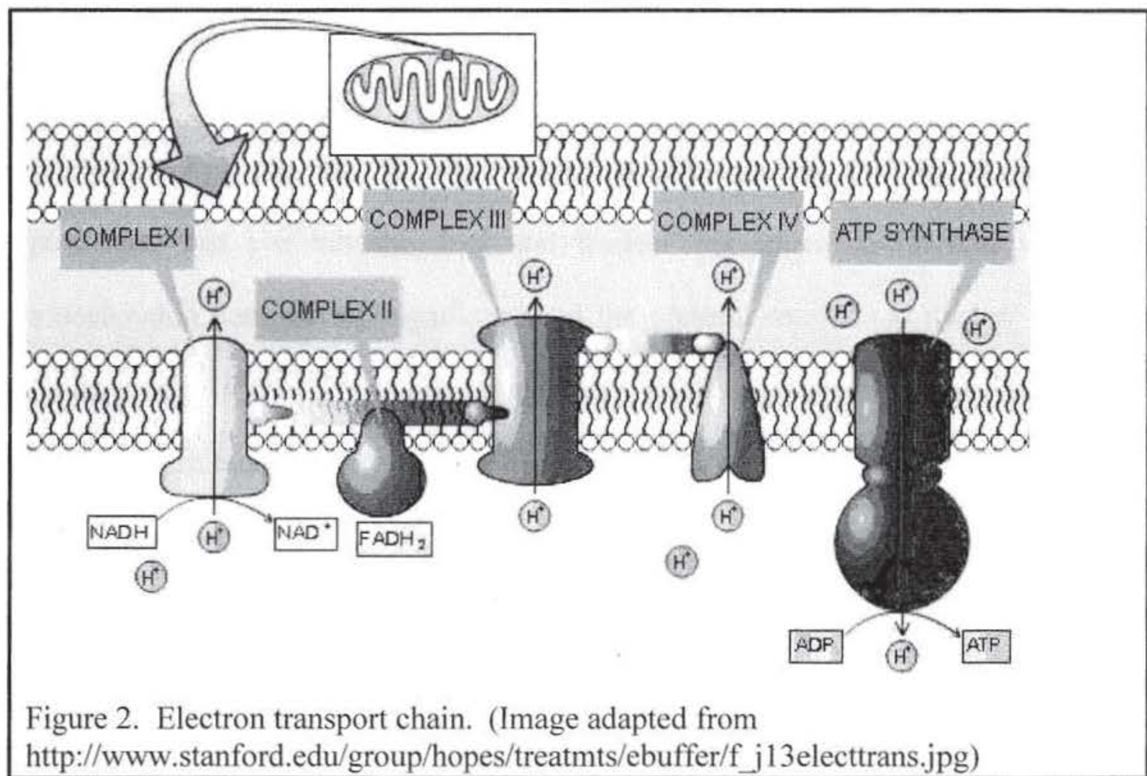

Figure 2. Electron transport chain. (Image adapted from http://www.stanford.edu/group/hopes/treatmts/ebuffer/f_j13electtrans.jpg)

Mitochondrial Evolution.

The first organisms that inhabited the earth were anaerobic microbes or primitive prokaryotes; chemosynthetic bacteria for example. The precise mode of transition to eukaryotic organisms is not clearly defined, but the origin of the mitochondrion has been traced through genomic analysis. The mitochondrion originated from the eubacterial domain, as seen through comparison of rRNA genes and protein-encoding genes in mtDNA (Lang et al., 1999). The endosymbiotic theory of mitochondrial evolution argues that a eukaryotic-type organism with a nucleus captured a bacterium which later evolved into the mitochondrion as we know it. This case is supported by the presence of extant a-mitochondrial organisms (Gray et al.,

2001) such as *Giardia intestinalis*, a parasite that is commonly known for causing intestinal discomfort.

Through the use of sequencing and phylogenetic analysis, it has been postulated that the mitochondrial and nuclear organelles co-evolved, with the mitochondria contributing significantly to the present state of the nuclear genome (Lang et al., 1999). This view is supported by the number of nuclear genes that encode for mitochondrial proteins. The mitochondrial genome may have lost these redundant genes over the course of its evolution.

The origin of the mitochondrion is still not completely resolved. However, our understanding of how this organelle may have appeared is considerably greater and increasing steadily through the advent of genomic studies.

Mitochondrial Genome.

It was not always common knowledge that mitochondria have their own DNA. Gottfried "Jeff" Schatz was a key investigator in the discovery of mtDNA in the early 1960s. His findings came about as a result of his curiosity about the origin of mitochondrial proteins. He decided to test the most logical rationale: that the mitochondria are producing their own proteins via their own genome. A new method of purifying yeast mitochondria yielded a small amount of DNA. Furthermore, this DNA could only be digested by DNase if the mitochondrial membranes were destroyed by a detergent (Schatz, 2001). Schatz had provided the first evidence of the existence of mtDNA.

Major discoveries usually bring about more questions than answers. With the discovery of mtDNA, many questions arose. What is the structure of mtDNA, what genes are represented within its sequence, how does replication occur, how is mtDNA inherited? These are just a few examples of the new problems that now needed to be investigated.

In the time since Schatz first discovered mtDNA, many organisms have had their mtDNA sequenced. Organisms from the taxa Nematoda, Insecta, Crustacea, Echinodermata, Cnidaria, Amphibia, Aves, Osteichthyes, and Mammalia are examples of Metazoans that now have their mtDNA completely sequenced. The mitochondrial genomes of all these organisms are circular and range in size from 13,794 bp (*Caenorhabditis elegans*) to 20,500 bp (*Meloidogyne javanica*) Mitochondria p. 51). Plant mitochondrial genomes are considerably larger and range from 200 to 2400 kb (Reviewed in Scheffler, 1999).

Saccharomyces cerevisiae.

Yeast is a unicellular organism in the Fungus Kingdom. *S. cerevisiae* is more commonly referred to as baker's yeast, brewer's yeast, or budding yeast. It can exist stably as a haploid (n) or diploid (2n). Haploid cells can be either a or α, which denotes mating type. When these types are placed in close proximity the result is a mating and a diploid of genotype a/α is generated. Both haploid (a or α) and diploid (a/α) cells can undergo mitotic division which occurs by budding (Reviewed in Herskowitz, 1988). *S. cerevisiae* has a replicative life span of about 30 divisions, meaning that each mother cell can give rise to thirty daughter cells (Arkin, 2004).

Haploid and diploid cells are specialized in that they only participate in activities appropriate to their ploidy. For instance, haploid cells mate but do not sporulate and diploid cells sporulate but do not mate. This activity is directed by the master regulatory locus also known as the mating-type locus (Reviewed in Herskowitz, 1988).

The mitotic cell cycle of *S. cerevisiae* progresses through four stages (Figure 3). DNA synthesis occurs during the 'S' phase. The 'M' phase signifies mitosis. In between these phases are two G stages. G1 and G2 stand for GAP1 and GAP2. During these times the cell produces the necessary components to proceed into the next stage. The termination of each of these GAP phases is associated with a check-point. Cyclin dependant kinases and cyclins regulate the cell's progression through the stages of the cell cycle (Reviewed in Schafer, 1998).

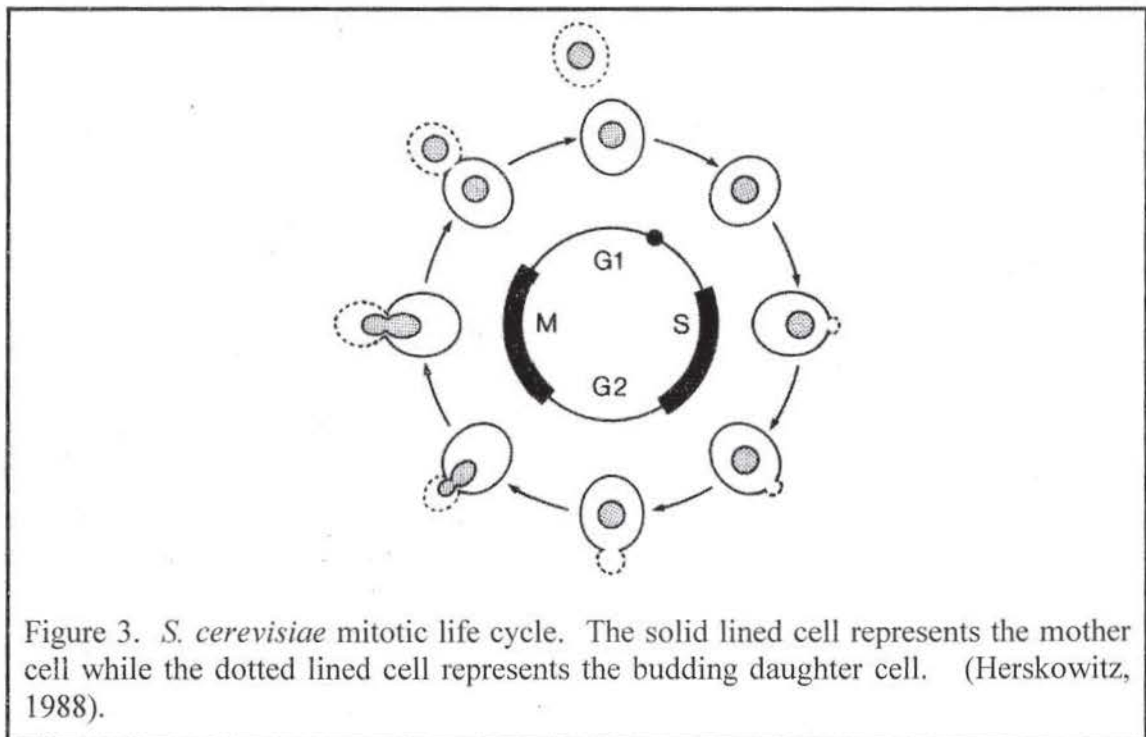

Figure 3. *S. cerevisiae* mitotic life cycle. The solid lined cell represents the mother cell while the dotted lined cell represents the budding daughter cell. (Herskowitz, 1988).

In a starvation environment, diploid yeast can undergo meiosis and spore formation. This occurs in the absence of nitrogen and the presence of a poor carbon source. Researchers often use sporulation media to manipulate diploid strains to undergo sporulation. The resulting structure following sporulation is an ascus with four haploid spores enclosed, known as a tetrad (Figure 4, Reviewed in Herskowitz, 1988). These four spores represent each product of meiosis now with one set of chromosomes, making genetic analysis of recombination a simple task.

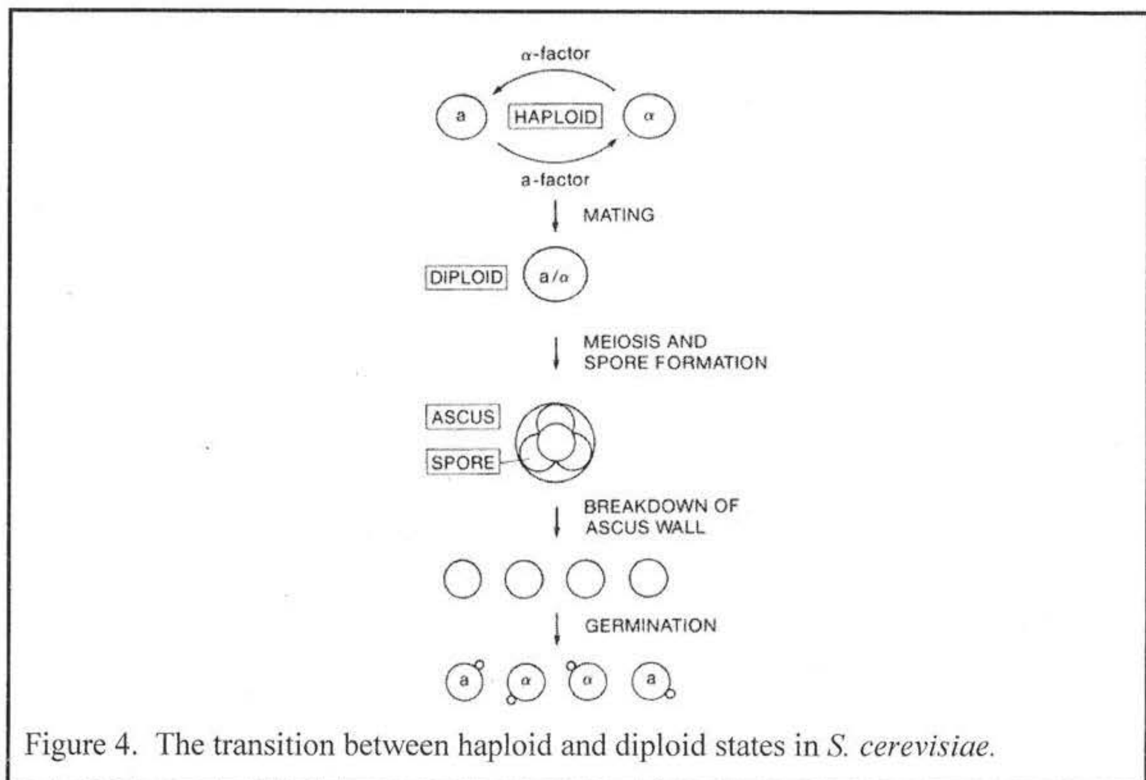

Figure 4. The transition between haploid and diploid states in *S. cerevisiae*.

Yeast as a Model Organism.

In 1996 *Saccharomyces cerevisiae* became the first eukaryotic organism to have its nuclear genome completely sequenced. The yeast nuclear genome contains 12,052 kb on 16 linear chromosomes. A total of 6,183 open-reading-frames (ORFs)

of over 100 amino acids long were reported upon completion of the sequencing (Sherman, 2002). The published sequence information is accessible through databases and allows researchers to procure information that would have previously taken months of bench work. For instance, an inclusive *Saccharomyces* Genome Database website on the internet has a compilation of identified genes. For each gene, there is cited literature, sequence analysis, protein information, localization, and much more available. This wealth of information makes yeast research even more powerful than ever (Christie et al., 2004).

To further facilitate the use of yeast in research, the *Saccharomyces* Genome Deletion Consortium was launched. The goal of this group is to delete open reading frames as they are identified through the genome sequencing project. These deletions are collectively known as the Yeast Knockout (YKO) Library. A new strategy developed by (Baudin et al., 1993) using a one step PCR amplification greatly simplifies this process. Previously, creating deletions was a tedious process requiring multiple cloning steps. The latest update on the progress of the YKO Library shows that 95% of approximately 6200 ORFs have been deleted.

The single cell nature of the eukaryotic *S. cerevisiae* makes it an ideal system for modeling higher, multi-cellular eukaryotes. Rapid growth, the ease of replica plating and mutant isolation, a well defined genetic system, and efficient DNA transformation capabilities also make yeast an invaluable organism (Sherman, 2002).

The evolutionarily conserved mechanisms and genes that yeast share with humans also make them desirable model organisms. *S. cerevisiae* played an integral

role in the discovery of cell cycle regulators. In 2001, Leland Hartwell was a co-recipient of the Nobel Prize in Physiology or Medicine for his discovery of a set of genes that control the cell cycle. During the course of his work with *S. cerevisiae*, he discovered more than 100 cell division cycle (CDC) genes. Of particular importance was a gene he called *CDC28* also known as "start." This gene was found to control the progression through the G1 phase. Hartwell also first coined the term 'checkpoint' in referring to how the cell cycle progresses. The mechanisms of the cell cycle are greatly conserved between eukaryotes throughout evolution, so these findings were of utmost importance (Hartwell et al., 1973).

Yeast and Human Mitochondrial Genomes: a comparison.

The yeast mitochondrial genome (mtG) is contained in a circular double stranded structure of approximately 85 kb (Foury et al., 1998). In comparison, the human mtG is 16.6 kb (Figure 5). This difference in size is the result of a low gene density in the yeast mtDNA and a high adenine and thymine (A+T) content. Intergenic spaces consist of A+T regions interrupted by more than 150 guanine and cytosine (G+C) rich sequences ranging from 10 to 80 bp (de Zamaroczy and Bernardi, 1986).

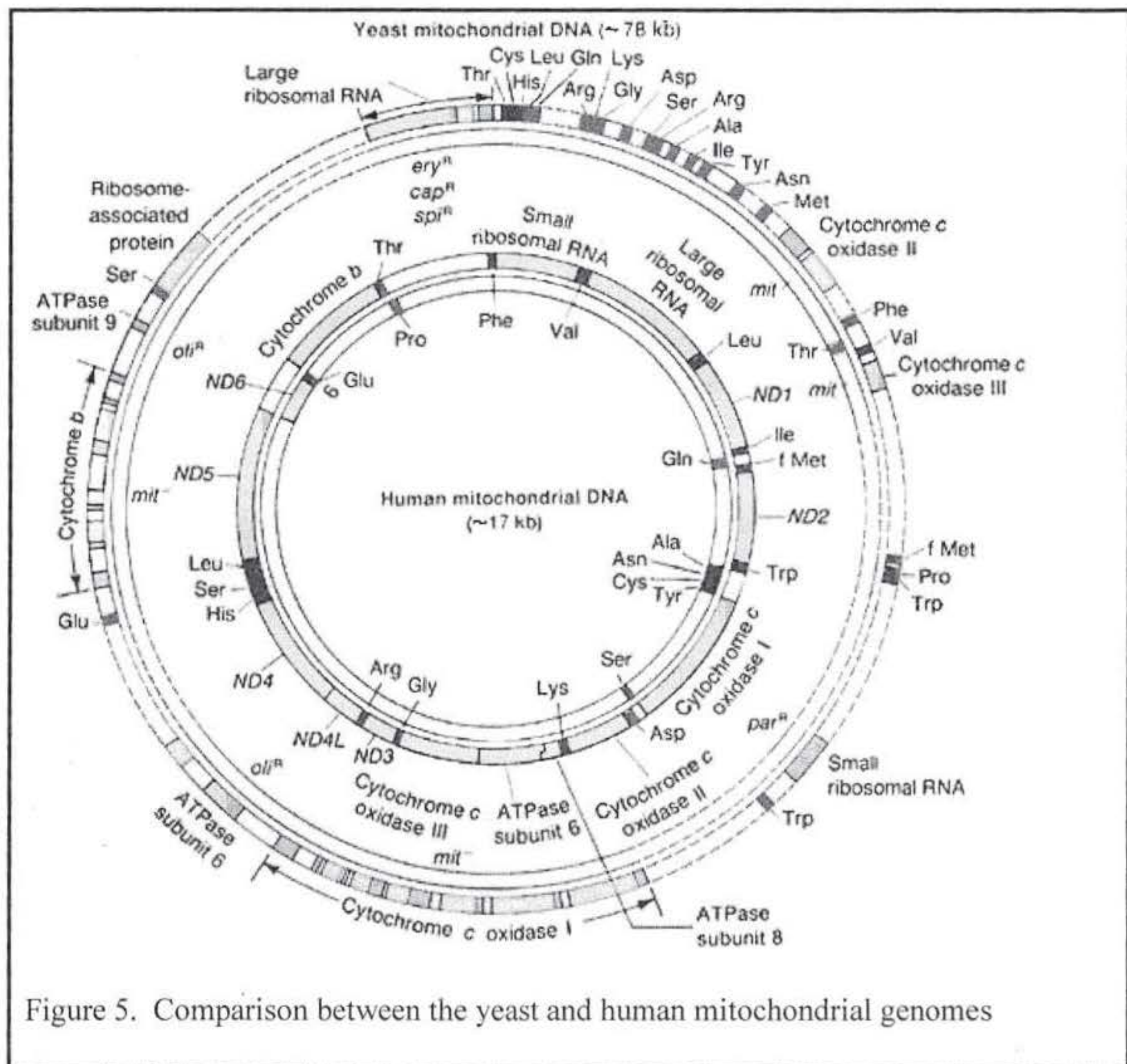

Figure 5. Comparison between the yeast and human mitochondrial genomes

The mtDNA of yeast encodes a total of eight stable proteins while human mtDNA encodes thirteen subunits of respiratory complexes (Schatz, 2001; Anderson et al., 1981). In humans, the thirteen polypeptides that are produced by mtDNA are: 7 subunits of NADH dehydrogenase, 3 subunits of cytochrome c oxidase, 2 subunits of ATP synthase, and cytochrome b. The stable proteins which are encoded for within the yeast mtDNA include cytochrome oxidase subunits I, II, and III which

make up complex IV of the electron transport chain. The yeast mtG is also responsible for the production of cytochrome b and components of ATP synthase; encoding subunits 6, 8, and 9. Mitochondrial genomes also encode for 24 tRNAs and 2 rRNAs (21s and 15s) which are essential for translation of its own DNA.

