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Nuclear Encoded Proteins Important in Mitochondrial Genome Stability

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Nuclear Encoded Proteins Important in Mitochondrial Genome Stability

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ABSTRACT

The mitochondrion is widely known to be the site of cellular respiration and the factory of cellular energy. Similar to the nucleus, mitochondria house genetic material (mtDNA), which is responsible for the production of proteins essential to mechanisms required for cellular respiration. Furthermore, if there is a mutation or deletion in the mtDNA there can be ramifications in terms of energy production, which will hinder cell viability. Additionally, mutations in the mtDNA are associated with certain neuromuscular diseases as well as contributing to the aging process. The focus of this research is to identify genes that contribute to the maintenance of the mtDNA. Our data from genetic assays indicate that loss of the Clu1p protein exhibits an increase respiration loss as well as increase spontaneous point mutations. In addition, loss of Clu1p alters mitochondrial morphology.

INTRODUCTION

The mitochondrion is an organelle of unique importance to the cell. Mitochondria are invaluable to the cell in the way that they provide the cell with chemical energy in the form of adenosine triphosphate (ATP) through the process of cellular respiration. Cellular respiration is the catabolic process by which the mitochondria aerobically metabolize particular carbon sources and convert the energy into usable form that can be stored in the high energy phosphate bonds of ATP.

With the mitochondria's functionality aside, there is one feature in particular that contributes to the mitochondrion's unique complexity: the mitochondrion possesses its own genome. This distinctive feature supports the endosymbiotic theory, which hypothesizes that mitochondria were once their own self-sustaining entities, but adopted an endosymbiotic relationship with prokaryotic cells and they evolved to become dependent on each other.

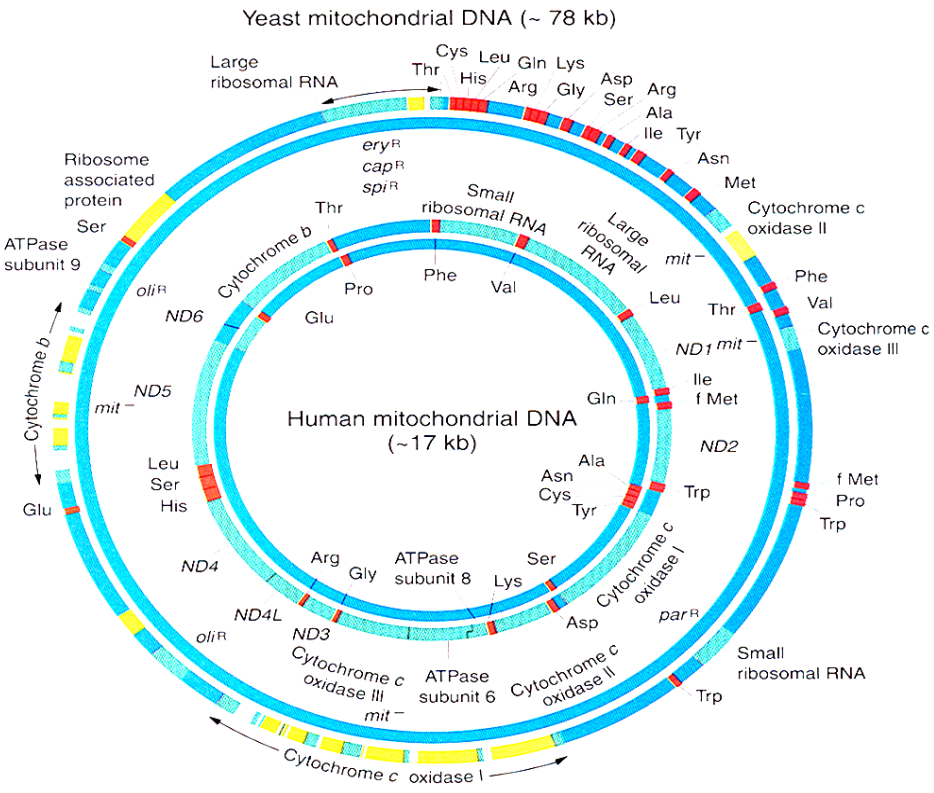

Figure 1. Mitochondrial DNA. The outside double stranded DNA depicts yeast mitochondrial DNA whereas the inside DNA depicts human DNA. Notice that the yeast mtDNA is significantly larger, relative to the human mtDNA. Many of the genes encoded by the mtDNA are conserved among yeast and humans. (Adapted from Griffiths 2000)

The length of the mitochondrial genome varies in length among species, for example the human mitochondrial genome is 16,569 base pairs (bp) long, but the coding regions of the mitochondrial genome are relatively conserved (Bianchi, Bianchi, & Richard, 2001). The proteins that the mitochondrial genes code for are primarily used for the electron transport chain, which is the final phase of cellular respiration that ultimately forms the high energy ATP molecules. Also encoded by the mitochondrial genome are transfer and ribosomal RNAs that are necessary for the translation of the aforementioned proteins. It is

important to note, however, that a majority of the proteins localized to the mitochondrion are encoded by nuclear DNA.