Within the yeast mitochondrial genome, approximately 33 to 35% of its entirety is coding sequence. The remaining sequences are non-coding yet still highly conserved (de Zamaroczy and Bernardi, 1985). The non-coding region is dispersed with seven to eight replication origin-like (ori) elements (de Zamaroczy and Bernardi, 1986). mtDNA is present in multiple copies. In a haploid yeast cell there are approximately 50 copies of mtDNA while in a diploid cell there are approximately 100 copies (Grimes, 1997).

Mitochondrial Proteome.

Many proteins that are involved in maintaining the stability of the mitochondrial genome and the function of the organelle itself are nuclear encoded genes. The mRNA transcripts of these genes are translated in the cytoplasm, and the resulting proteins are sent to the mitochondria. The proteins have a mitochondrial targeting signal (MTS) that directs the movement of the proteins to their proper locations (Reviewed in Roise and Schatz, 1988).

Prokisch et al. (2004) identified 546 mitochondrial associated proteins in yeast by applying liquid chromatography mass spectrometry to purified mitochondria. This technique identifies proteins in high concentrations. Using other genomic approaches such as expression profiling and computational analysis, which can identify proteins

present in lower concentrations, Prokisch et al. (2004) increased their prediction to approximately 700 mitochondrial associated proteins. According to the Mito2P database, there are 477 total mitochondrial proteins with 469 of these proteins being encoded by the nuclear genome (Andreoli et al., 2004).

The mitochondrial proteome varies under differing conditions. In yeast this variation is demonstrated during a process called carbon catabolite repression. The carbon source that is available to the yeast determines which genes will be transcribed and at what rate (Reviewed in Gancedo, 1998). Until recently, differences in respiratory and fermentative conditions were studied at the mRNA level. Now more research is being conducted on the proteomic deviations between these two metabolic states.

Ohlmeier et al. (2004) studied the composition of the yeast mitochondrial proteomes during the switch between fermentation and respiration, also known as diauxic shift. Yeast were forced into diauxic shift by transferring the cells from glucose to glycerol. After analysis of the proteomes of seven different extracts, it was determined that only 18 proteins were changed; 17 increased, while 1 decreased. The proteins that were affected during the switch were involved in the Krebs cycle and the respiratory chain (Ohlmeier et al., 2004).

Mitochondrial Mutations in Yeast.

Yeast are particularly valuable model systems to use in the study of mitochondria. In higher eukaryotes, a gene disruption that causes a loss of mitochondrial function will also lead to a loss of viability. This results in a complex

problem: how do we research gene function when null mutants are not viable? The answer can be found within *S. cerevisiae*. Yeast that lose the ability to respire are still viable because of their ability to utilize fermentable carbon sources for the production of energy. Aerobic respiration is a more efficient method of producing ATP, but the ability to switch to fermentation during anaerobic conditions enables survival. Organisms that operate in this manner are known as facultative anaerobes. Due to this feature, it should come as no surprise that yeast are highly utilized research tools in the study of the mitochondrion.

Mitochondrial mutations can be of two types. Yeast cells that have undergone large deletions in their mitochondrial genome (mtG) are termed *rho*$^{-}$ (Fox et al., 1991). *Rho*$^{+}$ and *rho*$^{-}$ refer to the mtG integrity of the yeast cell. Frequently, but not always, *rho*$^{-}$ cells have lost the ability to respire due to large portions missing from their mtG. A respiratory deficient cell exhibits a phenotype known as 'cytoplasmic petite' (Fox et al., 1991) so named because of the small colonies that result. This distinct phenotype helps to differentiate between cells with functional mtDNA and cells with defective mtDNA. *Rho-* mutations can occur spontaneously among yeast cells, but they can occur at higher frequencies in compromised yeast cells such as those exposed to mutagens. The second type of mutation affects single gene functions and are called *mit*$^{-}$. *Mit-* strains may be capable of respiration depending upon where the mutation occurs within the mtG (Fox et al., 1991).

Mitochondrial Structure and Morphology in Yeast.

Mitochondria are usually pictured as individual organelles situated within the cytosol of cells, comparable to isolated islands of energy production. However, it has been shown that mitochondria in yeast are arranged in continuous tubular networks (Figure 6), and these networks are dynamic in that they can undergo conformational changes and move throughout the cell (Okamoto and Shaw, 2005).

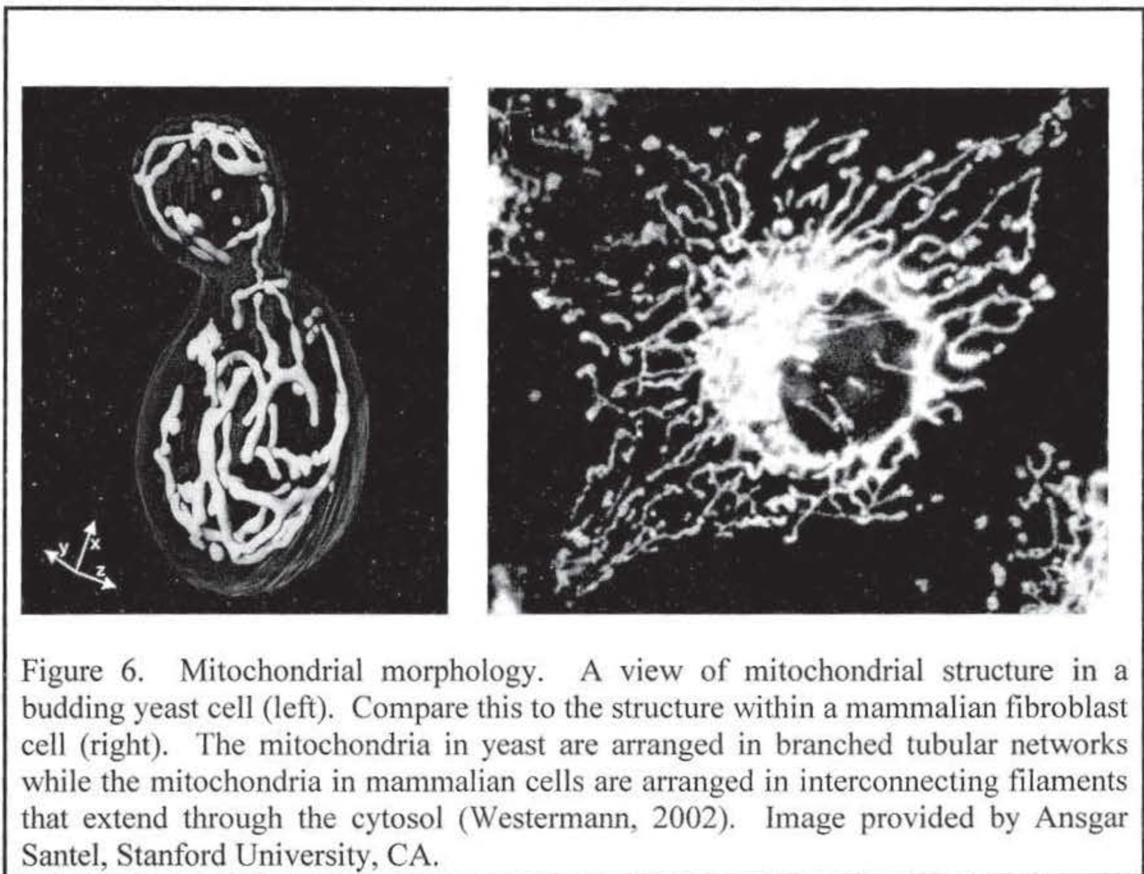

Figure 6. Mitochondrial morphology. A view of mitochondrial structure in a budding yeast cell (left). Compare this to the structure within a mammalian fibroblast cell (right). The mitochondria in yeast are arranged in branched tubular networks while the mitochondria in mammalian cells are arranged in interconnecting filaments that extend through the cytosol (Westermann, 2002). Image provided by Ansgar Santel, Stanford University, CA.

A class of proteins called mitochondrial distribution and morphology proteins (Mdm) is essential for the maintenance of morphology in yeast. Their role as intermediate filament proteins influences organelle morphology and positioning in a non-dividing cell (McConnell and Yaffe, 1993). Intermediate filaments in

conjunction with motor proteins are involved in organelle motility and morphology (Drubin et al., 19893).

It is essential for cells to maintain proper mitochondrial morphology, because morphology has been implicated in mitochondrial inheritance, segregation, and function. As stated earlier, yeast mtDNA encodes eight stable proteins. However, the mitochondria need many more proteins to function; therefore, it is necessary for protein products of nuclear genes to be localized to the mitochondria for various functions. In order to maintain proper trafficking of these proteins, morphology must remain stable (Stojanovski et al., 2006). When the import of these proteins is affected, the morphology of the organelle can be compromised, and vice versa, which results in a positive feedback cycle. Under normal circumstances, the general import pore (GIP) translocates precursor proteins across the mitochondrial membrane with the help of receptors (Wiedemann, 2004). The GIP and receptors form a collection of subunits, collectively called the TOM (translocase of the outer mitochondrial membrane) complex (Wiedemann et al., 2004). The TOM and TIM (translocase of the inner membrane) complexes facilitate the import of proteins across the outer mitochondrial membrane where they are further directed to their specific locations.

Mitochondrial Nucleoid Structure and Inheritance.

Nucleoids are the fundamental segregating unit of mtDNA (MacAlpine et al., 2000). Nucleoids consist of several copies of mtDNA along with associated proteins. The nucleoid associated proteins help to package the mtDNA into a shape that is more resilient in the highly oxidative environment of the mitochondrial matrix. (Sia

grant) Because the number of segregating units within the mitochondria was seen to be smaller than the actual copy number itself (Dujon, 1981), it was inferred that nucleoids are the segregating units of mtDNA inheritance (MacAlpine, 2000). Therefore the integrity of the nucleoid structure is imperative in the maintenance of the mitochondrial genome between generations.

As stated previously, it is essential for cells to maintain normal mitochondrial morphology. Two proteins involved in this process, Mdm10p (Mitochondrial Distribution and Morphology) and Mmm1p (Maintenance of Mitochondrial Morphology), are integral in maintaining morphology in non-dividing cells. They are also important in actively dividing cells for the role they play in organelle inheritance (Hermann, 1997). They are required for both nuclear and mitochondrial inheritance to daughter cells (McConnell and Yaffe, 1993). During the early S phase of the yeast cell cycle, a portion of the mitochondrial network is transferred from the mother cell to the bud with the aid of these maintenance proteins (Stevens, 1981).

Research of Stevens (1981) describes the passing of mitochondrial structures, not necessarily the DNA content of the mitochondria. Beyond knowing that the nucleoid is the unit of segregation, the precise mechanism by which mtDNA is divided and segregated is not known. However, many proteins have been found to be involved in this as yet unidentified process. Abf2p is involved in compacting the structure of mtDNA which aids in the formation of nucleoids (Friddle, 2004). Due to the importance of the nucleoid structure in segregation and inheritance, it stands that Abf2p is also integral in this process.

Recombination in the Mitochondrial Genome

The mitochondrial genome has long been considered a stable molecule with relatively few mutations. For this reason, and also because of its maternal inheritance, it has been used to trace evolution of humans in Western Europe through a matriarchal ancestry (Richards et al., 1999). Evidence of mitochondrial recombination events have brought the method of using mtDNA as an evolutionary marker into question (Thyagarajen et al., 1996; Maynard Smith and Smith, 1998). Can mtDNA be used to trace lineage if it undergoes recombination? Are all of the evolutionary studies up to this point now invalid? It has been difficult to challenge the popular dogma that mtDNA does not undergo recombination. Eyre-Walker (year?) notes that the field of molecular biology and human genetics has ceased debate on the subject, but evolutionary biologists still abide by strict mitochondrial clonality. Over the past decade, many studies have surfaced that lend evidence and support for mtDNA recombination.

Homologous recombination in the nucleus is evidenced as a mechanism to repair damaged DNA (Ling et al., 1995). The highly oxidative environment of the mtDNA makes it a good candidate for the use of recombination as a DNA repair mechanism. It has been shown experimentally in yeast that tolerance to oxidative stress may indeed involve recombination events. Yeast cells that were exposed to oxidants had a higher occurrence of recombination events (Doudican et al., 2005; Brennan et al., 1994).

In yeast, high mitochondrial homologous recombination activity has been shown following the mating of two haploids. The reason for this activity is not determined, but it does provide support for the phenomenon of recombination within mtDNA. Based on this knowledge, Ling et al. (1995) devised a novel method to examine mutant clones: 'mitochondrial crossing in haploid'. In this technique, mtDNAs with different genetic markers are allowed to recombine in cells with haploid nuclei. Nakagawa et al. (1992) found that post-mating recombination could be induced by endonucleases with mitochondrial specific cutting sites. Double stranded breaks are often associated with recombination, so it has been postulated that the endonuclease cutting sites are where the initiation of homologous recombination occurs (Nakagawa et al., 1992).

Many opponents still argue that these recombination events are primarily seen in plants, fungi, and protists which does not necessarily translate to similar function in humans. Using purified protein extracts from mitochondrial rich cellular fractions of mammalian liver cells to perform a homologous recombination assay, it was shown that higher eukaryotes can also share this process (Thyagarajan et al., 1996). This biochemical approach used a transformation of two DNA substrates into recombination deficient *E. coli* to demonstrate recombination activity. The DNA substrates alone encode defective heteroalleles of selectable markers, but when co-incubated with the liver protein extracts, recombination occurred to give rise to functional marker genes (Thyagarajan et al., 1996).

MacAlpine et al. (1998) postulate that recombination is also an important factor in the initiation of the replication of mtDNA. Abf2p is a high mobility group protein that could possibly arrange the conformation of mtDNA in a position that is conducive for strand exchange. Two-dimension gel electrophoresis shows that Abf2p promotes or stabilizes Holliday recombination junctions. Also an increase in Abf2p expression by 10-fold results in a dramatic increase in mtDNA recombination intermediates (MacAlpine et al., 1998). In support, Zelenaya-Troitskaya found that the absence of Abf2p reduces the efficiency of mtDNA recombinations as demonstrated by homozygous Δabf2 crosses (1998).

There is much evidence to support that, not only does recombination occur in mtDNA, but that it is also an important process in maintaining mtG stability.

Microsatellite Instability.

Microsatellites are regions of DNA that consist of many repeats of simple nucleotide sequences. Microsatellites are usually 20 to 60 base pairs in length (Sia et al., 2000). They are also known as Short (or Simple) Tandem Repeats (STR). An example of a microsatellite region is a sequence of alternating guanines and thymines. Such a sequence would be denoted as poly(GT) or $(GT)_n$ with n being the number of times the sequence is repeated. There is great variance in the number of repeats between individuals; therefore, microsatellites have had applications as genetic markers in humans. Microsatellite regions are used for identifying individuals, paternity confirmation, forensic evidence, and lineage studies.

Microsatellites have much higher mutation rates than non-repetitive regions in both humans and yeast (Ellagren, 2000; Sia et al., 2000; Brinkmann et al., 1998). The primary mechanism suggested for mutation is polymerase slippage (Figure 7), in which DNA strands disassociate during replication and reanneal incorrectly (Ellagren, 2000).

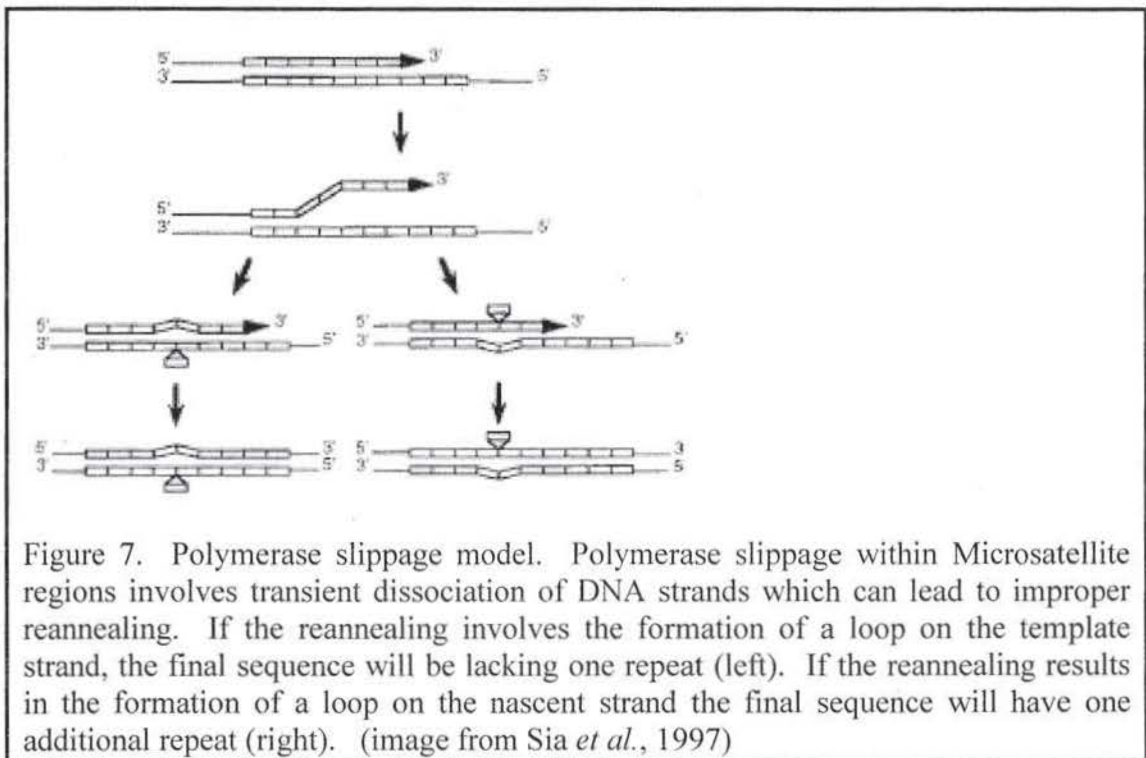

Figure 7. Polymerase slippage model. Polymerase slippage within Microsatellite regions involves transient dissociation of DNA strands which can lead to improper reannealing. If the reannealing involves the formation of a loop on the template strand, the final sequence will be lacking one repeat (left). If the reannealing results in the formation of a loop on the nascent strand the final sequence will have one additional repeat (right). (image from Sia *et al.*, 1997)

The Stepwise Mutation Model proposes that the microsatellite region only changes by one unit as a consequence of either the addition or deletion of one repeat (Ohta and Kimura, 1973). Sia et al. (2004) found that deletions occur more often than additions for both poly(AT) and poly(GT) tracts. In comparison, poly(AT) and poly(GT) tracts in nuclear DNA revert at the same rate with additions being the most common events.