Multiple copies of mitochondrial DNA (mtDNA) are tightly associated with proteins within mitochondrial matrix to form a nucleoid structure. These nucleoid structures consist of about 5 copies of mtDNA and more than 20 proteins. One of the poorly understood mechanisms is how the nucleoid structures replicate and segregate, but is becoming progressively better understood (Bielawski & Gold, 2002). There are many variables that influence the segregation patterns of the nucleoids including number of copies of the mtDNA and the organization of the nucleoid within the cell. The method by which the cell is able to control and synchronize mtDNA replication with cell division is still unclear (Trinei, Berniakovich, Pelicci, & Giorgio, 2006).

It is understood that mtDNA replicates via DNA Polymerase gamma (Foury, 1989). This nuclear encoded enzyme has a catalytic component that is highly conserved (Foury, 1989). Additionally, Pol gamma has a 3' to 5' exonuclease activity that has proven to contribute significantly to the integrity of the synthesis (Roberts & Kunkel, 1996). In yeast there are multiple mtDNA origins of replication and replication is considered to proceed bidirectionally (Baldacci, Cherif-Zahar, & Bernardi, 1984) & (de Zamaroczy, Faugeron-Fonty, Baldacci, Goursont, & Bernardi, 1984).

When there are replication errors or errors induced by other processes, certain mechanisms exists to repair the mistakes. The proteins and pathways involved in repair mechanisms are still widely unclear, but mtDNA is believed to be able to utilize a mechanism homologous to the base excision repair (BER) used by nuclear DNA (Croteau & Bohr, 1997). In this pathway, erroneous bases are recognized and the bases are cleaved

from their sugar base. The now abasic site can be recognized by a specific endonuclease, which can excise the base along the phosphodiester backbone. After the excision, the DNA can be synthesized again (Croteau & Bohr, 1997). Mitochondrial specific glycosylases necessary for the cleavage of the base from the sugar have been identified (You, et al., 1999). Expectedly, glycosylase null mutations have displayed increased spontaneous point mutation formation.

As yet, no analogous pathway to the nuclear mismatch repair (MR) pathway has been found in the mitochondria. MR is similar to BER, but recruits different enzymes and follows a different mechanism to mend post-replication errors. There have been some proteins studied that, upon deletion, seem to directly influence mitochondrial integrity and drastically increase spontaneous point mutation (Chi & Kolodner, 1994). However, the exact implication in MR, if there is one, is still in need of further investigation. Even more poorly understood is a nucleotide excision repair (NER) pathway to mend large DNA mistakes. There is no evidence thus far that supports a mitochondrial homolog to this pathway.

Just as nuclear DNA is exposed to mutagenic agents, mtDNA has similar mutagenic threats, which require the employment of the partially understood repair pathways discussed previously. The mtDNA's proximity to the electron transport chain mechanism within the mitochondrial membranes, however, poses a particular mutagenic threat. In the electron transport chain, oxidative phosphorylation produces a notable amount of reactive oxygen species (ROS), which can damage the nearby mtDNA (Singh, Sigala, Sikder, & Schwimmer, 2001). Mutations within the mitochondrial genome can cause a multitude of

mitochondrially genetic disorders (Kujoth, et al., 2005). Alterations in the mitochondrial genome has also been linked to certain cancers, apoptosis, and aging (Kujoth, et al., 2005).

Another event that DNA is susceptible to is recombination (Dujon, 1981). Recombination is vastly better understood in a nuclear setting, but mtDNA exhibits high activity of recombination as well. This recombination is encouraged because the yeast mitochondrial genome is plagued with direct repeats, which are 'hot spots' for recombination. Some suggest that these recombination events excise portions of mtDNA that lead to dysfunctional mtDNA (Gaillard, Strauss, & Bernardi, 1980).

As mentioned before, mtDNA is packaged into nucleoids after replication. The nucleoids must then be segregated from each other to be packaged into the respective daughter cells (Meeusen, et al., 1999). It is during replication and segregation that mtDNA may be particularly sensitive to the aggressive oxidative exposure, as mentioned before (Meeusen, et al., 1999).

In order to for the nucleoid and mitochondria to segregate they must be tethered to actin cables (Boldogh, Fehrenbacher, Yang, & Pon, 2005). Many proteins have been identified to mediate this tethering process. A disruption in the tethering process has been identified in disorganization of the nucleoid structure and altered mitochondrial morphology. These studies indicate that there may be a correlation between mitochondrial morphology and nucleoid organization.