From these data, it was concluded that it is not the structure of DNA that impacts microsatellite stability, but rather organelle specific factors and interacting sequences.

Point Mutations.

Point mutations are single base pair substitution that can occur spontaneously within the genome. At the DNA level, base substitutions can be transitions (purine to purine or pyrimidine to pyrimidine) or transversions (purine to pyrimidine or vice versa). Point mutations can be silent, missense, nonsense, or frameshift mutations. The presence of mutagens such as UV-radiation increases the occurrence of point mutations. Mutations can also occur as a result of error in DNA replication and spontaneous lesions (Griffiths et al., 1999).

Point mutations that contribute to a variety of diseases have been identified.

Oxidative Stress.

The normal function of the electron transport chain leads to the production of reactive oxygen species (ROS) in the form of superoxide anions, hydrogen peroxide, and hydroxyl radicals. These molecules have unpaired electrons that cause them to be highly chemically reactive. ROS are not entirely harmful to the cell. They are beneficially produced in response to bacterial infections by phagocytic cells in order to kill invading pathogens. To some extent, ROS are cleared away by superoxide dismutase (mnSOD or SOD2), catalase, and glutathione peroxidase. However, when left unchecked ROS can lead to damage in cellular membranes, macromolecules, and of particular interest to my research, both nuclear and mtDNA. mtDNA is especially prone to damage by ROS because of its proximity to the electron transport chain.

ROS are released into the matrix of the mitochondria which is where mtDNA is located.

The structure of and packaging of mtDNA into nucleoids results in protection from ROS. Abf2p, which was previously shown to be involved in nucleoid formation, inheritance, and recombination, is also important in protecting the mtDNA (Friddle et al., 2004). However, the nucleoid structure of mtDNA is certainly not as well protected as the more densely packaged chromosomes of the nuclear DNA. Superoxide dismutase, catalase, and glutathione peroxidase are three enzymes that catalyze the conversion of ROS to less harmful substances such as diatomic oxygen and water (Arkin, 2004). Other proteins are also indirectly involved in the oxidative stress response of the cell. *PIF2 and NTG1* are involved in the mitochondrial base excision repair (BER) pathway. *pif1*Δ and *ntg1*Δ strains were seen to have compromised oxidative damage resistance. An intact BER pathway is important during cellular responses to oxidative stress (Doudican et al., 2005). In yeast it is also shown that tolerance to oxidative stress may involve recombination events (Doudican et al., 2005). Therefore, the proteins involved in the recombination repair pathway would also be important in combating oxidative stress.

Studies have shown that intact mitochondrial ROS removal systems may be sufficient to completely neutralize even the highest levels of ROS production (Andreyev et al., 2005). However, in mitochondria that are structurally or enzymatically compromised, ROS production exceeds ROS removal. This net accumulation of ROS can be a source of oxidative damage. This is thought to be due

to either a change in the solutes of the matrix or an impairment in the membrane transport of proteins into the mitochondria (Andreyev et al., 2005).

UV Sensitivity.

Experiments that demonstrate the result of photolysis on NADH reveal that this process generates two free radicals, NAD+ and a free electron. When NADH photolysis is performed on isolated mitochondria, the resulting free radical production does not affect the respiration and oxidative phosphorylation of the mitochondria. This shows that in a background of intact scavenging pathways UV radiation does not impact cellular processes (Joubert et al., 2004). However, this experiment was performed under low levels of photolysis, so a threshold of tolerable UV radiation was not established. What would happen if the UV radiation level was increased and the cell had a compromised repair system?

Ultra-violet radiation has been established as an inducer of apoptosis through the disruption of the mitochondrial membrane potential in keratinocytes (Denning et al., 2002). The mitochondria is responsible for the regulation of the apoptotic signal through the release of the molecule cytochrome c in all cells, not just skin cells. Using the mitochondria in the signaling pathway for apoptosis is beneficial, because functional mitochondria are necessary for the viability of the cell.

MtDNA has a repair pathway for UV damage that is similar to the established nuclear repair pathway. UV radiation most commonly causes the formation of thymine dimers. An important protein in the direct repair (DR) pathway of mtDNA is *PHR1*. *PHR1* encodes a photolyase that is capable of directly reversing UV damage.

PHR1 activity is present in *S. cerevisiae*, but no photolyase activity has been observed in mammalian cells (Larsen et al., 2005). Methylguanine methyltransferase (MGMT) is the major DR protein in the nucleus of mammalian cells. There is a splice variant of MGMT which acts in the mitochondrion. This protein acts to remove methyl and ethyl groups from the O6 position of guanine (Larsen et al., 2005).

Mitochondrial DNA Repair.

MtDNA, like nDNA, is susceptible to errors during its replication. It is also housed in a highly oxidative environment which can contribute to DNA alterations. Specific proteins which defend against damage to mtDNA have been identified. The yeast mtDNA polymerase subunit, *MIP1,* has 3'-5' exonuclease activity to correct replication errors (Foury and Vanderstraeten, 1991). Msh1p has been identified as a DNA binding protein that is involved in mismatch repair in yeast (yeastgenome.org).

In a recent review, Larson et al. (2005) present a compilation of studies on mtDNA repair and compare these findings to what is established in the field of nDNA repair (Figure 8). nDNA is repaired through six major pathways: direct reversal (DR), nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), recombinational repair (RER), and translesion synthesis (TLS). Mitochondria share these basic pathways with the exception of NER. However, the pathways are not entirely similar. For instance, MMR in the mitochondrion does not distinguish between daughter and template strand. This is thought to lead to the higher frequency of mutation that is seen in mtDNA (Larson et al., 2005).

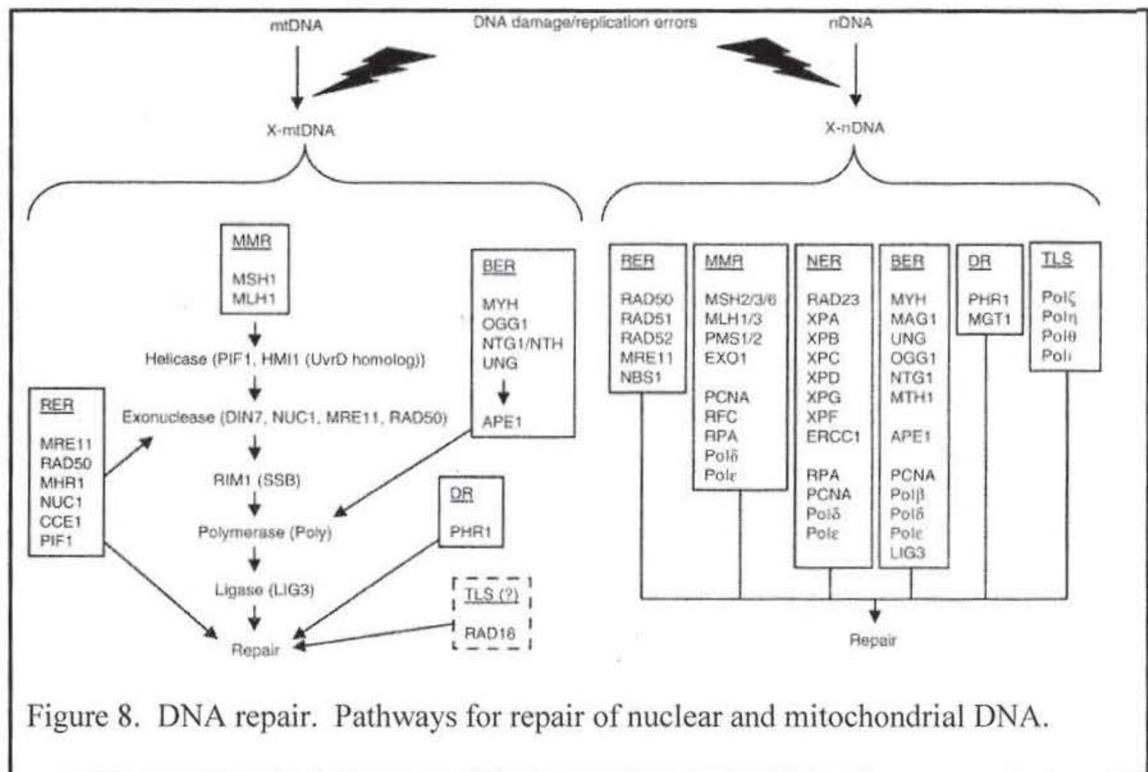

Figure 8. DNA repair. Pathways for repair of nuclear and mitochondrial DNA.

Mitochondrial Diseases.

Mitochondrial function is implicated in a great number of human diseases. These pathologies arise from many different pathways, but all are united by the fact that the mitochondria play a role in degeneration. Parkinsons is associated with a loss of catalytic activity in the *COX1* gene of the electron transport chain (Keeny et al., 2006). Mitochondrial diabetes is an inherited disorder that accounts for 1% of all cases of diabetes. The mtDNA of these patients contain mutations or deletions and also exhibit heteroplasmy, in which not all copies of the mtG are identical (Wiederkerh and Wollheim, 2006). Point mutations in the mitochondrial tRNA genes

are responsible for many different types of mitochondrial encephalomyopathies (Yasukawa et al., 2000).

The implications of microsatellite regions in terms of replication were previously discussed. Microsatellite regions are also associated with neurodegenerative disorders. Tri-nucleotide repeats have been implicated in Fragile X syndrome and Huntington's disease (Schlotterer, 2000).

Researchers in Japan were working towards the creation of a transgenic mouse to model Chronic Progressive External Ophthalmoplegia (CPEO). CPEO is a mitochondrial disease characterized by mtDNA deletions caused by defects in mtDNA polymerase (POLG). Humans with this mitochondrial disease show altered mood disorders. The mouse models that were produced also display behavior comparable to manic-depressive disorder. This study lends support to the hypothesis of mitochondrial dysfunction causing bipolar disorder (Kasahara et al., 2006). It is apparent that the effects of mitochondrial malfunction cover a wide range of pathologies, with more associated diseases being uncovered as research progresses.

Finally, mitochondria are involved in the universal phenotype: aging. The mitochondrial theory of aging implicates the production and accumulation of reactive oxygen species (ROS) as a by-product of cellular metabolism as the key causative factor in aging. These ROS cause oxidative damage to mtDNA that leads to decreased respiratory function and a cascading event of deleterious effects (Arkin, 2004).

PRELIMINARY STUDIES

Nuclear DNA replicates in a well understood fashion. Semi-discontinuous, semi-conservative, bi-directional are all accepted as the general mechanisms by which nDNA replicates (Campbell and Reece, 6th ed. 2002). Even the repair processes that maintain the integrity of the nDNA are clearly defined. Not only are the pathways understood, but also the major proteins that are involved in these processes have been identified.

In contrast, replication and repair of the mitochondrial genome remain relatively unknown. Few proteins have been identified and implicated in maintaining mitochondrial genome stability. With such a modest amount of information available on this topic, it is hard to begin creating comprehensive pathways to describe the processes that occur. Each new protein that is classified as being important in mitochondrial function is a new part of the larger puzzle. These proteins can be used to piece together models for how the mitochondrial genome is regulated.

Ilv5p.

Ilv5p is a bifunctional protein (Zelenaya-Troitskaya et al., 1995). It is a nuclear encoded protein that localizes to the mitochondria where it is involved in branched-chain amino acid synthesis (Bateman et al., 2002). *ILV5* encodes the mitochondrial matrix enzyme acetohydroxy acid reductoisomerase which is a catalyst involved in the biosynthesis pathways of isoleucine, leucine, and valine (Peterson et al., 1983). Ilv5p has a second role in maintaining mtDNA stability. This function was found to be independent of its role in branched chain amino acid synthesis

because deletion of *ILV2* (also involved in branched-chain amino acid synthesis) resulted in no deleterious effects on mtDNA stability (Zelenaya-Troitskaya et al., 1995). This shows that a functional branched chain amino acid synthesis pathway is not required for mtDNA stability. Also point mutations in *ILV5* that resulted in either branched-chain amino acid synthesis malfunction or mtDNA instability were identified. Taken together, these data show that Ilv5p is a bifunctional protein, and its effects are not just a result of branched chain amino acid function (Bateman et al., 2002). Ilv5p has also been demonstrated to be required for packaging mtDNA into the nucleoid structure based on the response to general amino acid control (MacAlpine et al., 2000).

In *S. cerevisiae,* Bateman et al. examined a+D- Ilv5p mutants (2002). These mutants are *rho*$^{+}$ but exhibit phenotypes of mtDNA instability. It was shown that a+D- mutants give rise to unstable mtDNA phenotypes and also display altered localization within the mitochondria as compared to wildtype Ilv5p. Furthermore, the unstable mtDNA was found to be a result of the altered organization of the mutant forms of Ilv5p (Bateman et al., 2002; Zelenaya-Troirskaya et al., 1995).

The proper function of Ilv5p is important to the mitochondria because it directs nucleoid structure and, consequently, segregation and inheritance due to general amino acid control (MacAlpine et al., 2000). Their group found that activation of the general amino acid response pathway in *rho*$^{+}$ and *rho*$^{-}$ petite yeast cells results in an increased number of nucleoids without an increase in mtDNA copy number. Increases in nucleoid number increase the transmission of mtDNA to

daughter cells. This mechanism is thought to operate during times of amino acid starvation as a means to enhance inheritance. Specifically, in *ilv2Δ* and wild-type strains, the nucleoid number increased after activation of the general amino acid response. However, in *ilv5Δ* strains the nucleoid number remained the same, lending strong support that Ilv5p has an integral role in regulating nucleoid structure, segregation, and inheritance in response to general amino acid control (MacAlpine et al., 2000).

Yeast 2-hybrid to identify Ilv5p interactors.

The importance of Ilv5p in mitochondrial maintenance in yeast can be used in the identification of other integral proteins. Yeast 2-hybrid assays are a valuable research tool in identifying protein-protein interactions. Fields and Song (1989) developed this strategy to supplement existing techniques of crosslinking, co-immunoprecipitation and co-fractionation by chromatography. The GAL4 protein is a DNA-binding transcription factor required for the activation of the GAL genes in response to galactose (yeastgenome.org). GAL4 consists of two domains: a DNA binding domain and a transcriptional activator domain. The binding domain is typically fused to one protein, known as 'the bait.' The activation domain is fused to a cDNA library. Only proteins that are able to interact will bring the two domains of GAL4 together which activates transcription. This transcription is detected by growth on selective media. In this case it would be galactose (Fields and Song, 1989) but other reporters can be utilized as well.

Our lab performed a yeast 2-hybrid assay with Ilv5p fused to the LexA binding domain and a cDNA library fused to the GAL4 activation domain. Eighty-seven interactors were discovered, of which 25 have been identified as open reading frames. Our lab has further investigated *VMA8, YOL057w, FMP35, GYP7,* and *CLU1.*

Clu1p.

My work will focus on *CLU1*. *CLU1* was first discovered via the *cluA* gene in *Dictyostelium discoideum*, a soil amoeba. *cluA* was found to impair cytokinesis and cause a clustering phenotype of the mitochondrial network. After searching the GenEMBL database, a homolog to *cluA*, *CLU1*, was detected as an open reading frame in *S. cerevisiae*. *CLU1* encodes for a protein that is 27% identical and 50% similar to *cluA.* A deletion of *CLU1* did not affect the viability of yeast cells, but it did promote an abnormal clustering of the normally branched mitochondrial network. (Fields and Clark, 1998)

Despite its affect on mitochondrial morphology a *clu1* deletion does not impact mitochondrial inheritance. *CLU1* is one of only a few known proteins with this feature, as many times affecting mitochondrial morphology severely affects inheritance. Dnm1p is another of these proteins. It functions as a GTPase that is structurally similar to dynamin. A disruption of *DNM1* displays similar phenotypes to *clu1*Δ which could help in further classification of *CLU1*. The mitochondrial network in these mutants is collapsed to one side of the cell, but morphology and distribution of other organelles are not affected. (Otsuga *et al.*, 1998) Although

images have shown the transfer of mitochondria from the mother to the daughter bud, the exact number of copies of mtDNA that are also transferred has not yet been quantified.

TIF31 is a gene that encodes for p135; a 135-kDa protein which is one of eight proteins associated with yeast eIF3 (Vornlacher et al. 1999). eIF3 (Figure 9) functions as an intermediate to establish the interaction between the 40s ribosome and eIF4F, which is more commonly known as the G-cap binding complex (Vornlacher *et al.* 1999). Preliminary studies by Vornlacher et al. (1999) did not find a definitive role for p153 in yeast. However, they noted that it was 27% identical and 50% similar to a 150-kDa protein found in *D. discoideum.* The simultaneous discovery of *CLU1* and *TIF31* shows the competitive nature of research. Fields and Clark (1998) cloned and named *CLU1* while Vornlacher's manuscript about *TIF31* was still in preparation.

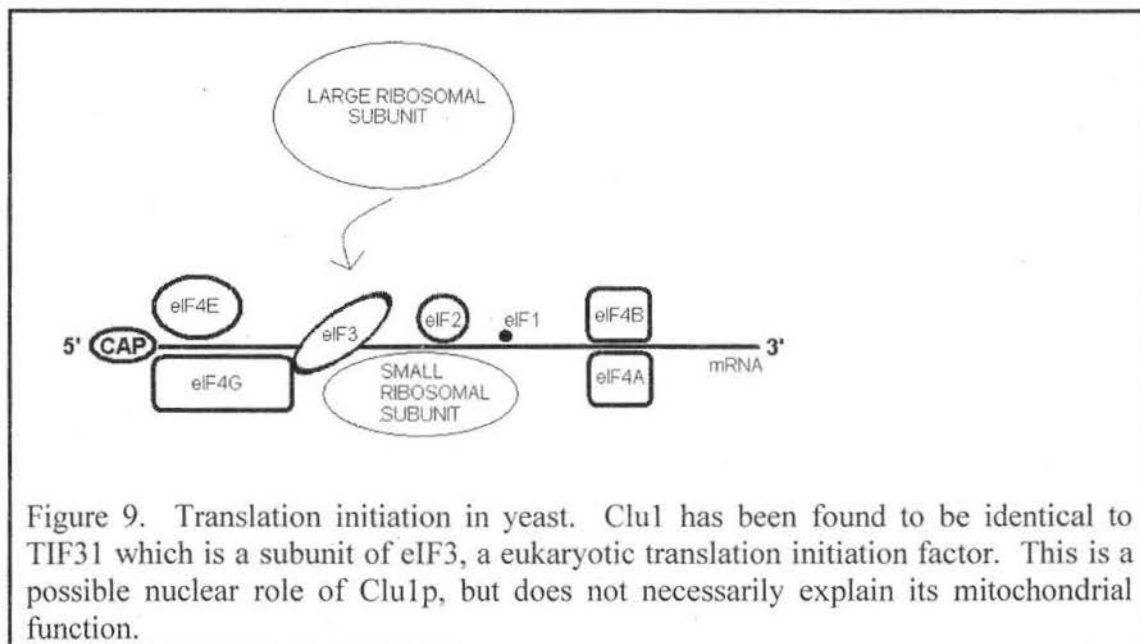

Figure 9. Translation initiation in yeast. Clu1 has been found to be identical to TIF31 which is a subunit of eIF3, a eukaryotic translation initiation factor. This is a possible nuclear role of Clu1p, but does not necessarily explain its mitochondrial function.

Contradicting existing research, Browning *et al.* (2001) found that Clu1p homologues do not co-purify with eIF3 subunits from plant or human cells. This datum, along with the fact that a deletion of Clu1p does not affect eIF3 activity (Vornlacher *et al.,* 1999), brings uncertainty to the classification of Clu1p as a putative eIF3 subunit (Logan *et al.,* 2003).

Despite the ambiguity in classifying Clu1p, it is important to note that there are *cluA* homologues in the form of open reading frames in every eukaryotic genome sequenced as of 2003 (Logan *et al.*, 2003).

GENETIC ASSAYS AND MITOCHONDRIAL REPORTERS

My research focuses on characterizing the phenomena and phenotypes that a *clu1* deletion strain displays. Through a yeast 2-hybrid assay Clu1p was found to interact with Ilv5p, a known mitochondrial protein. In order to ascertain that the interaction between Clu1p and Ilv5p is not solely an *in vitro* occurrence, and that Clu1p does indeed have an impact on mitochondrial function, an initial respiration loss assay was performed. If this experiment shows a deviation from wild-type function, it is reasonable to further investigate the mechanisms by which Clu1p causes this change in respiration loss.

Through the use of various mitochondrial reporters and fluctuation analysis assays, I investigated microsatellite slippage, direct-repeat mediated deletions, point mutations, oxidative stress and UV resistance as possible pathways leading to compromised mitochondrial function in *clu1* deletion strains.

Due to the previous reports that *CLU1* is highly involved in the formation of a normal mitochondrial structural network, it was of extreme importance that my research also included microscopy studies. In addition to observing the mitochondrial membranes, the nucleoid structure of the mtDNA under the influence of a *clu1* deletion was also studied.

Respiration Loss Assay.

Yeast are facultative anaerobes; therefore, they are able to survive without the ability to respire as long as they are provided with a fermentable carbon source such as YPD (Yeast-Peptone-Dextrose) medium (Campbell 6th ed, 2002). Only colonies

that have functional mitochondria and can respire are able to grow on non-fermentable carbon sources such as YPG medium. This is the premise of the respiration loss assay (Figure 10). A mixed medium of YPG + 0.1% dextrose is used to allow complete growth of respiring colonies, while the respiration deficient colonies will grow until the supply of dextrose is exhausted. The respiration loss frequency is calculated by determining the occurrence of phenotypically petite colonies as compared to the total number of colonies.

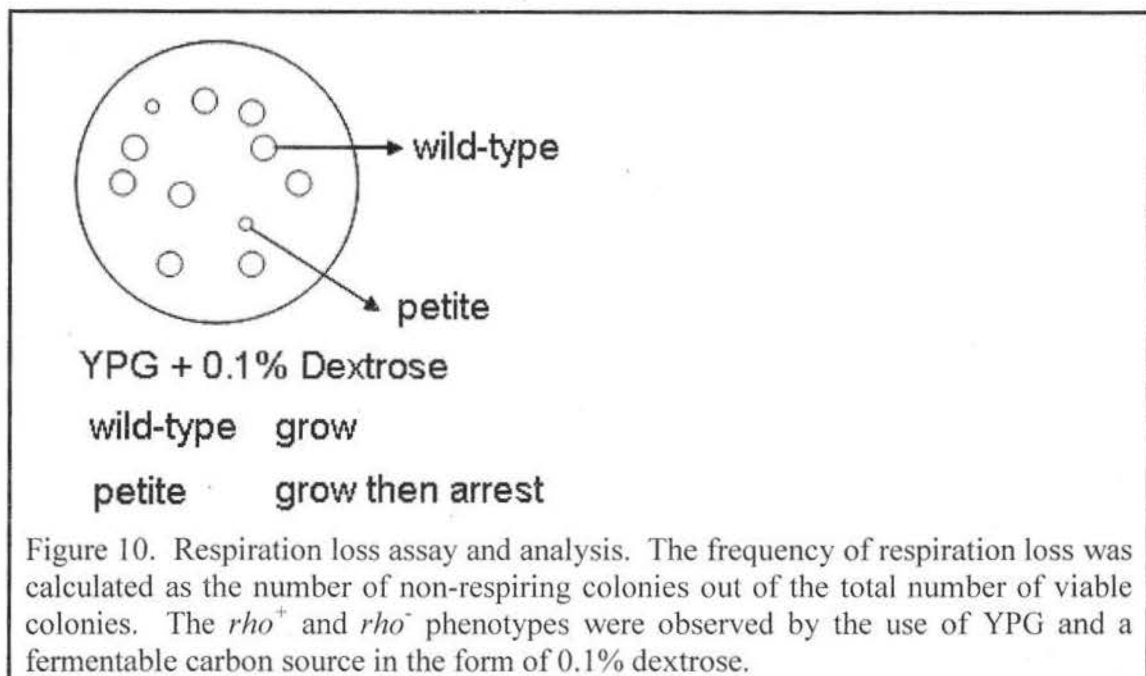

Figure 10. Respiration loss assay and analysis. The frequency of respiration loss was calculated as the number of non-respiring colonies out of the total number of viable colonies. The *rho*$^+$ and *rho*$^-$ phenotypes were observed by the use of YPG and a fermentable carbon source in the form of 0.1% dextrose.

Mitochondrial Reporters.

The advent of mitochondrial reporters has made studying mtDNA mutation events more accurate than ever. These reporters allow researchers to study microsatellite mutations and direct-repeat mediated deletion events in viable yeast

cells. Plasmids with the reporter construct of interest are cotransformed into *rho*^{0} yeast strains by microprojectile bombardment (Fox et al., *1988*)

Microsatellite Instability Reporter

One such reporter is used to quantify the rate of mutations in microsatellite regions (Sia et al., 1997, 2000, 2004). *ARG8*^{m} is an important gene involved in both of the mitochondrial reporters used in our lab, so it is necessary to have an understanding of its origin. *ARG8* is a nuclear gene which encodes for an acetylornithine aminotransferase, a protein necessary for the fourth step in the biosynthesis of the arginine precursor ornithine (yeastgenome.org). Nuclear genes are unable to be expressed within the mitochondria due to genetic code differences. Steele et al. (1996) resolved this issue by creating a synthetic gene, *ARG8*^{m}, which was able to be expressed in the mitochondria. This success was confirmed by the ability of *ARG8*^{m} to complement a nuclear *arg8* deletion.

The microsatellite reporter contains the *ARG8*^{m} gene fused to the first 8 codons of the *COX3* mitochondrial gene (Figure 11). A Poly(GT) tract of 16 units was inserted into the construct at 20 bp downstream of the start codon thereby disrupting the *COX3-ARG8*^{m} reading frame and causing a +2 frameshift (Sia *et al.*, 1997). The polyGT tract mimics naturally occurring microsatellite sequences that are very common in the mitochondrial genome. With this reporter integrated, yeast cells are incapable of synthesizing arginine (Arg$^-$). Cells are also deficient in respiratory ability due to the disruption of *COX3* by the Poly$(GT)_{16}$ tract. If these repeats are lost, the cells become arginine proficient (Arg$^+$). The strain fails to grow in the

absence of arginine unless the frameshift is restored. The occurrence of frameshift mutations is monitored by using complete media lacking arginine.

The restoration of the Arg+ phenotype is thought to occur through polymerase slippage events (Sia et al., 1999). The slippage corrects the reading frame to encode a functional Arg8p by misreading and producing a 'gain' of one base pair or a 'deletion' of two base pairs. The actual DNA sequence of the microsatellite region remains intact, but the way in which the polymerase reads the sequence changes. A high occurrence of polymerase slippage could be one factor contributing to lessened mtDNA stability.

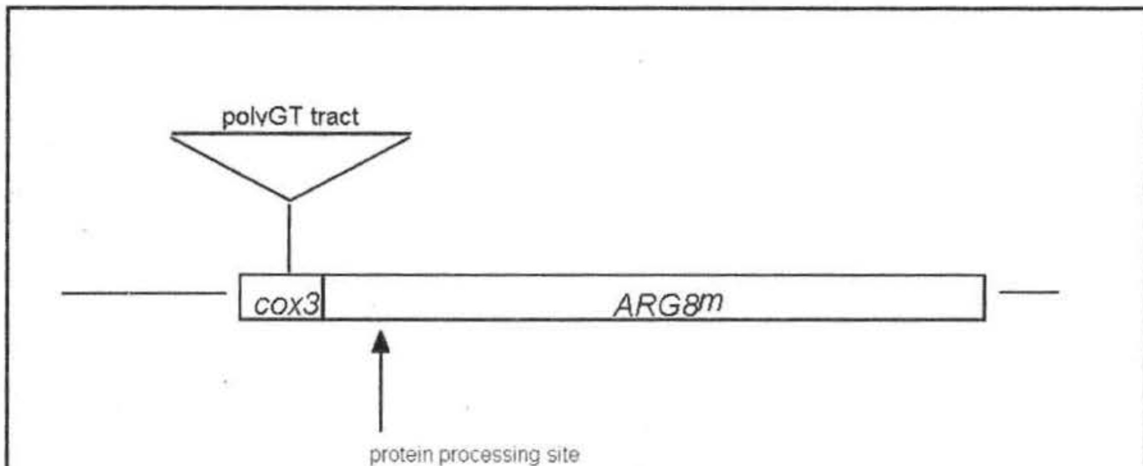

Figure 11. Microsatellite reporter. The mitochondrial genome contains many repetitive sequences called microsatellites. The stability of these regions is directly related to the effectiveness of DNA mismatch repair mechanisms or fidelity of the mitochondrial DNA polymerase. The microsatellite frameshift reporter is constructed with a polyGT +2 frameshift inserted into the *COX3-ARG8^{m}* reading frame. The strain fails to grow in the absence of arginine unless the reading frame is restored. Restoration of the frameshift is monitored using dextrose containing media lacking arginine.

Direct Repeat-Mediated Deletion Reporter

Another reporter generated in conjunction with our collaborator's lab (E. Sia, University of Rochester) is used to observe the frequency of direct repeat-mediated deletion events (Figure 12). As its name suggests, regions of homology can be involved in the deletion of the region of DNA between these repeats resulting in the generation of *rho*$^-$ cells. Again the *ARG8*^{m} gene is used in this reporter to select for cells containing the reporter. The reporter consists of the *ARG8*^{m} gene fused to the first 99 bp of the *COX2* gene including the ATG start codon. The entire *COX2* gene (without the ATG start codon) follows this construct (Phadnis et al., 2005). *COX2* encodes for cytochrome c oxidase subunit II which is an integral membrane protein of the electron transport chain. Therefore cells that have this reporter integrated at the *COX2* locus are unable to respire due to the disruption of the *COX2* gene but are Arg$^+$ due to the presence of *ARG8*^{m}. The regions of *COX2* homology surrounding *ARG8*^{m} provide a site for the occurrence of direct repeat-mediated deletion. Only after this recombination event occurs are cells able to respire. The *COX2* gene comes into proximity of the promoter as *ARG8*^{m} is excised; thereby making these cells unable to synthesize arginine and also respiratory efficient. Recombination events are monitored using non-fermentable carbon source media to determine the number of respiring colonies.

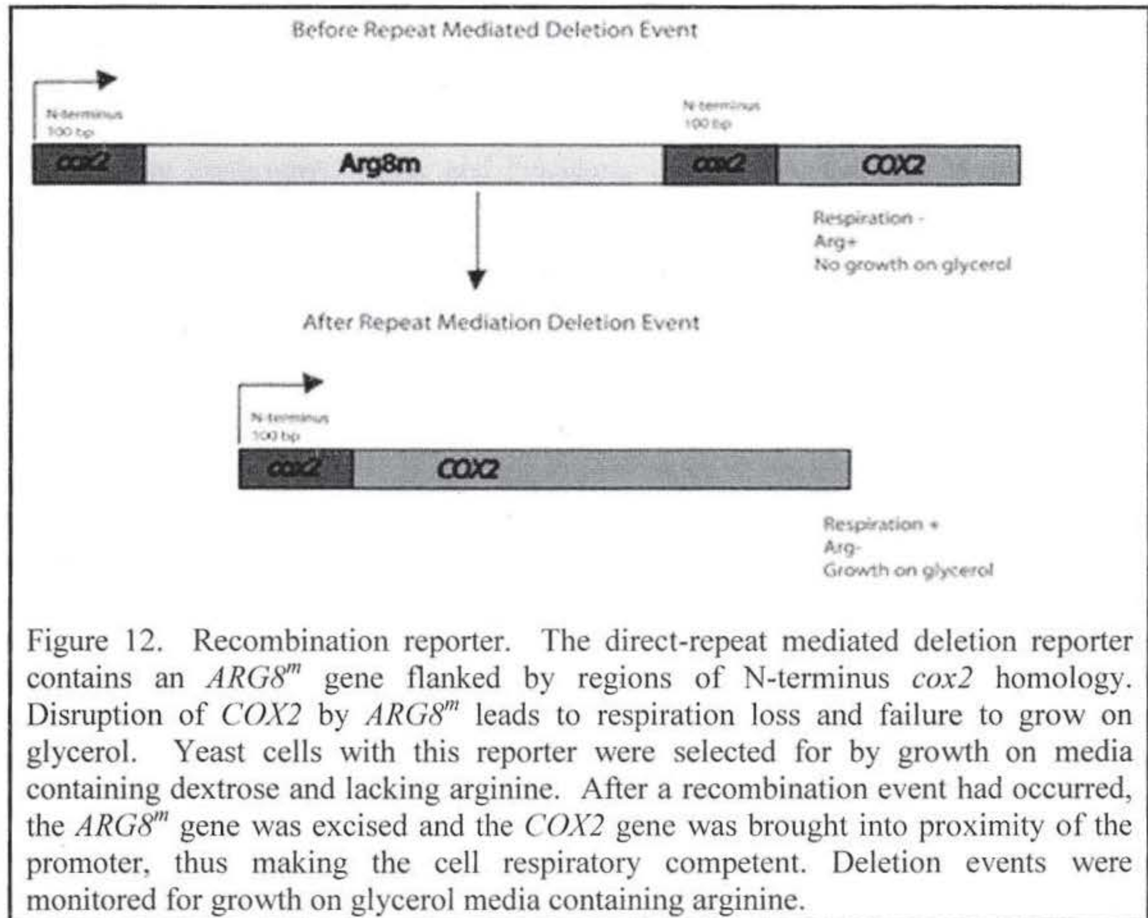

Figure 12. Recombination reporter. The direct-repeat mediated deletion reporter contains an *ARG8^m* gene flanked by regions of N-terminus *cox2* homology. Disruption of *COX2* by *ARG8^m* leads to respiration loss and failure to grow on glycerol. Yeast cells with this reporter were selected for by growth on media containing dextrose and lacking arginine. After a recombination event had occurred, the *ARG8^m* gene was excised and the *COX2* gene was brought into proximity of the promoter, thus making the cell respiratory competent. Deletion events were monitored for growth on glycerol media containing arginine.

Phadnis et al. (2005) classified the types of deletions that arise with this reporter. The types of deletions were determined by analyzing the products that appear after strains containing the mitochondrial reporter are released from selection for the functional Arg8 protein. This study revealed that deletions are generated by at least two different pathways. One of the pathways generates only deletions and the other pathway produces both deletions and reciprocal products of recombination.

Point Mutation Assay.

The antibiotic erythromycin stops translation in yeast by binding to the P-site and blocking elongation and peptide exiting (Figure 13). The rationale behind this

assay is that there are two specific point mutations that can occur within the 21s rRNA that will prevent translation from being disturbed, thus making the yeast cells resistant to erythromycin (Sor and Fukahara, 1982, 1984; Cui and Mason, 1989). Recently, a new study by our collaborators further characterized the spectrum of mutations conferring erythromycin resistance at one of these loci (Kalifa and Sia, 2007). Using our wild-type strain (a reporter is not necessary for this assay) I can find the frequency of these occurrences by plating on media containing erythromycin. Only those cells which have one of the beneficial point mutations will be able to grow on this media.

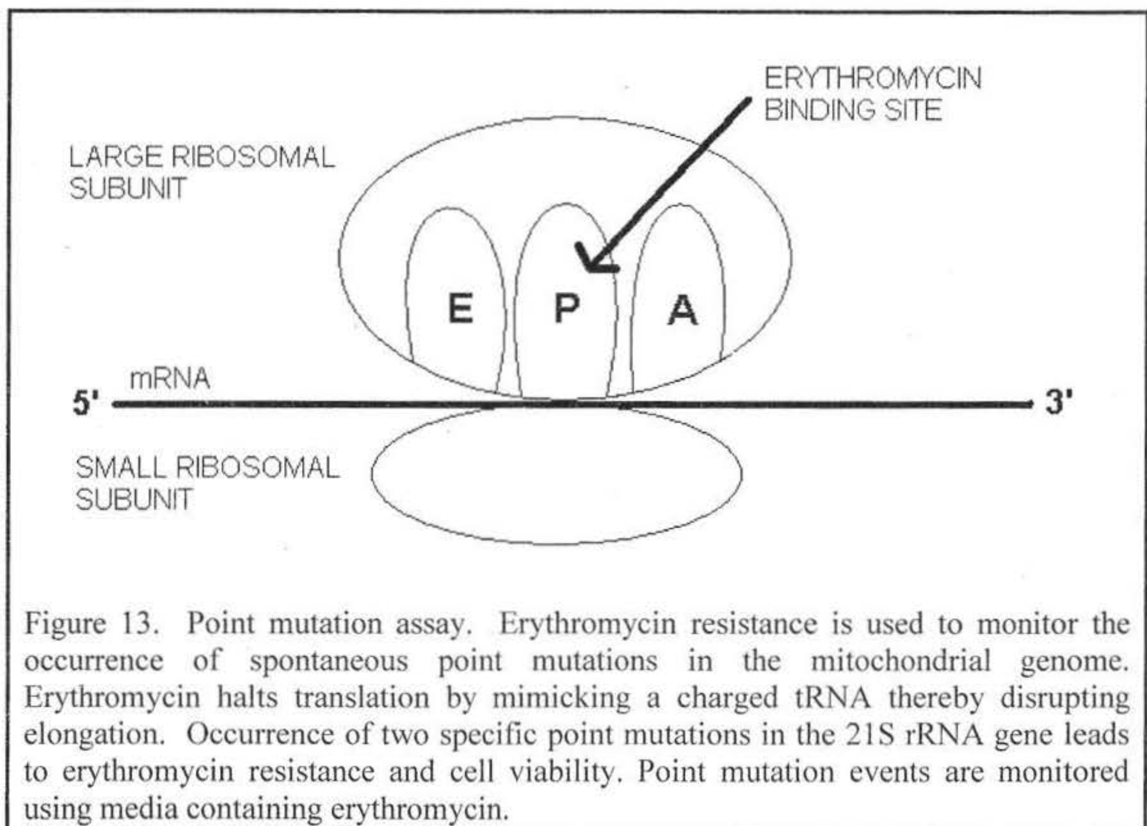

Figure 13. Point mutation assay. Erythromycin resistance is used to monitor the occurrence of spontaneous point mutations in the mitochondrial genome. Erythromycin halts translation by mimicking a charged tRNA thereby disrupting elongation. Occurrence of two specific point mutations in the 21S rRNA gene leads to erythromycin resistance and cell viability. Point mutation events are monitored using media containing erythromycin.