In order to study the effects that mtDNA mutations would have on an organism, it was important to select a model that could tolerate any potential loss of mitochondrial function. *Saccharomyces cerevisiae*, or more commonly known as baker's yeast, is a facultative anaerobes meaning the organism can make ATP via aerobic respiration if

oxygen is present, but is also capable of fermentation, which does not require the machinery of the mitochondria. Therefore, mutation to the mitochondrial genome can be tolerated because the yeast cells can still survive through fermentation. All the colonies will grow nearly identically until an mtDNA mutation renders them unable to continue cellular respiration. The non-respiring colonies are distinguishable from their wild-type counterparts because energy production methods of fermentation are not as effective as the methods of cellular respiration, which changes the phenotype of the colony to a noticeably smaller size. These non-respiring colonies are referred to as "*petites*". Baker's yeast also proves to be an excellent model organism for the study of genes involved in cellular respiration because exogenous DNA can be incorporated into the yeast mitochondrial genome with relative ease.

The nonrespiring '*petite'* cells can develop their phenotype in a variety of ways. One way is through a loss of the entire mitochondrial genome termed rho^0 (ρ^0), and another way is through a deletion in the mtDNA termed rho^- (ρ^-). (Functional mitochondrial genomes are referred to as rho^+.) After analysis of the deletion junction, it is not unlikely that some of ρ^- attributes could have arisen via direct-repeat mediated deletion events (Dujon, 1981). This event is characterized by DNA sequences that are repeated throughout a segment of DNA that facilitate a recombination event which results in DNA deletion.

It has been shown that mitochondrial point mutations and deletions accrue as a function of time (Phadnis, Sia, & Sia, 2005). For example, it is not unusual to find a specific 4977 base pair mtDNA region flanked by direct repeats in aging individuals or individuals with particular mtDNA genetic disorders. Additionally, this specific deletion was found in individuals with Kearns-Sayre syndrome and chronic external ophthalmoplegia. Both these

genetic diseases are characterized by loss of motor function of the eyelids and oculo-motor muscles. Thus far, 66% of mitochondrial DNA discovered deletions have implications of direct-repeat mediated deletion events based on their possession of direct repeats at their junctions (Bianchi, Bianchi, & Richard, 2001).

Two particular genes of interest in this research are *CLU1* and *DNM1*. *CLU1* encodes a protein that has unknown function, but displays homology to a eukaryotic translation initiation factor eIF3 (Vornlocher, Hanachi, Ribeiro, & Hershey, 1999). A CLU1p characteristic of particular interest is *CLU1* knockout strains seem to display altered mitochondrial morphology (Dimmer, Jakobs, Vogel, Altmann, & Westermann, 2005) (Fields, Conrad, & Clarke, 1998). *DNM1* codes for a dynamin-related GTPase that has been shown to control mitochondrial morphology.

The focus of this study is to investigate the role particular nuclear encoded proteins play in the stability and morphology of the mitochondrial genome.

MATERIALS AND METHODS

Yeast strains and growth media

The growth used media included YPD (2% dextrose), YPG (2% glycerol), YPG with 0.1% dextrose, YPG with 200 mg/l of Geneticin, YG with 4.0 mg/ml of erythromycin, complete synthetic dextrose medium (2% dextrose), complete synthetic glycerol medium (2% glycerol), synthetic dextrose medium lacking tryptophan or arginine and uracil.

The strains used in this study were DFS188 *MATa ura3-52 leu2*-3, 112 *ly2 his3 arg8::hisG* (Sia *et al.*2000), RAS 501 DFS188 *clu1Δ::kanMX*, RAS 502 DFS188 *clu1Δ::kanMX*, and RAS 503 DFS188 *clu1Δ::kanMX*. LKY196 DFS188 Rep96::*ARG8^{M}::cox2* Rep96*::URA3::trp1* (Kalifa, Beutner, Phadnis, Sheu, & Sia, 2009), RAS504 LKY196 *clu1Δ::kanMX*, RAS505 LKY196 *clu1Δ::kanMX*, and RAS506LKY196 *clu1Δ::kanMX*.

Creating *clu1Δ::kanMX* strains

In order to construct the clu1 knockout strains, a knockout cassette was amplified via polymerase chain reaction (PCR) from a *clu1Δ::kanMX* disruption allele from the yeast deletion bank library. The CLU1 forward and reverse primers used to amplify the knockout cassette were 5'-GGATAGCTGAGAAACTTGTGC-3' and 5'-GGGAATTGGTGATTCCTTAGC-3', respectively. Non-coding CLU1 flanking sequences approximately 550 base pairs upstream and downstream were included in order to target the disruption cassette as shown in Figure 2. With the disruption cassette amplified, it was transformed into DFS188 and LKY196 yeast strains using a high efficiency yeast transformation protocol. Knockout strains were verified by PCR using genomic DNA. To ensure proper transformation, the presence of the disruption allele was verified using the aforementioned *CLU1* forward primer and the *kanMX* reverse primer 5'-CGGATGTGATGTGAGAACTGTATC-3'. To confirm

the absence of the wild-type *CLU1* gene, the *CLU1* forward primer and an internal *CLU1* reverse primer 5'-GCAAATCCGATGAAATCGCG-3' were used. The PCR products were electrophoresed on a 1% agarose gel to confirm the state of the transformants.