MATERIALS AND METHODS

Yeast strains and growth media.

The *Saccharomyces cerevisiae* yeast strains used in this study are isogenic with DFS188 (*MATa ura3-52, leu2-3,112 ly2 his3 arg8::hisG*). DFS188 is the background strain used due to its naturally low rate of spontaneously occurring petite, non-respiring cells.

Strain	Relevant Genotype	Reference	Assay
DFS188	*MATa ura3-52 leu2-3,112 lys2 his3 arg8::hisG*		Respiration loss & point mutation
EAS748	DFS188 Rep96::*$ARG8^m$::cox2*	Phadnis *et al.*, 2005	Direct-repeat mediated deletion
CAB193	*MATa* [pEAS22*, poly(GT), +2 reading frame]	Sia *et al.*, 2000	Microsatellite instability
NRY088	DFS188 Rep33::*$ARG8^m$::cox2*	Phadnis *et al.*, 2005	Direct-repeat mediated deletion
DFS188clu1	*MATa ura3-52 leu2-3,112 lys2 his3 arg8::hisG clu1Δ::URA3*	This study	Respiration loss & point mutation
EAS748clu1	DFS188 Rep96::*$ARG8^m$::cox2 clu1Δ::URA3*	This study	Direct-repeat mediated deletion
CAB193clu1	*MATa* [pEAS22*, poly(GT), +2 reading frame] *clu1Δ::URA3*	This study	Microsatellite instability

*pEAS22, 5′-A(GT)$_{16}$AC and 5′-CTGT(AC)$_{15}$A

Table 1. Strains used in this study

Growth media included yeast peptone glycerol (YPG), YPG + 0.1% dextrose, YPD, YG + Erythromycin (4 mg/ml), synthetic dextrose media lacking arginine (SD-Arg), and SD-Ura. Unless otherwise noted, each assay was performed at least twice with 10 independent colonies for both genotypes.

Creating *clu1Δ::URA3* disruption cassette

A disruption cassette was created by utilizing Polymerase Chain Reaction (PCR) using *clu1*/URA hybrid primers. The pRS406 plasmid (containing the *URA3* gene) functioned as the template. The purpose of this construct is to amplify the URA3 gene and have it be flanked by regions of *CLU1* homology for ease of transformation (Figure 14). The nuclear background of the yeast strains used in this research is uracil minus, so future integration of the disruption cassette can be verified by selecting colonies that have gained the ability to synthesize uracil (shown by plating on SD-Ura).

The reagents for this PCR protocol are as follows: 1µl pRS406 (template), 1µl *Thermus aquaticus* (*Taq*) Polymerase (Fermentus), 2.5µl $MgCl_2$, 1.5µl 10mM dNTPs, 36µl dH_2O, 1.5µl of 20 µM up and down primers, and 5µl buffer. All reagents are added to the reaction mixture. It is important to add the *Taq* Polymerase last so that its 5' endonuclease activity does not affect the template or primers. The reaction is then placed in a thermocycler and run on the expand program. This program has an extension stage that increase 5 seconds per cycle starting at 20 cycles. All reagents in this PCR are supplied by Sigma Aldrich with the exception of the primers. The primers used in this reaction are CLU1 pRSup 5'-GCT TCT TTT TAT TGT TGG TTT TGT TTT-3' and CLU1 pRSdn 5'-GCG TAG TGT TAC AGT TTA TCT GAG AC-3' (Integrated DNA Technologies).

To verify the PCR disruption cassettes, 10µl of PCR product, 3µl 6X loading buffer (Promega), and 7µl dH_2O were run on a 1% agarose gel. To make the gel,

0.5g of Agarase Multi-Purpose Molecular Grade (BIOLINE) was weighed and dissolved in 50ml of 1X TAE (Tris-Acetate-EDTA) buffer. 2.5µl of EtBr (ethidium bromide) was added to aid in the visualization of the nucleic acid bands. This mixture was poured into an electrophoresis mini-gel tray (a comb was added to form wells) and allowed to solidify. The PCR product with loading buffer was added into the wells. Hyperladder I (BIOLINE) served as a reference marker. 99 volts were applied as the electrical charge. After the DNA migrated sufficiently through the agarose gel, the gel was removed from the tray and placed on a transilluminator to visualize the results. Bands of ~1200 base pairs indicated a successful creation of the disruption cassette.

The resulting disruption cassette consisted of the URA3 gene flanked by regions of clu1 homology.

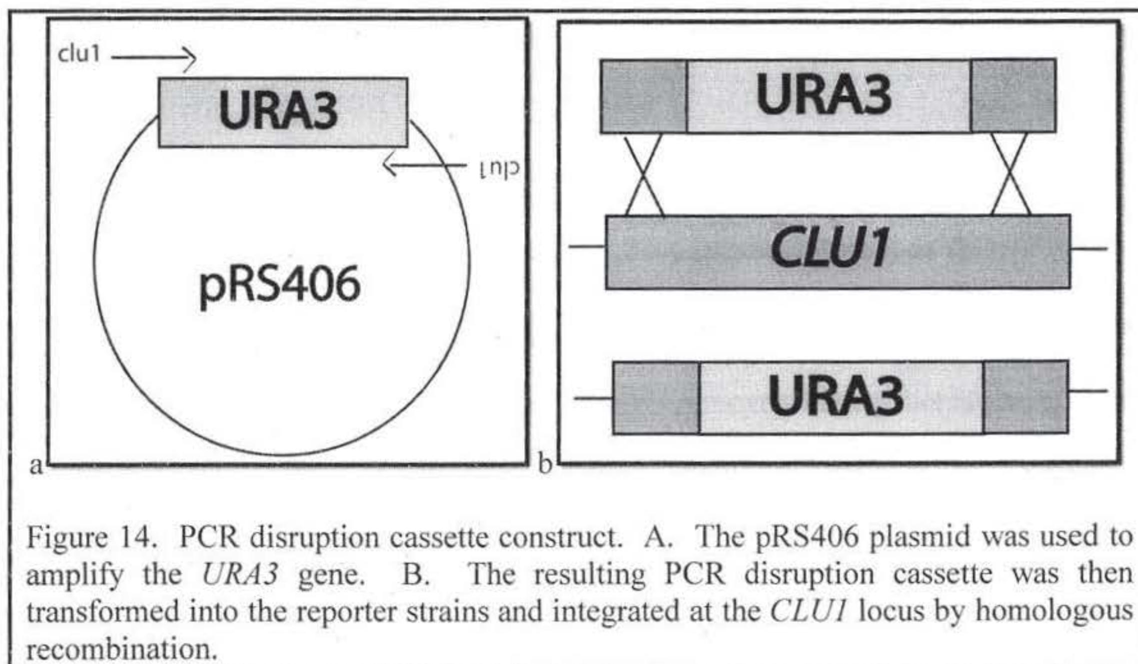

Figure 14. PCR disruption cassette construct. A. The pRS406 plasmid was used to amplify the *URA3* gene. B. The resulting PCR disruption cassette was then transformed into the reporter strains and integrated at the *CLU1* locus by homologous recombination.

Transformation

CLU1 was subsequently disrupted by the method of one-step gene transplacement. In this method, homologous flanking regions are added to a selectable marker (Wach, 1996). A high efficiency lithium acetate transformation protocol was used to introduce clu1Δ::URA3 into the strains DFS188, EAS748, and CAB193. 50ml of cells were grown to an optical density of 0.3. Cells were spun at 3,000 rpm for 5 minutes and washed with sterile water. They were then resuspended in 1ml 100mM LiOAc and transferred to a 1.5ml microfuge tube. Cells were spun quickly to pellet and the liquid was removed. The cells were resuspended in 400μl LiOAc. At this point, single stranded (SS) DNA in the form of 10mg/ml salmon sperm was boiled for 10 minutes to promote strand dissociation and placed on ice immediately to prevent reannealing. The resuspended cells are vortexed and divided into 50μl aliquots for each transformation. The following solutions are added in precise order: 250μl 50% PEG (polyethylene glycol), 36μl 1M LiOAc, 10μl SS DNA, 0.1 to 10μg DNA, and sterile water to bring the total volume to 360μl. This mixture is vortexed to resuspend the pellet. Two incubation periods follow: one at 30°C for 30 minutes and a second at 42°C for 30 minutes. After these incubations the mixture is spun once more, and the supernatant is removed. The pellet is resuspended in 400μl water by gently pipetting up and down. 200μl is plated on 2 uracil omission plates. These plates are incubated at 30°C until colonies appear.

Verification of transformants.

Transformants were patched onto a master plate of SD-URA and verified using whole cell PCR with a downstream clu1 primer (CLU1 F-93 5'- CAC AGA GTA GTA ATT GAG CTG -3' and an upstream URA3 primer (URA3 5'- CCG TGT GCA TTC GTA ATG TC -3') (Integrated DNA Technologies). The reagents for whole cell PCR are 12.5μl of BioMix Red (BIOLINE), 1.5μl of 20μM forward primer, 1.5μl of 20μM reverse primer, 9.5μl dH_2O, and whole cells from each transformant colony for the DNA. These 25μl reactions were run in a thermocycler on the expand program.

The whole cell PCRs were run on a 1% agarose gel (made as previously described). Each PCR was mixed with 3μl 6X loading buffer (Promega)

Respiration loss analysis.

The DFS188 strain was streaked on YPG for respiring independent colonies. This plate was incubated at 30°C for 3 days. Twenty independent single colonies were selected and serially diluted. 100 μl of 10^{-4} were plated on YPG with 0.1% dextrose. These plates were incubated at 30°C for 3 days. Respiration loss rates were then calculated.

Direct repeat mediated deletion assay.

EAS748 strains were patched onto SD-Arg to verify the integration of the mitochondrial reporter. Cells were then streaked onto SD-Ura (to ensure the presence of a *clu1* deletion) and grown at 30°C for 3 days to allow for recombination events. Twenty independent single colonies were selected and serially diluted. 100μl of 10^{-2}

were plated on YPG as the experimental and 50μl of 10^{-4} were plated on YPD as a control. These plates were incubated at 30°C for 3 days. After 3 days, colony counts were obtained for all plates.

Microsatellite assay.

The CAB193 strain was streaked onto SD-Ura for singles. Twenty individual independent colonies were selected and serially diluted. 95ul of the undiluted sample was spread on SD-Arg plates to function as the experimental value. 50ul of 10-4 cells were plated onto YPD for the control. These plates were allowed to incubate at 30°C for 3 days. Colony counts were obtained for each plate.

Point mutation assay.

Using a technique adapted from Mookerjee *et al.* (2004), DFS188 strains were streaked onto YPG for respiring single colonies. Twenty independent individual colonies were inoculated in 2.5 ml of YPG growth media and grown to saturation at 30°C. After dilution, 50μl of 10^{-5} were plated on YPG and 200μl of undiluted were plated on YG with phosphate buffer and 4.0g/l Erythromycin. The control YPG plates were incubated at 30°C for 3 days and the experimental plates were incubated at the same temperature for 7 days. After the incubation period, colony counts were determined for each plate.

Ultraviolet sensitivity assay.

The DFS188 strain was streaked onto YPG to select for independent respiring single colonies. Individual colonies were picked and grown in 2.5 ml overnight YPG cultures at 30°C. The culture was spun down, and the cells were resuspended in

500μl dH_2O. Cultures were serially diluted. 50μl or 100μl of a 10^{-4} dilution were plated on YPG + 0.1% dextrose. Plates were subjected to UV-B radiation at time intervals of 0, 20, 40, 60, and 80 seconds. Plates were then incubated in the dark, to prevent photorepair processes, for 3 days at 30°C. Cell viability as a percentage was quantified by comparing the total number of colonies (petites and respiring cells) on plates treated to UV-B to the total number of colonies on plates that were not exposed to UV-B (0 seconds). Respiration loss was also quantified (as previously described). The timepoints were performed in triplicate, and each strain was tested two times.

Oxidative stress assay.

The DFS188 strain was streaked onto YPG and grown at 30°C for 3 days to select for respiring colonies. Individual colonies were diluted in 100μl H_2O_2 then incubated at 30°C for 1 hour. After the incubation period, 1 ml of d H_2O was added immediately. Cells were spun down for 30 seconds, the supernatant aspirated, and the pellet resuspended in 100μl d H_2O. These suspensions were serially diluted to 10^{-4}. 200μl was plated on YPG + 0.1% dextrose and incubated at 30°C for 3 days. To quantify the results of oxidative stress, respiration loss was determined as described above.

Calculation of frequencies and mutation rates.

To determine the frequency of respiration loss in *clu1Δ::URA3*, the respiration loss of each plate was calculated as the number of petite colonies appearing on the plate divided by the number of total colonies. After obtaining a frequency for each

plate, the mean of all of the frequencies was calculated for the ultimate respiration loss frequency.

For the remaining fluctuation assays, the method of the median (Lea and Coulson, 1949) was used to calculate the rate of mutations per cell division. A Microsoft Excel program is utilized to enter colony counts for both control and experimental plates (Figure) The first average was taken from the values entered for the control plates. One-half the average and 2X average were computed. From here, any values outside of this range were disregarded and a new average (the 2nd average) was calculated. The median of the experimental plate colony count was determined from the remaining data groups.

The units for my data are mutations per cell division. The number of cell divisions (denominator) can be found by multiplying the 2nd average and the dilution factor of the control. The number of mutations (numerator) is a multi-step calculation involving the use of a chart devised by Lea and Coulson (1949). r_0 is established by calculating the product of the median and the dilution factor of the experimental.
The chart is then used to determine a corresponding r_0/m for that particular r_0 value. r_0 divided by r_0/m gives the number of mutations.

In some instances, the exact r_0 is not represented on the chart (it falls between two posted r_0 values); therefore, there is no corresponding r_0/m. The adjusted r_0/m can be computed mathematically. I will illustrate using the hypothetical r_0 value of 1.7. (a)

Using the table below (Table 2), an r_0 of 1.7 falls between an r_0 of 1.6 and 1.9. Therefore it is sensible that the corresponding r_0/m would fall between 1.4 and 1.5. This will act as a check to ensure that the calculations are performed correctly upon completion.

First, 1.6 (the next lowest r_0 value) is subtracted from 1.7 to yield 0.1.

$1.7 - 1.6 = 0.1$ (b)

Then the difference between the two r_0's that surround where 1.7 would be located on the chart is taken. (This difference is actually included on the chart in the first column.)

$1.9 - 1.6 = 0.3$ (c)

The second number is then divided by the first (b ÷ c)

$0.1 \div 0.3 = 0.333\ldots$ (d)

This quotient is then multiplied by 0.1 (d x 0.1)

$0.333 \times 0.1 = .0333\ldots$ (e)

This number is then added to the r_0/m of the number directly below where the actual r_0/m would fall. (e + low range r_0/m)

$.0333 + 1.4 = 1.4333$ (f)

To double check, 1.4333 is indeed between 1.4 and 1.5 which we originally predicted.

Again the number of mutations is determined by dividing r_0 by r_0/m. (a ÷ f)

$1.7 \div 1.4333 = 1.186$

Now, we have both the numerator (mutations) and the denominator (# of cell divisions), so it is a matter of performing the division and converting the answer to scientific notation.

	r_0	r_0/m
	1.4	1.3
0.2		
	1.6	1.4
0.3	*	*
	1.9	1.5
0.4		
	2.3	1.6

Table 2. Representative portion of the Lea and Coulson (1949) chart.

Fluorescence microscopy.

In my research, I used two specific molecular probes which are important in visualizing mitochondrial morphology. DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) binds specifically to AT regions of DNA and is used to visualize the location of the mtDNA or nucleoids. Mitotracker comes in a variety of colors and binds to mitochondrial membranes based on membrane potential. This is useful in observing the mitochondrial network. The stain utilized in this research was Mitotracker Red (Invitrogen) which fluoresces upon oxidation.

Wild-type DFS188 and DFS188 clu1Δ::*URA3* were used to observe the effects of a clu1 disruption and compare the phenotypes of the mutant to the wild-type. To prepare the cells for microscopy I used a protocol provided by Mookerjee *et*

al. (2005). An overnight culture was started then diluted and grown to mid-log phase. I observed yeast cells grown in both synthetic media containing dextrose (SD-Ura) and glycerol (YPG). 1 ml of this preparation was added into a microfuge tube and 0.1 ul of 1mM Mitotracker was added. The Mitotracker was allowed to stain for 1 hr at 30°C on a shaker. At the end of the hour, 1µl of 10mM DAPI solution was added to the cells and allowed to stain for 5 minutes. The cells were then spun down, washed with 200µl dH2O, and resuspended in 200µl dH_2O. Approximately 6-7 µl of vortexed cells were added to a Poly-Lysine treated slide. These slides were then observed and photos were captured with a Zeiss Axioplan 2 microscope with standard DAPI and Mitotracker settings.

RESULTS

Identification of Clu1p.

ILV5 has been found to be important in mtDNA stability (Bateman et. al, 2002; Sia et. al, 2003; MacAlpine et. al, 2000). Thus, Ilv5p was utilized as the bait in our yeast 2-hybrid assay to find other proteins involved in this process.

The results of this preliminary screen with Ilv5p fused to the Gal-4 binding domain identified Clu1p as an Ilv5p interactor. *CLU1* is a nuclear encoded protein that localizes to the mitochondria. It received its name because the appearance that null mutants display: a clustering of the mitochondrial network. (Fields et al., 1998) *CLU1* has also been identified as *TIF31*, a 135 kilodalton subunit of Translation Initiation Factor eIF3 (Vornlacher *et al.* 1999), although it is not yet known whether it can be properly classified as a putative eIF3 subunit (Logan *et al.*, 2003). Its systematic name is YMR012w. *CLU1* consists of approximately 3.8 kilobase pairs located on chromosome XIII. *CLU1* encodes for a 1,277 amino acid protein with a molecular weight of approximately 145 kilodaltons. In terms of protein structure, Saccharomyces Genome Database indicates predicted transmembrane domains at the 379-407 and 833-855 amino acid coordinates (yeastgenome.org).

Creation of a CLU1 disruption cassette.

In order to study the phenotype of a *CLU1* disruption, it was necessary to construct a functional knockout. This goal was achieved through the use of one step gene displacement. A *CLU1* disruption cassette was created by one step gene replacement and verified by running the PCR products on a 1% agarose gel

(BIOLINE). The PCR products were compared against the HyperLadder I molecular ladder (BIOLINE). The presence of a band between 1,000 and 1,500 bp (1.3 kb) indicates the correct product (Figure 15).

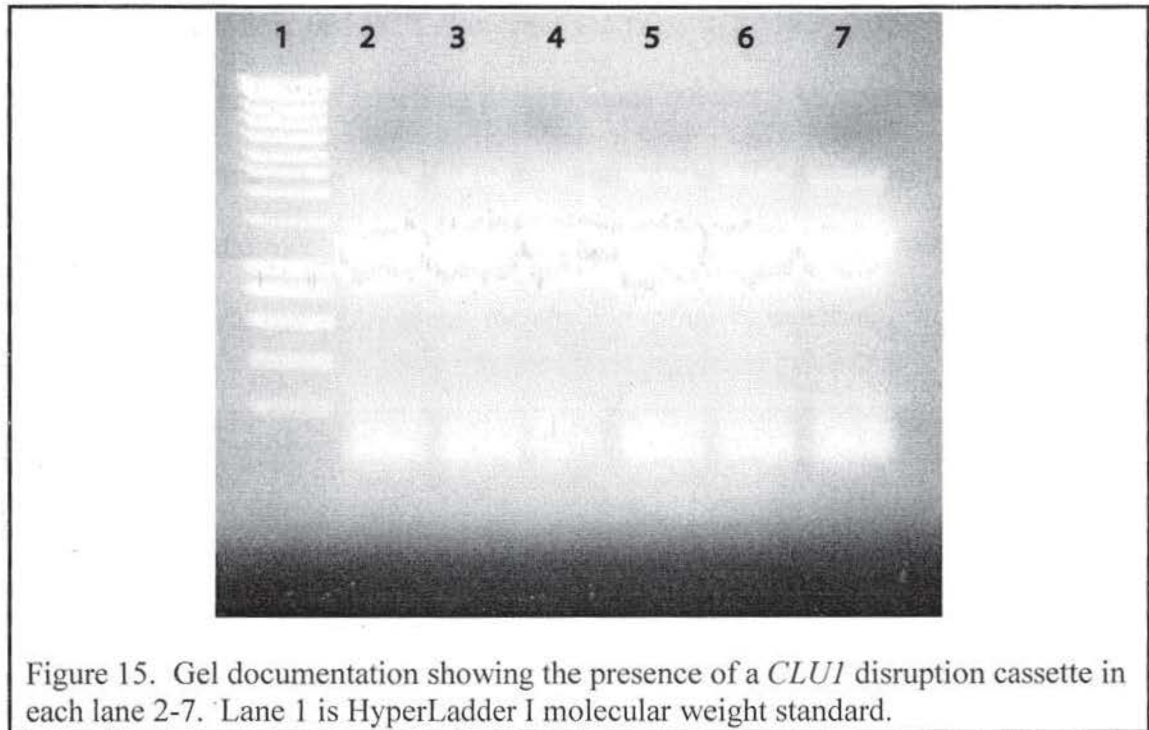

Figure 15. Gel documentation showing the presence of a *CLU1* disruption cassette in each lane 2-7. Lane 1 is HyperLadder I molecular weight standard.

***CLU1* Disruption.**

In order to observe the effects of a *CLU1* deletion in specific mitochondrial activities, the *CLU1* disruption cassette was introduced into various strains carrying reporter constructs. The *CLU1* disruption cassette was transfected into a wild-type strain (DFS188) well known for its low occurrence of spontaneous mitochondrial mutations, a strain containing a microsatellite frameshift reporter (CAB193), and a strain containing a direct repeat-mediated deletion reporter (EAS748). Both EAS and CAB strains are isogenic with DFS188. The strains used in this study have a

background that is Ura -. Therefore SD-Ura plates were used to select for colonies that had integrated this construct. Proper transfection efficiency was further verified through whole cell PCR. Whole cell, or colony, PCR is much like regular PCR except that grown cells as opposed to purified genomic DNA is used as the template. Opting to use whole cell PCR eliminates the need to perform genomic preparations, and therefore makes the screening process much quicker. An upstream *URA3* primer and a downstream *CLU1* specific primer were used in this reaction. The presence of a band at approximately 700 bp (Figure 16) indicated that the disruption cassette had been integrated at the *CLU1* locus, thereby disrupting its function.

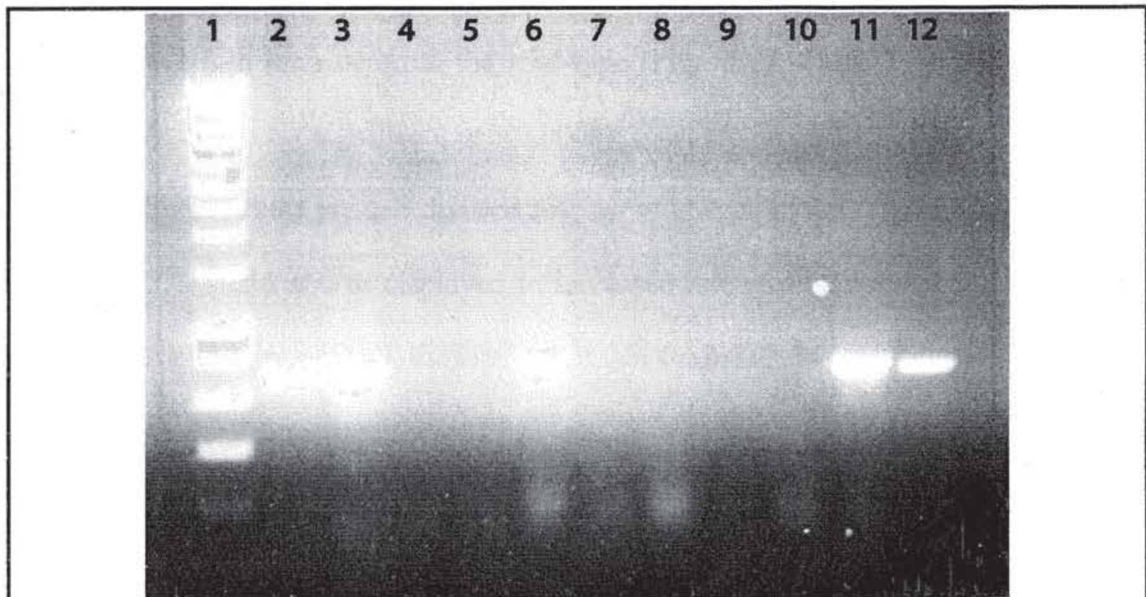

Figure 16. Gel documentation showing a successful transfection of the *CLU1* disruption cassette into the EAS748 strain. Lanes 2, 3, 6, and 11 show the presence of EAS748 *clu1Δ::URA3* product. Lane C (at the far right) serves as the positive control for the PCR. Lane 1 contains HyperLadder I as the molecular weight standard.

Clu1p is required for normal cellular respiration.

The primary function of the mitochondria is to perform respiration. In testing the effects of knocking out a mitochondrial protein, the first logical experiment is one which tests for respiration capability. Therefore, to determine if my Clu1p mutants have proper mitochondrial function, an initial respiration loss assay was performed.

The distinguishing phenotypes of petite versus large colonies allowed us to quantify the frequency of respiration loss on mixed media of YP Glycerol + 0.1% dextrose. Wild-type has a natural low background of spontaneously arising non-respiring petite cells. The rate of respiration loss for my wild-type strain is 0.2%. In comparison, petite colonies arise in *clu1Δ::URA3* strain at a frequency of 3.23%. This is a 9.2-fold increase from the wild-type (Figure 17, Table 3; p. 76), which is statistically extremely significant ($p = 0.0006$).

The mutations per cell division rate for wild-type in the respiration loss assay is 3.9×10^{-4}. *clu1Δ* strains displayed an increased rate of mutations of 9.5 fold. The number of mutations per cell division rate of this strain is 3.74×10^{-3} (Table 3; p. 76).

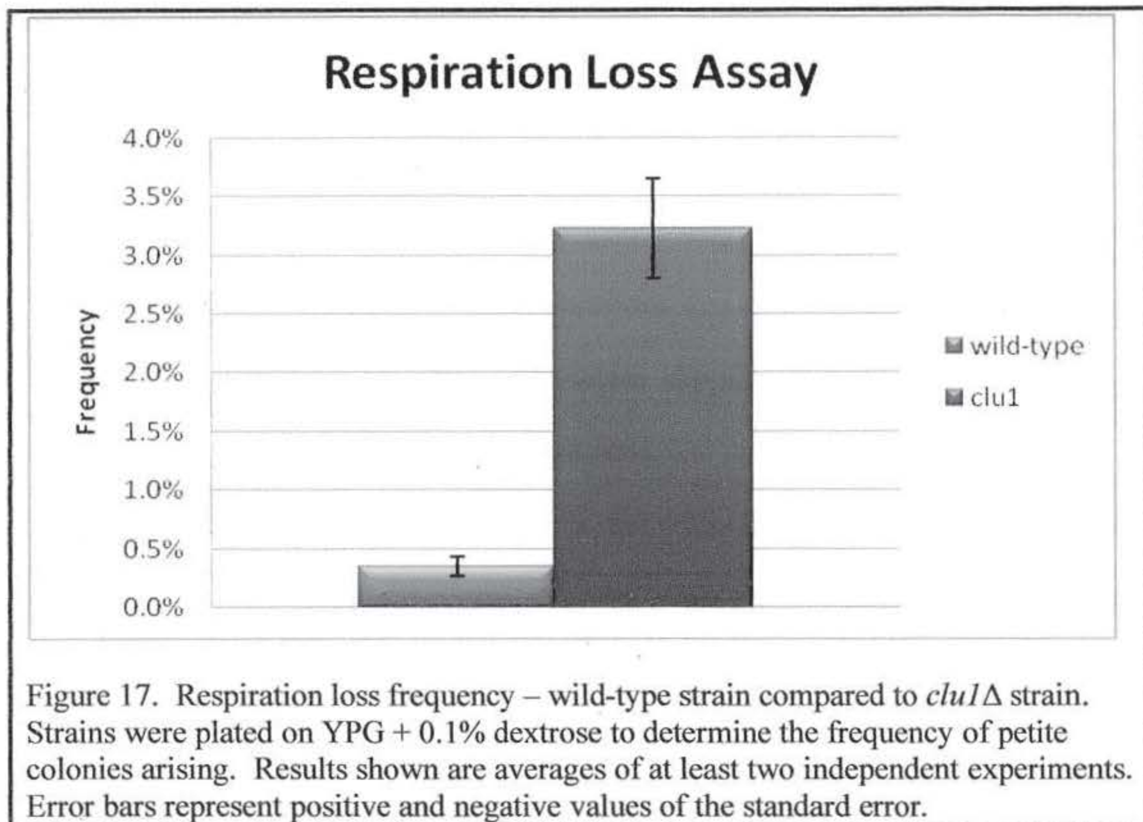

Figure 17. Respiration loss frequency – wild-type strain compared to *clu1*Δ strain. Strains were plated on YPG + 0.1% dextrose to determine the frequency of petite colonies arising. Results shown are averages of at least two independent experiments. Error bars represent positive and negative values of the standard error.

These data show that a *clu1* deletion results in an increase in respiration loss in *clu1* mutant strains as compared to wild-type strains. The difference in the fold changes between mutations per cell division and respiration loss frequency show the dynamic nature of mitochondrial function. Many different single mutations and combinations of mutations can lead to a loss of respiratory function in the mitochondria. It is not an easily defined relationship. Subsequent assays will help to elucidate what is causing the high rate of respiration loss.

***clu1* mutants display no significant differences in polymerase slippage.**

Based on my initial respiration loss assay, *CLU1* somehow contributes to maintaining respiratory function in yeast. One of the possible mechanisms by which yeast can lose respiratory capability is a malfunction during the replication process of

mtDNA. Mistakes in replication can lead to mutations in necessary mitochondrial genes, and consequently to improperly functioning proteins, i.e. respiratory complex proteins. To test if the respiration loss seen in *clu1Δ* strains is a result of this phenomenon, a microsatellite reporter (previously described) was utilized to determine the frequency of frameshift mutations that occur in *clu1* deletion strains. This assay is a measure of DNA polymerase slippage during replication along a Poly(GT) tract within the mitochondrial genome.

The use of this reporter showed that the wild-type strain (CAB193) exhibited a rate of 2.25×10^{-6} mutations per cell division. *clu1Δ::URA3* strains exhibited a rate of 3.50×10^{-6} mutations per cell division (Figure 18, Table 3; p. 76). This is a 1.6 fold increase, which is not significant ($p = 0.2999$) in implicating Clu1p as a major factor in microsatellite instability.

This finding suggests that Clu1p is probably not involved in the process of mtDNA replication. However, the possibility that Clu1p is involved in replication cannot be completely excluded; perhaps it has a function which is not essential to the fidelity of the process.

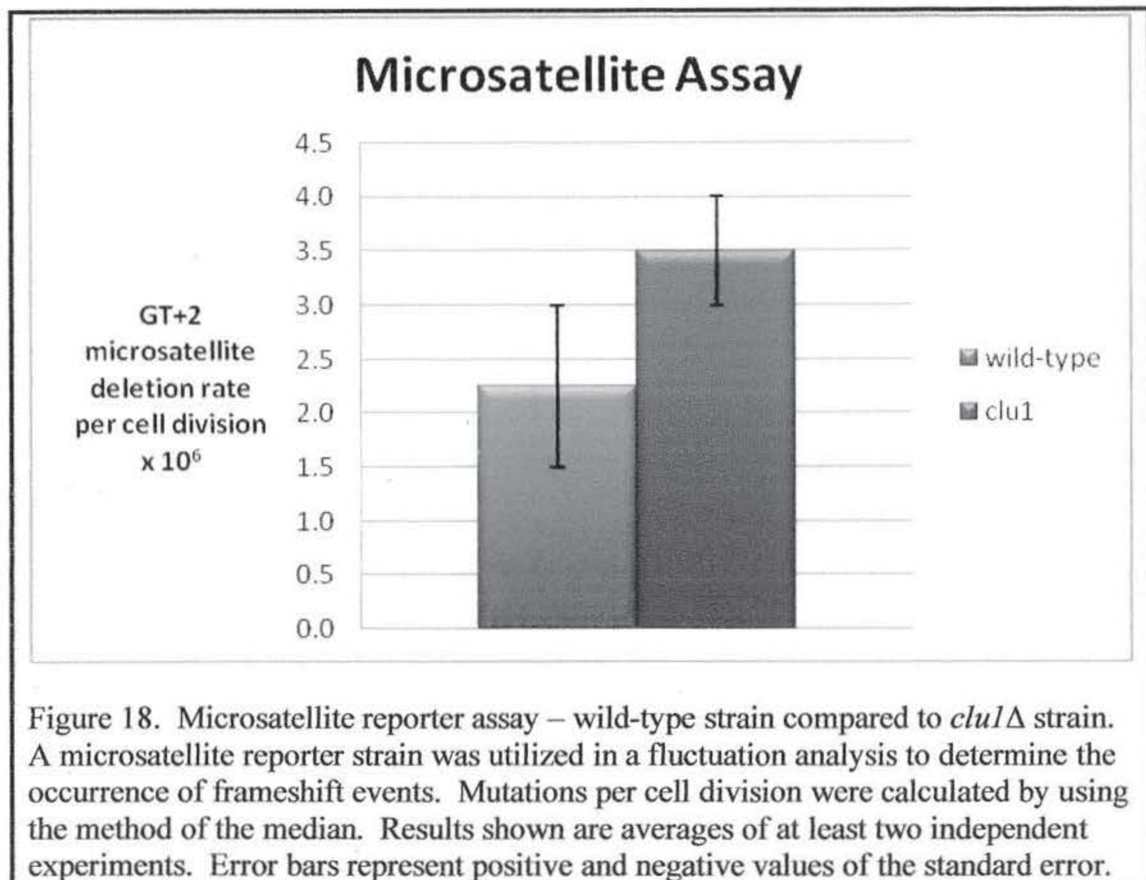

Figure 18. Microsatellite reporter assay – wild-type strain compared to *clu1*Δ strain. A microsatellite reporter strain was utilized in a fluctuation analysis to determine the occurrence of frameshift events. Mutations per cell division were calculated by using the method of the median. Results shown are averages of at least two independent experiments. Error bars represent positive and negative values of the standard error.

Clu1p plays a role in direct repeat-mediated deletion.

After eliminating the possibility of polymerase slippage as the means by which respiration loss arises in *clu1* mutants, I hypothesized that perhaps Clu1p is involved in alteration of the mtDNA by alternative methods. Another source of genome instability within mtDNA is the deletion of regions of the genome itself. The direct-repeat mediated deletion reporter is used to determine how often a segment of mtDNA located between two regions of homology is deleted.

*clu1*Δ*::URA3* strains showed a 5.3 fold decrease in direct-repeat mediated deletion events when compared to wild-type EAS748 strains. The rate of mutations per cell division of a *clu1*Δ*::URA3* is 1.55E-05 while the rate for wild-type is 8.15 x

10^{-5} mutations per cell division (Figure 19, Table 3; p. 76). The p-value for this assay is 0.0455 which is considered significant.

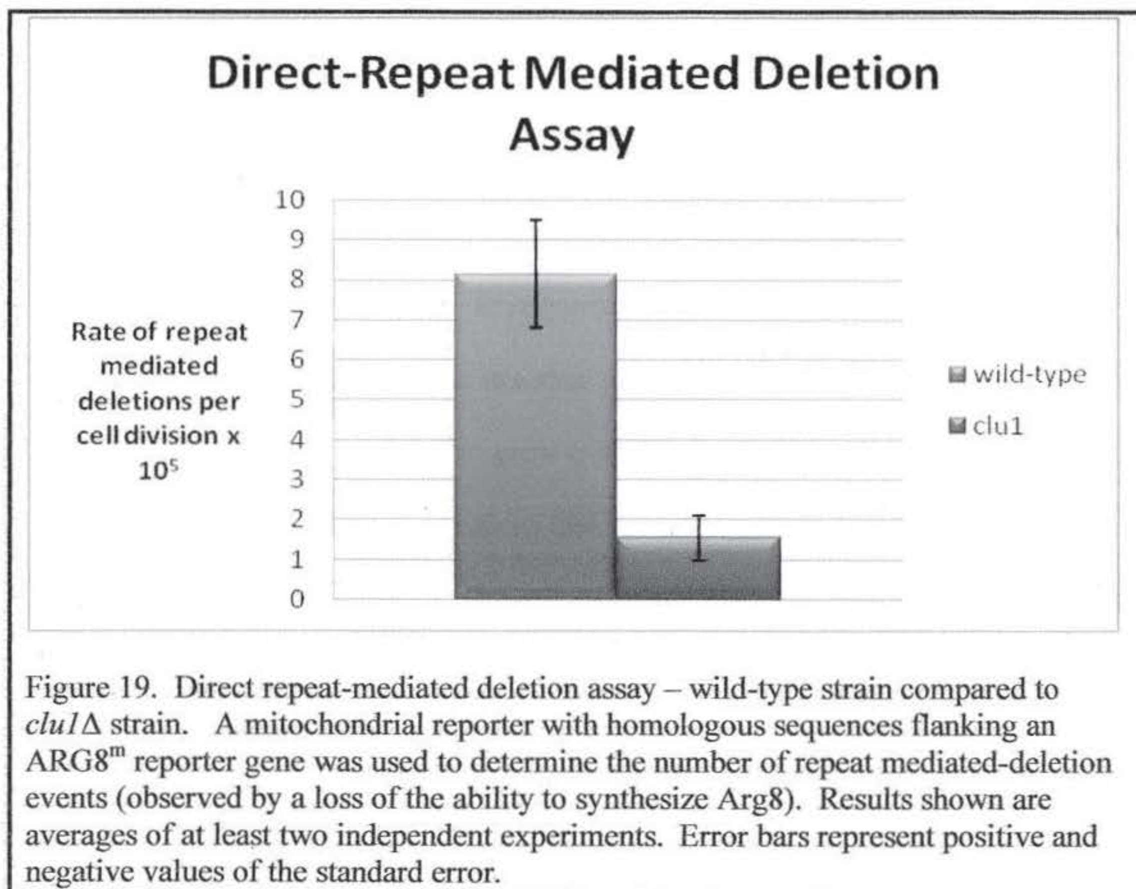

Figure 19. Direct repeat-mediated deletion assay – wild-type strain compared to *clu1*Δ strain. A mitochondrial reporter with homologous sequences flanking an ARG8^{m} reporter gene was used to determine the number of repeat mediated-deletion events (observed by a loss of the ability to synthesize Arg8). Results shown are averages of at least two independent experiments. Error bars represent positive and negative values of the standard error.