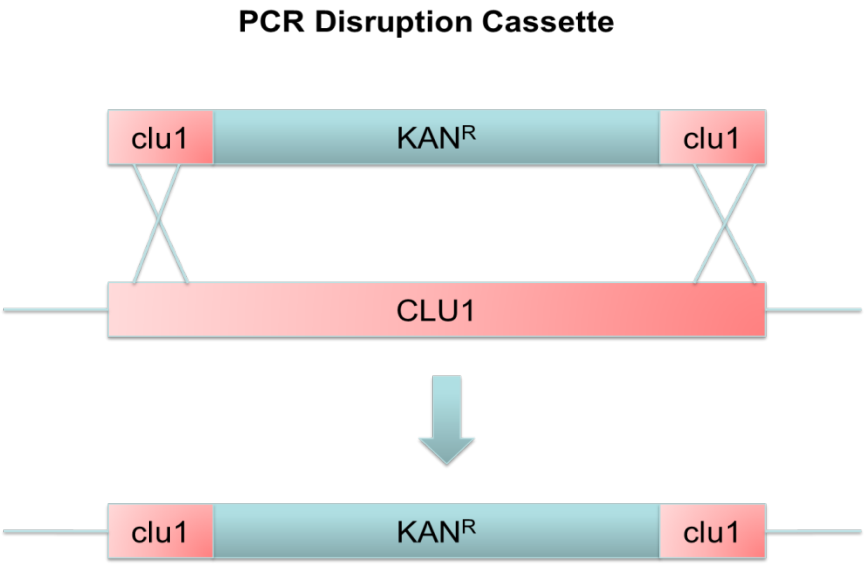

Figure 2. Disruption Cassette. *CLU1* deletion strains were constructed using a PCR disruption cassette introduced into yeast strains. The disruption cassette contains the *KAN*^{R} gene that confers resistance to Geneticin surrounded by two *clu1* regions that direct recombination to the endogenous *CLU1* locus.

Respiration Loss Assay

To confirm a population of respiring cells, wild-type (DFS188) and *clu1Δ* strains were grown on YPG (a non-fermentable carbon source) medium for two days at 30°C. Cells were then streaked for singles on YPD (a fermentable carbon source) for three days at 30°C. Fifteen independent colonies of both wild-type and knockout strains were isolated and serially diluted in sterile deionized (DI) water to 10^{-5}. After dilution 20 µl of each colony was plated on YPG containing 0.1% dextrose. The plates were incubated at 30°C for

three days. Due to the carbon source variation both respiring and non-respiring cells arise. These non-respiring, or petite, cells are phenotypically smaller because their growth arrests when the fermentable carbon source (dextrose) is exhausted. The respiring cells are able to retain their ability to metabolize the non-fermentable glycerol when all the dextrose has been consumed. To calculate the respiration loss frequency the number of petite colonies was divided by the total number of colonies for each plate. Duplicate assays were completed for each strain. The frequencies of all the assays were then averaged and the error was described by the respective standard deviations.

Ultraviolet sensitivity assay

Identical to the method described in the respiration loss assay, individually diluted colonies were plated on YPG containing 0.1% dextrose. Prior to incubation, cells were subjected to 1 $J/m^2/sec$ of UV-B radiation for 50 seconds. Plates were then incubated in the dark for three days at 30°C. Duplicate assays were completed for each strain. The average frequency of respiration loss was calculated and compared to the frequency of the cells that were not exposed to the UV-B radiation.

Mitochondrial DNA isolation and electrophoresis

To study the possibility of rearrangement and/or deletion of mtDNA, wild-type and non-respiring cell DNA was isolated using a method adapted from Defontaine A. *et al.* (1991, Nucleic Acids Research). Once the mtDNA once isolated it was cleaved with the restriction enzyme Cac81. DNA fragments were separated via 0.8% agarose gel electrophoresis.

Direct repeat mediated deletion assay

In order to determine the rate of both nuclear and mitochondrial direct repeat-mediated deletion (DRMD) events, a different yeast strain (LKY196) was used. This special strain was useful because it possesses unique reporters that can directly measure the rate of both nuclear and mitochondrial DRMD events. To select for cells with the appropriate reporters, cells were patched on synthetic dextrose medium that lacked arginine and uracil. Cells were then streaked for singles on YPD and were incubated at 30°C for three days to allow for spontaneous DRMD events. Fifteen single colonies for both the wild-type and knockout strain were isolated and individually diluted in 100 µl of sterile DI water. Each colony was then serially diluted to and plated on the appropriate media to select for the desired DRMD event, either mitochondrial or nuclear. To determine the rate of mitochondrial DRMD events the diluted colonies were plated on YPG. In order to determine the rate of nuclear DRMD events, diluted colonies were plated on synthetic media lacking tryptophan. Additionally, diluted colonies were plated on YPD to determine the total number of cells in the colony. Plates were incubated for three days at 30°C. Using the Lea and Coulson method of the median, the rate of DRMD events for each assay was determined (Lea & Coulson, 1949). Rates were then averaged and error was described using their respective standard deviations.