This decrease indicates that *clu1* is important in the process of recombination within the mitochondrial genome. This effect could be a direct effect of the loss of *clu1* or an indirect effect via another gene product. Further testing would be necessary to determine the exact role of *clu1* in this process.

***clu1* cells have an increased occurrence of spontaneous point mutations.**

Many genes that contribute to a loss of respiratory function in mitochondria have been linked to point mutations (Kalifa and Sia, 2007). This relationship led us

to question whether my *clu1* mutant strain had a higher incidence of point mutations, ultimately resulting in increased respiration loss.

Point mutations, if left unrepaired, can create dysfunction within the genome. Certain point mutations in the mitochondrial genome have been associated with specific diseases. The rate of point mutations was determined with the use of the wild-type background containing no reporters. Yeast cells were plated onto media with the antibiotic erythromycin. Erythromycin halts translation by binding to the P-site of the large ribosomal subunit and thus preventing further elongation and protein production. Yeast cells will fail to grow in the presence of erythromycin. However, there are three specific point mutations that confer resistance to erythromycin (Ery^R) (Sor and Fukahara, 1982, 1984; Cui and Mason, 1989). By determining how often Ery^R arises in a population of cells, I can measure the rate of point mutations that have occurred.

Wild-type strains have a rate of 2.54×10^{-7} point mutations per cell division. *clu1Δ::URA3* strains have a rate of 4.26×10^{-8} point mutations per cell division. This is a 2.2 fold increase (Figure 20, Table 3; p. 76), which is considered not significant ($p = 0.3283$).

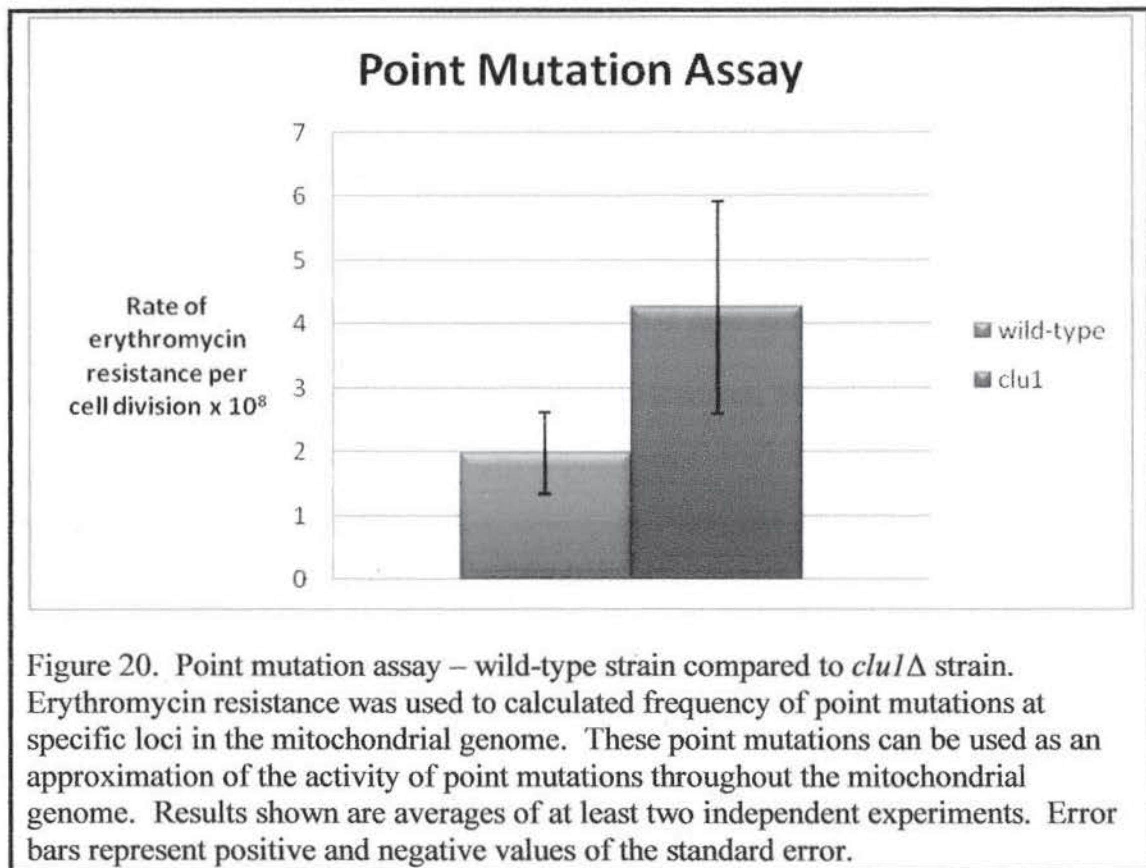

Figure 20. Point mutation assay – wild-type strain compared to *clu1*Δ strain. Erythromycin resistance was used to calculated frequency of point mutations at specific loci in the mitochondrial genome. These point mutations can be used as an approximation of the activity of point mutations throughout the mitochondrial genome. Results shown are averages of at least two independent experiments. Error bars represent positive and negative values of the standard error.

The role of Clu1p in UV repair is unclear.

UV radiation is used to introduce a higher rate of mutations into the mtDNA. The viability and respiration loss of yeast depends on its ability to repair the damage caused by this mutagen. Due to the inability of *clu1*Δ to maintain normal levels of respiratory function, I decided to test if UV radiation could further exacerbate the loss of respiratory capacity.

Wild-type strains were plated and exposed to ultraviolet radiation for 6 different time points; 0, 20, 40, 60, 80, and 100 seconds. One control strain and two different *clu1Δ::URA3* strains were used. The *clu1* strains used were different transformants that were obtained from the initial transformation process. Two strains

were used to ensure the accuracy of the results. Sensitivity to UV radiation was quantified by two parameters: respiration loss and viability. These parameters were determined for the different time exposures and compared to the wild-type control (DFS188).

My results show that the absence of *CLU1* does not affect cell viability when exposed to ultraviolet radiation (Figure 21). However, the absence of *CLU1* does affect the respiration loss of yeast cells (Figure 22).

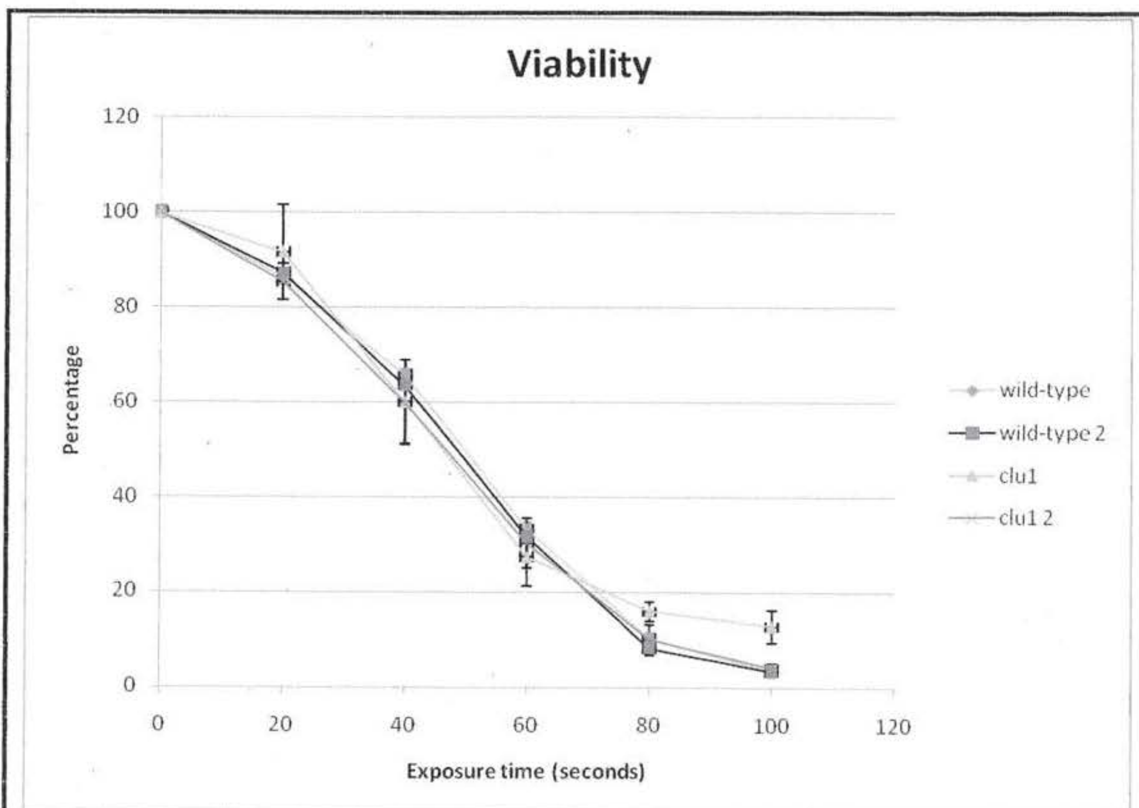

Figure 21. UV sensitivity – viability versus time exposure. Wild-type and *clu1*Δ strains were exposed to UV irradiation for 0, 20, 40, 60, 80, and 100 seconds. Viability was determined by normalizing the number of colonies present at the 0 second exposure timepoint to 100%. The viability of subsequent timepoints was compared to this baseline number. Two independent experiments were performed with each timepoint performed in triplicate. Error bars represent positive and negative values of the standard deviation.

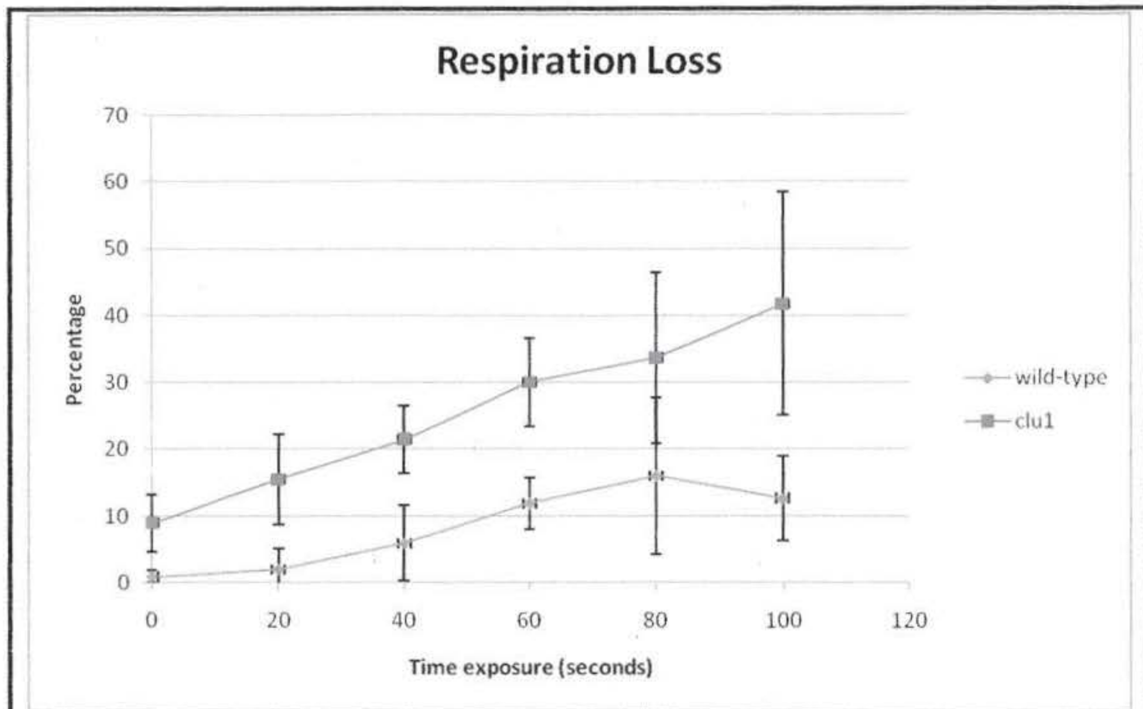

Figure 22. UV Sensitivity – Respiration loss versus time exposure. Wild-type and *clu1*Δ strains were exposed to UV irradiation for the timepoints previously noted. Respiration loss was calculated as previously described. Each experiment was performed twice with each timepoint being carried out in triplicate. Error bars represent positive and negative values of the standard deviation.

In the untreated timepoint, *clu1*Δ strains start off with an average of 9.0% frequency of respiration loss while the wild-type strains start off with an average of 0.8%. This is a fold change of 11.2 which is not quite as high, but consistent with my initial respiration loss assay results. The wild-type strains showed a peak of 22% respiration loss at the highest exposure time of 100 seconds. *clu1Δ::URA3* strains had a 53% respiration loss at that same time exposure. This indicates a 2.4 fold increase in the occurrence of petite colonies under UV exposure for *clu1*Δ*::URA3* strains. The rate of respiration loss is very similar, based on the slope of the curve, for both the control

and *clu1*Δ strains. Therefore, even though the respiration loss frequencies are consistently higher in *clu1*Δ strains, it is difficult to say that *CLU1* is having a protective effect on the repair of UV induced mutations. If *CLU1* were protective, I would expect to see an increased rate of respiration loss in *clu1* mutants. However, other possible roles of *CLU1* in this process cannot be ruled out, as there are other indicators of mtDNA stability besides respiratory function.

However, at this point I can say that *CLU1* does not appear to have a deleterious role in UV repair. If this were the case, I would expect to see, at the very least, a partial rescue of respiration loss in *clu1* mutants.

Taken together, these results suggest that *CLU1* may have a protective effect during UV radiation, but more studies are needed. If there were a relationship between *CLU1* and UV repair, this effect could be due to the morphology of the mitochondrial DNA, in which *CLU1* is known to be involved. The effect could also be due to a possible role for *CLU1* in repair of UV damage in the mitochondrial genome. A potential experiment to test the hypothesis that *CLU1* is involved in UV repair could be to expose *clu1*Δ strains to UV irradiation to see if point mutation frequency is affected. Also, epistasis analysis with known UV repair proteins could help to resolve this question.

Oxidative Stress.

The mtDNA is arranged in nucleoid structures within the matrix of the mitochondrion. Due to its proximity to the reactive oxygen species (ROS) generated by the process of oxidative phosphorylation, the mtDNA is subjected to high levels of

oxidative stress. Clu1p is involved in the morphology of the mitochondrial network (Fields and Clark, 1998) which could influence the compaction and positioning of the mtDNA within the mitochondrion. For this reason, I decided to test whether *CLU1* is involved in protecting the mtDNA from damage by ROS. This was accomplished by treating the cells with hydrogen peroxide (H_2O_2)

The results of my oxidative stress assays are preliminary. More studies are needed to determine the effects that oxidative stress has on mtDNA. Our lab is still working on finding a protocol that will accurately reflect this phenomenon in this model system. With that being said, preliminary studies have shown that an oxidatively stressed environment leads to an increase in microsatellite instability in my control strains. The effects of oxidative stress on direct-repeat mediated deletion are also being tested, but it remains to be shown whether the increase in repeat-mediated deletions that I see in my control strains is significant (data not shown) .

Calculation of Mutation Rates.

After using the method of the median (Lea and Coulson, 1949) to analyze the colony counts of the various assays, a table was constructed to summarize the collective data. All statistical analysis was performed with InStat 3 for Macintosh (GraphPad Software, Inc., San Diego, CA).

Assay	*clu1Δ*	Std. Dev.	Wild Type	Std. Dev.	Fold Diff.	P-value
Respiration Loss Freq.	3.23%	0.60	0.2%	0.17	+9.2	0.0006*
Respiration Loss	3.74e-3	-	3.90e-4	-	+9.5	-
Microsatellite Reporter	3.50e-6	7.1e-7	2.25e-6	1.1e-6	+1.6	0.2999**
Direct Repeat Mediated Deletion	1.55e-5	7.8e-6	8.15e-5	1.9e-5	-5.3	0.0455***
Point Mutation	4.26×10^{-8}	2.3e-8	1.98×10^{-8}	9.1e-9	+2.2	0.3283**

Table 3. Mutations per cell division rates. The method of the median was employed to compare mutations per cell division of wild type and *clu1Δ::URA3* strains. The fold difference was then calculated to determine whether *clu1Δ::URA3* had an increased or decreased effect on that particular assay. The p-value was determined by a student's t-test using a two-tailed, two sample-unequal variance test.
* This value is considered extremely significant.
** This value is not significant.
*** This value is significant.

The t-test assumes that both sets of data, wild-type and *clu1Δ*, have equal standard deviations. This assumption was tested, and it was calculated that the difference between the two standard deviations was not significant for all assays. Therefore, the p-value obtained from the t-test was verified to be valid.

***clu1* mutants have abnormal mitochondrial morphology.**

Clu1 has been shown to be involved in mitochondrial morphology in the model organism *D. discoideum*. Therefore I thought it was integral to my study to observe the effects of a clu1 deletion on the morphology of the yeast mitochondrion.

Two molecular markers, DAPI and mitotracker red, were used to visualize mitochondrial and nuclear structure, respectively, in *clu1*Δ::*URA3* strains. DFS188, or my wild-type strain, showed normal reticular mitochondrial networking, as indicated by mitotracker red. The nucleoids were observed in their normal punctate structures. In *clu1*Δ::*URA3* strains, the mitochondrial networks were abnormal in that they appeared to be clumped, or "clustered" as previously described, especially towards the periphery of the cell (Figure 23, 24, 25). The nucleoids in the mutant strains do not appear to be severely affected except in their new positioning at the cell edge in conjunction with the mitochondrial network.

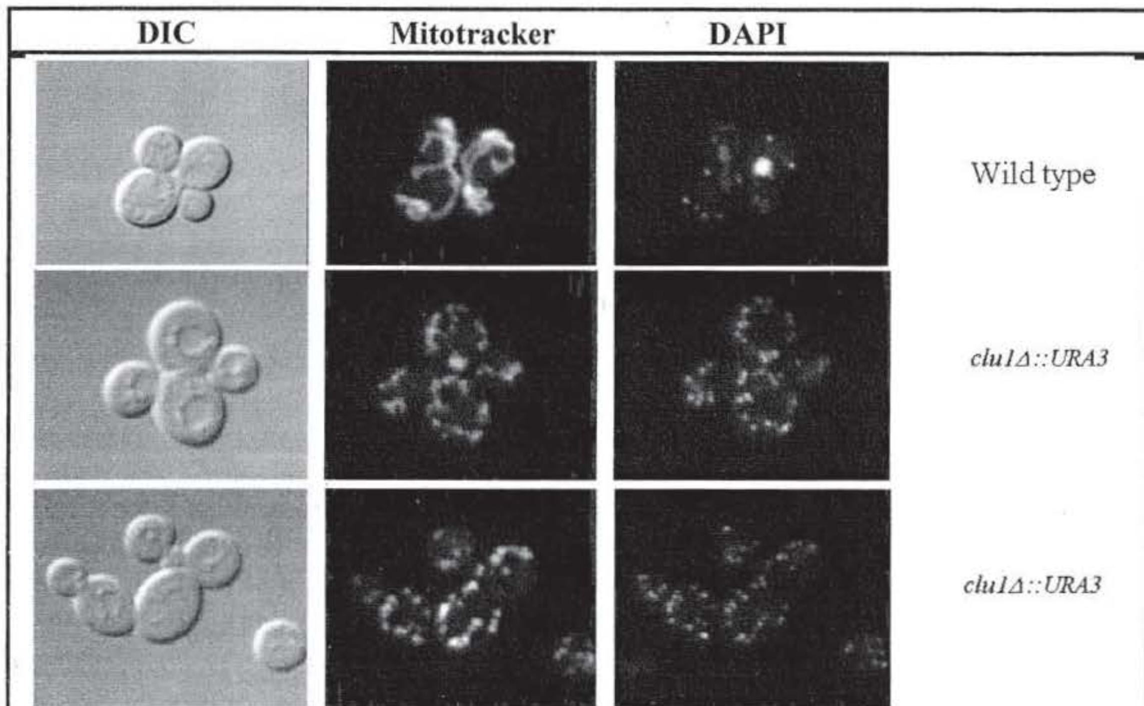

Figure 23. Microscopy imaging of mitochondrial and nucleoid structures in yeast. Yeast cells were treated with mitotracker and DAPI staining to visualize nucleoid structures and mitochondrial networks. The first row is DFS188 and the second and third rows are *clu1*Δ::*URA3*
The first column shows differential interference contrast images. The second column shows mitotracker staining and the third column shows DAPI staining.