Point mutation assay

A method adapted from Mookerjee *et al.* (2005) was used to determine the rate of spontaneous point mutations. Stains were streaked for singles onto YPG. Fifteen singles were selected and inoculated into 5 mL of YPG until saturation. Cells were isolated from media and then resuspended in 1 mL of sterile DI water. Cells were then plated onto YG

with 4.0 mg/mL of erythromycin. After dilution cells were plated on YPG to determine the number of cells in the liquid cultures and incubated for seven days at 30°C. Assay was done in duplicate. Rates were determined use the Lea and Coulson method. Average wild-type and *clu1Δ* were calculated.

Statistical analysis

To ensure statistical significance of all data, InStat 3 for the Macintosh was recruited to provide statistical analyses. Unpaired *t*-tests were used to calculate a two-tailed P value in the comparison of average or median rates and frequencies.

Fluorescent microscopy

Both wild-type and non-respiring *clu1Δ* were inoculated into synthetic complete media containing 2% glycerol and incubated overnight at 30°C. The overnight culture was diluted 20-fold into synthetic complete media with 2% glycerol and incubated for 3-4 hours at 30°C. Cells were then administered 150 pM Mitotracker for 45 minutes at 30°C, followed by 10 nM DAPI for 10 minutes at 30°C. Incubations were all done while shaking. Cells were washed with 1X PBS (phosphate buffered saline) pH 7.4 once. To observe cells, a Zeiss Axioplan 2 fluorescent microscope was used under 100x oil immersion.

RESULTS

The function of Clu1p is not exactly known, but it has been described as having a homology to a component of the eukaryotic translation initiation factor eIF3. Although it was previously determined by Fields *et al.* the deletion of the Clu1p has no effect on cell viability, growth, sporulation, or increased petite formation, altered mitochondrial morphology was observed.

cluΔ strains display increased respiration loss

Although it was previously determined that *CLU1* seems to have an effect on mitochondrial morphology, the goal of this study was to determine whether *CLU1* had an effect on mitochondrial genome stability.

Respiration loss is an indirect indictor of the status of the mitochondrial DNA. If the mtDNA becomes unstable and mutates, the proteins needed for oxidative phosphorylation, which the mtDNA codes for, would not be transcripted or translated. There is also the possibility that the protein is translated but mutations cause the protein to form an unusual conformation and is unable to perform its function. Either case, the cells would not be able to respire, hence 'respiration loss'. The respiration loss experiment is depicted in Figure 3.

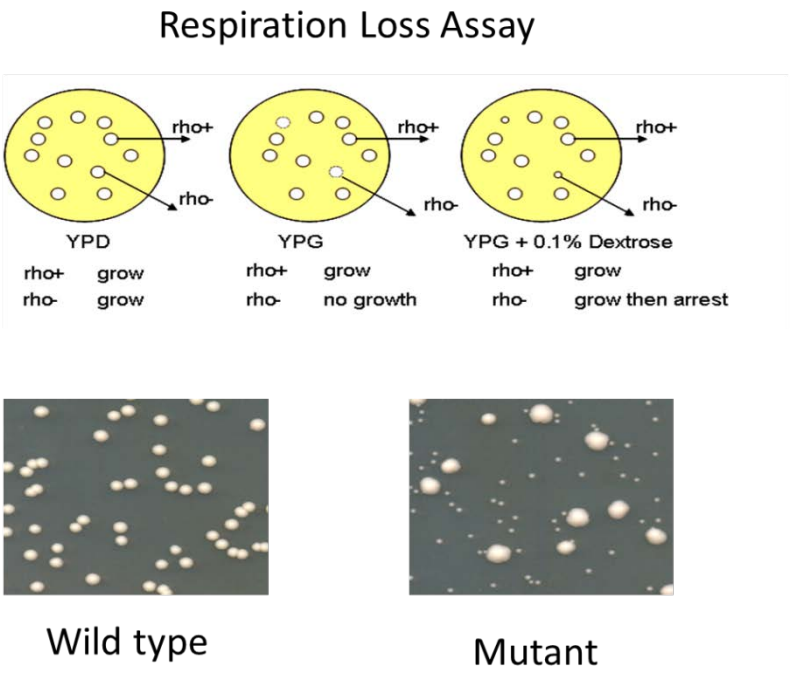

Figure 3. Respiration loss assay. Monitors the rate at which cells lose the ability to respire by quantitating petite colony formation on media containing 2% glycerol and 0.1% dextrose. Glycerol is a non-fermentable carbon source. Cells must have functional mitochondria (rho^+) in order to grow on media containing glycerol as the sole carbon source. Dextrose is a fermentable carbon source. If cells are lacking functional mitochondria (rho^-), they can use an anaerobic metabolic pathway to remain viable.

If cells are plated on YPD, where dextrose is the carbon source, cells can grow regardless of the mitochondrial genome status. Even if cells lose their mtDNA functionality (rho^-) they can survive because dextrose is fermentable carbon source. Similarly, cells that maintain their mtDNA function are indistinguishable because yeast cells prefer to ferment and will therefore ferment when a fermentable carbon source such as dextrose is available. Conversely, if yeast cells are plated on YPG, the carbon source present is non-fermentable glycerol. Therefore, if cells have a spontaneous loss of mtDNA (rho^-) and are unable to respire, there will be no growth because cells will not able to ferment (because glycerol is

non-fermentable) or respire. The assay done in this study combines the two concepts just described.

On YPG + 0.1% dextrose, all the yeast cells will grow because the cells prefer to ferment and thus will metabolize the fermentable dextrose. The dextrose, however, has limited availability and will soon be completely consumed leaving only glycerol. Cells that spontaneously lose their ability to respire through an mtDNA event will exhibit a growth arrest once the dextrose is depleted because they won't be able to metabolize glycerol. Furthermore, cells that retain their mtDNA function and ability to respire will not arrest and are phenotypically larger. The observed differences are seen in Figure 3, where respiring cells are distinguishably larger than non-respiring (petite) cells.

The results of the respiration loss assay are displayed in Figure 4. The loss of Clu1p exhibited nearly a 2-fold increase (p=0.0006) in the frequency of petite formation compared to wild-type.

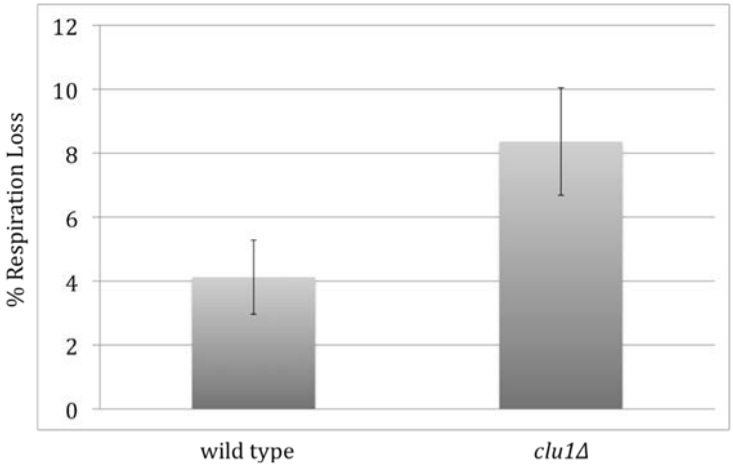

Figure 4. Respiration Loss Assay Data. *clu1Δ* strains exhibited almost two-fold increased loss of respiration compared to the wild type strains.

This data suggest that Clu1p may play a role in the preserving the proficiency of cellular respiration. Although this supports Clu1p's role in cellular respiration, it is unknown what events occur that lead to the increased respiration loss. A better understanding of the nature of the mitochondrial defects may allow an explanation for cause of the increased respiration loss. In an effort to measure the rate at which DNA mutations occur, whether by point mutation or through a direct repeat mediated deletion (DRMD) event, to genetic assays are used. Some insight into whether or not the mtDNA is rearranging and mutating could provide an explanation as to why *clu1Δ* experience an increase in respiration loss.

clu1Δ strains appear more susceptible to induced DNA damage

Before with the respiration loss assay, that rate of spontaneous DNA mutation was measured as a function of respiration proficiency. There was also an interest in whether Clu1p plays a role in mtDNA response to induced DNA damage. For the instances described in this study, the DNA-damaging agent was ultraviolet light. UV light primarily causes pyrimidine dimers, particularly thymine-thymine dimers. Unless repaired, these dimers prevent DNA from replicating.

To determine the sensitivity of Clu1p deletion strains to induced DNA damage, a respiration loss assay was performed similar to the respiration loss assay conducted previously. The difference for this study was that once the cells were plated, they were exposed to UV light for 50 seconds to induce DNA damage. After the incubation period on YPG + 0.1% dextrose, the plates were analyzed and compared to the frequency of petite formation when DNA damage was not induced. The results are shown in Figure 5.