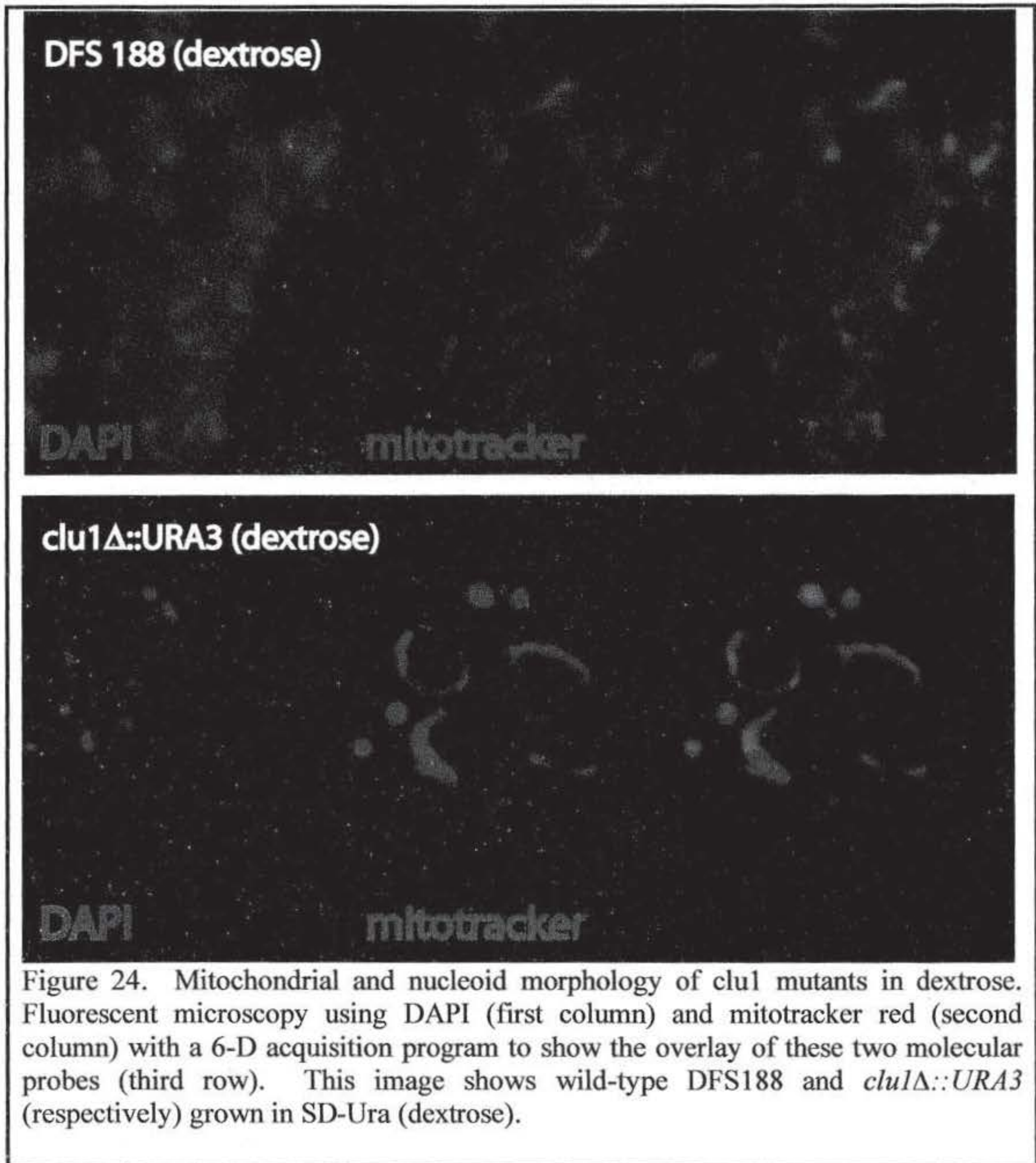

Figure 24. Mitochondrial and nucleoid morphology of clu1 mutants in dextrose. Fluorescent microscopy using DAPI (first column) and mitotracker red (second column) with a 6-D acquisition program to show the overlay of these two molecular probes (third row). This image shows wild-type DFS188 and *clu1Δ::URA3* (respectively) grown in SD-Ura (dextrose).

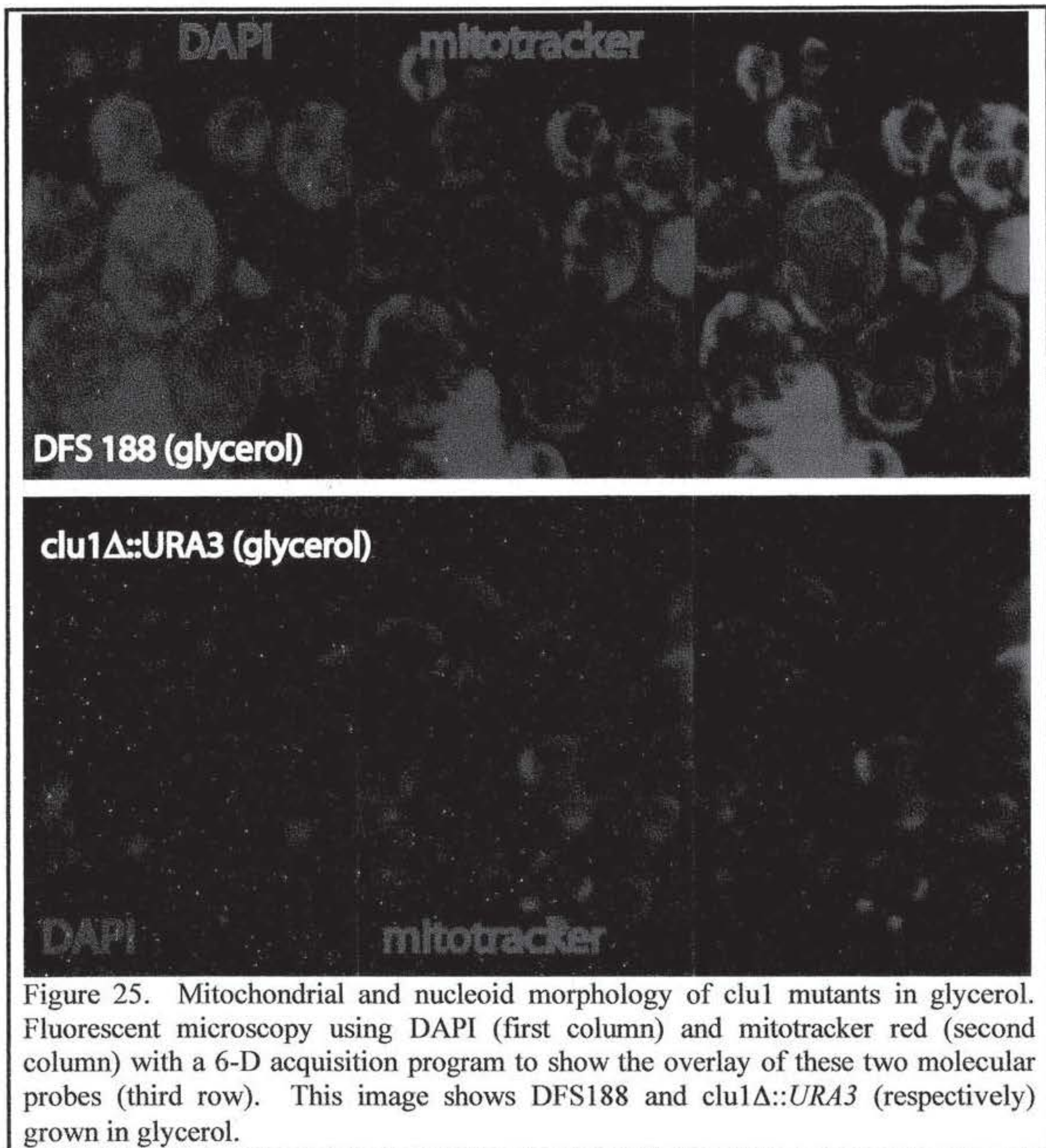

Figure 25. Mitochondrial and nucleoid morphology of clu1 mutants in glycerol. Fluorescent microscopy using DAPI (first column) and mitotracker red (second column) with a 6-D acquisition program to show the overlay of these two molecular probes (third row). This image shows DFS188 and clu1Δ::*URA3* (respectively) grown in glycerol.

This experiment lends further support to the current knowledge of the involvement of clu1 in maintaining proper mitochondrial morphology. Specifically, my study shows that *CLU1* is essential to retain normal mitochondrial structure in yeast.

DISCUSSION

In this research, I describe the analysis of a protein that our group found to interact with the known mitochondrial protein Ilv5p. Using standard yeast techniques I created three knockout strains to determine the effects on respiratory capability and mitochondrial morphology. This analysis included the use of genetic reporters to measure the effects of a clu1 deletion on the integrity of the mtDNA. A newly developed direct repeat-mediated deletion reporter aided in revealing the largely undescribed phenomenon of recombination within mtDNA.

Mitochondrial dysfunction can arise from many factors. It could be the end result of a cascade of occurrences, or it could be the result of a single event. This research in particular focused on the implications of microsatellite instability via polymerase slippage, direct-repeat mediated deletions, and point mutations in bringing about genome instability and respiration loss. Morphology is also related to maintaining the yeast cell's ability to respire.

Respiration Loss.

Cellular respiration is an integral metabolic activity in the cell. The catabolism of glucose in the presence of oxygen to produce ATP powers a multitude of cellular activities. As facultative anaerobes, yeast can survive without respiring by producing ATP through fermentation at a lower efficiency. Genes that are necessary to maintain respiration can be elucidated by performing respiration loss assays with null mutants to determine which strains cause an increase in the frequency of formation of petites.

The respiration loss that I have seen in clu1Δ::URA3 strains is a significant increase from my wild-type strain (31 fold). Dimmer et al. (2005) classified *CLU1* as a Class II mutant. According to their definitions, Mdm20p, Ptc1p, and Yme1p are included in this class. These proteins share similarity in the fact that they influence mitochondrial morphology defects but only under certain conditions.

In order to maintain respiration in yeast an intact and properly formed mitochondrial network must be in place. The results of disturbances in mitochondrial morphology are extensive. It interrupts the trafficking of proteins into the mitochondria. It also disrupts components of the electron transport chain, by changing the integrity of the inner membrane.

Studies have shown that proteins that are involved in mitochondrial morphology maintenance are also required for respiratory capabilities of the yeast cell.

Mitochondrial compartments in *yme1* (**y**east **m**itochondrial DNA **e**scape) deletion cells lack the normal elongated and reticulated structures found in wild-type cells and instead are swollen, punctate structures (Hanekamp et al., 2002). Mutations in *MMM* and *MDM* genes also result in this phenotype.

Microsatellite Instability.

With *clu1Δ::URA3* strains exhibiting only a 1.6 fold increase in microsatellite instability as compared to the wild-type strain, it can be inferred that *CLU1* is not playing a significant role in this phenomenon. Microsatellite instability is believed to arise by the event of polymerase slippage (Sia et al., 2000). Mip1p is the catalytic

subunit of DNA polymerase γ in the mitochondrion (yeast genome.org). Clu1p has not yet been identified as an interactor of Mip1p, but it could affect polymerase slippage in an indirect manner. Polγ is frequently oxidized by ROS (Larsen et al., 2005). This oxidation leads to a decrease in catalytic function of Polγ. This can, in turn, contribute to reduced replication fidelity and, ultimately, instability in mtDNA integrity and maintenance (Graziewicz et al., 2002). If Clu1p were involved in mtDNA repair mechanisms, errors in replication caused by ROS would be propagated.

Direct-repeat mediated deletion.

Through the use of a new direct-repeat mediated deletion reporter I am now able to identify proteins which are involved in generating deletions within the mitochondrial genome. By studying the intermediate structures formed during deletion events through the use of this reporter, Phadnis et al. (2005) postulated several pathways that could be in operation. Homologous recombination based deletion events were deduced based on the presence of reciprocal products generated by intra- or inter-molecular recombination. Single strand annealing, polymerase slippage, and recombination-dependant pathways were other hypothesized mechanisms (Phadnis et al., 2005)

With *clu1Δ::URA3* strains exhibiting a 5.3 fold decrease in deletion events, it is clear that Clu1p plays a role in regulating deletion events within mtDNA. A consequence of this decrease in recombination could be a decreased efficiency of *clu1* mutants in repairing double-stranded breaks (DSBs). DSBs occur in both nuclear and

mtDNA. The mechanisms of repair are non-homologous end joining (NHEJ) and homologous recombination. Multiple copies of mtDNA in the cell aid in the process of homologous recombination as a repair mechanism (Larsen et al., 2005).

Point mutations.

Point mutations do not seem to be severely affected in the absence of Clu1p, but a 2.2 fold increase of point mutations in *clu1Δ:URA3* compared to wild-type DFS188 is still a significant change. Because these cells were not subjected to any external stressors, the increase in point mutations can most likely be attributed to a decrease in the efficiency of DNA repair mechanisms.

Oxidative Stress.

With the limited results obtained from my assays, it is difficult to demonstrate a direct connection between *CLU1* and resistance to oxidative stress. However, I found that *clu1* mutants are deficient in recombination or deletion events, which are known to be protective during times of oxidative stress (Doudican *et al.*, 2005). Therefore, it can be postulated that *clu1Δ::URA3* would have decreased resistance to oxidative stress due to decreased ability of recombination repair.

CLU1 could also operate to reduce oxidative stress via alternative mechanisms. Using measurements of light scatter, Wilson et al. (2005) found that mitochondrial swelling can occur as a result of oxidative stress. Is there any evidence of the opposite phenomenon: mitochondrial morphology affecting a cells resistance to oxidative stress? If so, *clu1Δ::URA3* strains with their initial defects in mitochondrial morphology would have a definite disadvantage in combating oxidants.

UV sensitivity.

The apoptotic activity of the mitochondria protects cells that are severely damaged by the effects of UV radiation. UV radiation starts a caspase cascade that signals to the cell that it cannot properly function anymore. When these caspases are inhibited, UV-induced apoptosis is blocked. (Sitailo)

In my experiments, I show that *clu1Δ::URA3* strains exhibit an increase in respiration loss compared to wild-type. However, the viability does not change between the wild-type and *clu1* strains.

CLU1 may have a protective affect against UV radiation by a number of mechanisms. In terms of its mitochondrial function, it could be involved in the nucleoid structure that functions to package the mtDNA into a more condensed, protected structure. Due to its involvement in recombination as witnessed by my direct repeat-mediated deletion assay, it is possible that Clu1p is involved in a recombinational repair pathway.

The effect of Clu1p on translation initiation.

With *CLU1* having a possible function as a subunit of eIF3, it cannot be ruled out that disrupting a component of the translational machinery may have further implications. Any number of downstream effects could be witnessed with a dysfunction in translation. Vornlacher *et al.* (1999) saw that eIF3 was not entirely necessary for the initiation of translation. However, translation can still occur, albeit at a lower rate, with the loss of certain proteins. A disruption or change in the rate of translation initiation may affect mRNA stability (Barnes, 2003). Mutations in

components of the translation initiation machinery can also contribute to destabilized mRNAs. Prt1p has been identified as a subunit of eIF3. A *prt1* mutation inhibits the association of the initiator tRNAi and the tRNAi (Barnes, 2003). If Clu1p is indeed a subunit of eIF3, *clu1* cells could share this phenotype.

Mitochondrial Morphology.

It is in the area of mitochondrial morphology that Clu1p exerts its largest observed effects. Zhu *et al.* (1997) first discovered *CLU* in the soil amoeba *Dictyostelium discoideum*. *CluA* was the first in a group of novel proteins. Previously, proteins that were involved in mitochondrial morphology were divided into two groups: motor proteins and cytoskeleton structural elements (Zhu et al., 1997). *CluA* did not fit neatly into these definitions. *CluA* mutants did not exhibit an altered cytoskeleton nor did they contain homology with known motor proteins (Zhu *et al.,* 1997). Fields *et al.* (1998) continued the study of *CLU1* by observing its effects in yeast. *CLU1* is one of very few proteins that alter mitochondrial morphology but not inheritance.

A good starting point in elucidating the function of Clu1p in the cell is to study proteins with similar features. Another mutant that shares the characteristic of altered mitochondrial morphology but not inheritance is *dnm1* (Gammie *et al.,* 1995). *dnm1* mutants cause a clustering of the mitochondrial network in the same manner as *clu1* mutants (Shaw, 1995). *DNM* encodes for a dynamin-like protein (Gammie *et al.,* 1995). Dynamin-like proteins are a subdivision of the large group of Dynamin proteins which function as GTPases. These GTPases have many responsibilities,

ranging from vesicle transport to division of organelles. Although *CLU1* does not have significant homology to *DNM1*, as Fields et al. (1997) hypothesize it does not rule out the possibility of Clu1p operating similarly to, or in conjunction with, these dynamin-like proteins.

Church and Poyton (1998) determined that mitochondrial morphology is not dependent on respiration. By using a strain with a nuclear mutation that lacked respiratory competence but was otherwise undisturbed (no mtDNA mutation), they determined that neither cytochrome c oxidase nor respiration was necessary for normal mitochondrial morphology, distribution, or volume. This suggests that the abnormal morphology of my *clu1Δ::URA3* strains is based on factors other than its loss of respiration.

Conclusion.

Through the use of molecular mitochondrial reporters I am able to determine genes that are involved in various aspects of mitochondrial genome stability. Performing these fluctuation analyses on null mutants that have decreased respiratory function can help to obtain a better understanding of the processes that occur to keep the mitochondria functioning. Through the characterization of many different genes, commonalities which would otherwise remain unnoticed start to emerge. From these initial screens the ultimate goal is to elucidate pathways of protein interaction and mechanisms of mitochondrial replication, repair, and recombination.

Specifically, in my studies, I found that Clu1p is necessary for normal respiratory function. This decrease in respiratory function that is displayed by clu1

mutants may be a result of its role in direct-repeat mediated deletion events. Importantly clu1 mutants also display aberrant mitochondrial morphology as previously reported.

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