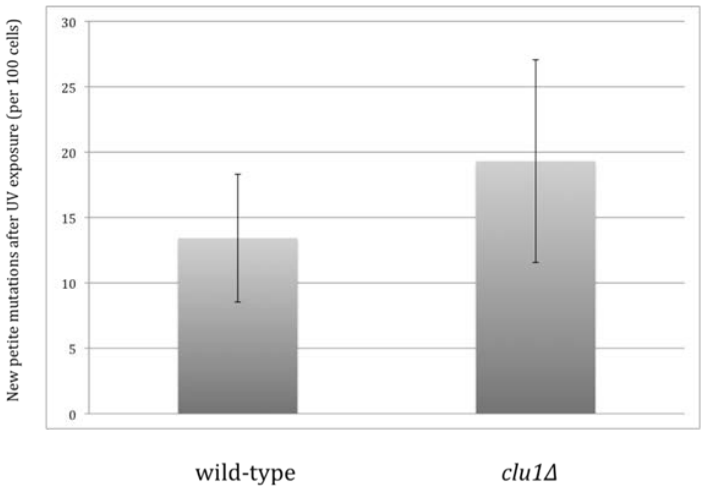

Figure 5. New petite formation after induced DNA damage via UV radiation. *clu1Δ* displayed an increase in new petite formation after UV exposure compared to wild-type.

Although the error bars indicating the standard deviation are relatively large, the data still indicate that *clu1Δ* strains are more susceptible to DNA damage. New petite formation in wild-type was 13.4 *petite* mutants per 100 cells compared to *clu1Δ* strains that exhibited 19.3 *petite* mutants formation per 100 cells. This data indicates that Clu1p may play a role in prevention of induced DNA damage or mtDNA repair.

It seems that Clu1p played a role in maintaining proficient cellular respiration, but the Clu1p contributions to cell viability was also determined. Figure 6 shows the percent cell viability of both wild type and *clu1Δ* compared to the percent cell viability of both strains after UV exposure. Without UV exposure both wild-type and *clu1Δ* presented 100% cell viability. As expected, when each strain was exposed to UV light, both displayed a noticeable decrease in cell viability. Interestingly, however, there was a negligible difference in percent loss of cell viability between wild-type and *clu1Δ* exposed to UV light, which indicates that Clu1p does not contribute to nuclear DNA response to UV-induced DNA damage.

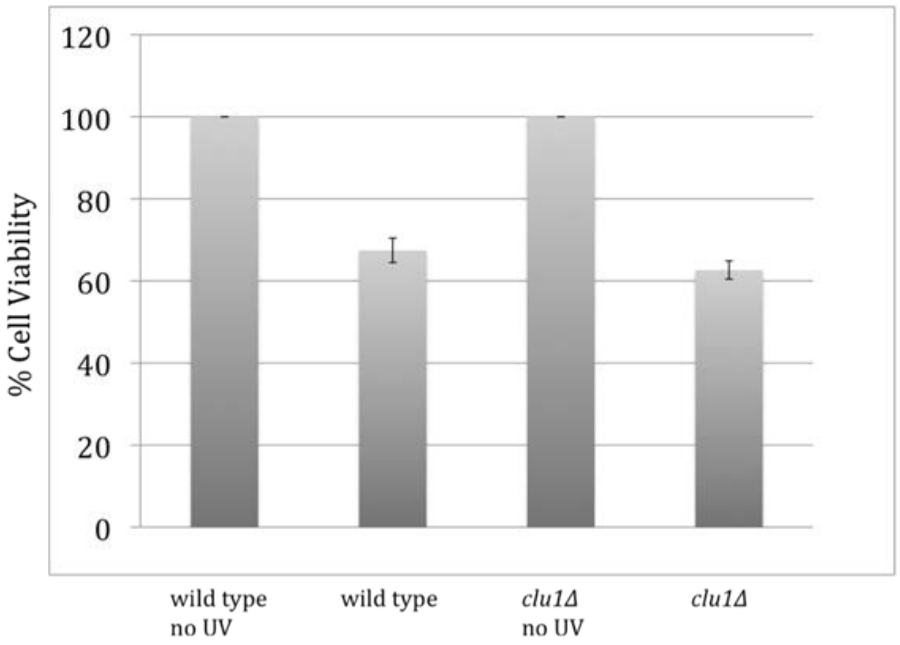

Figure 6. Cell viability. The percent cell viability was calculated for both wild-type and clu1Δ with and without UV exposure. Wild-type and clu1Δ both exhibited near identical cellular viability.

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clu1Δ strains have incomplete mitochondrial genomes

To answer the question of whether or not the mitochondrial genome may be involved in the observed respiration loss, the mtDNA was isolated and cleaved at specific palindromic sequences by a restriction enzyme. MtDNA is relatively conserved and if the same restriction enzyme was used in all samples, the same or at least similar size DNA segments should result. The wild-type mtDNA, once cleaved, displayed a particular banding pattern as shown in Figure 7. However, when the non-respiring cleaved mtDNA was compared to wild-type there is little pattern. The DNA fragments are small and often complete miss a wild-type band. This data is indicative that certain events are occurring in the mtDNA that are causing DNA excision.

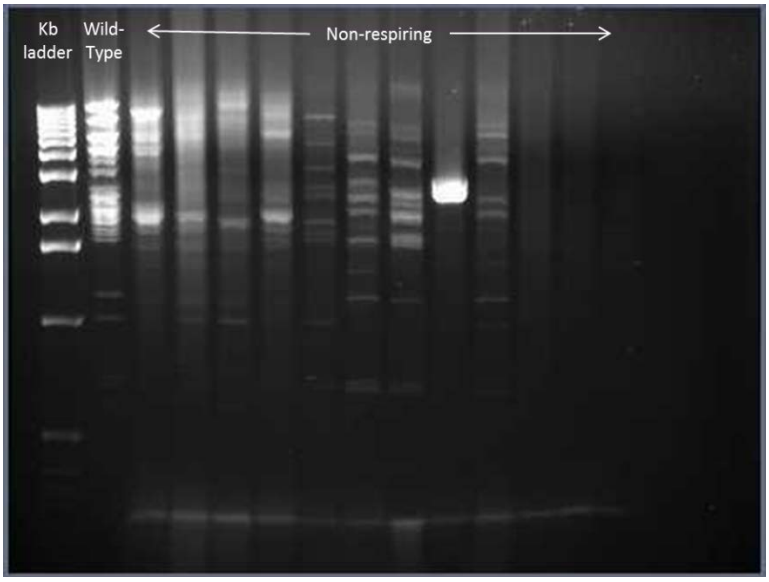

Figure 7. Digested mtDNA. 0.8% agarose gel showing the separation pattern of mtDNA digested with restriction enzyme

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clu1Δ strains show increased rate of spontaneous point mutations

To investigate the possible cause of the observed respiration loss, the rate at which *clu1Δ* mtDNA develop spontaneous point mutations was determined. This was done using an assay that monitors spontaneous resistance to the antibiotic erythromycin as a function of spontaneous point mutations. The erythromycin antibiotic imitates a charged tRNA and inhibits translation by preventing the peptide chain from elongating. Resistance to this lethal inhibition is provided by a point mutation in that mitochondrially encoded 21S rRNA gene. Therefore, spontaneous resistance to erythromycin is indicative of spontaneous point mutations and can thus be measured. As shown in Figure 8, erythromycin resistance was 3.9-fold high in *clu1Δ* strains than wild-type strains. This observed increased rate of spontaneous point mutation could provide an explanation for the increased loss of respiration loss in *CLU1* knockout strains.

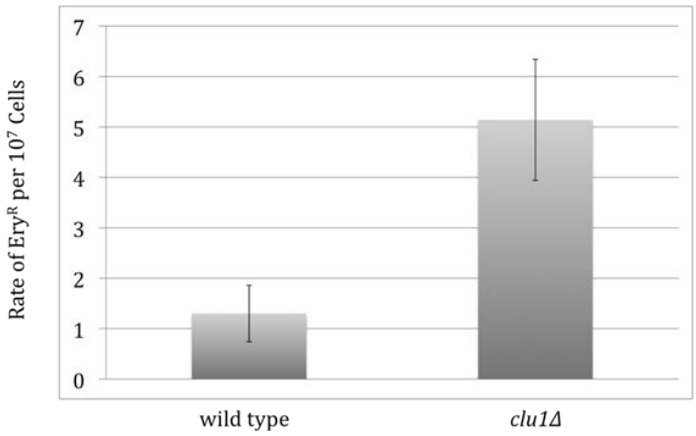

Figure 8. Rate of spontaneous point mutation. As a function of erythromycin resistance, the rate of the spontaneous point mutations in the 21S rRNA gene was determined. The rate in *clu1Δ* was nearly 4-fold high compared to wild-type.

Clu1p does not seems to play a significant role in direct-repeat mediated deletion events

The yeast mitochondrial genome is highlighted with direct repeats, or specific sequences of DNA that repeat in the same orientation. These regions of DNA encourage deletion because the areas of homology are hot-stops for recombination events. The accumulation of direct-repeat mediated deletion (DRMD) events could be the source of the respiration loss observed previously. As mentioned in the materials and methods, the rate at which DRMD events occur can be measure through the use of two genetically engineered reporters.

The schematic of the two reporters is summarized in Figure 9. Figure 9a shows the mitochondrial reporter; the COX2 gene is interrupted by the ARG8^m gene. COX2 is a mitochondrial gene necessary for respiration, but with the ARG8^m interruption the gene is unable to be properly transcribed and therefore is unable to respire. The reporter can be selected for by plating cells on media lacking arginine and with a fermentable carbon source because the cells are unable to respire. Once certain that cells possess the reporter, the cells are plated on a non-fermentable carbon source, such as glycerol. Only cells that

undergo a recombination event will enable the COX2 gene necessary for oxidative phosphorylation. The ARG8^{m} interruption site allows the gene to be flanked by 96 bp repeats indicated in black.

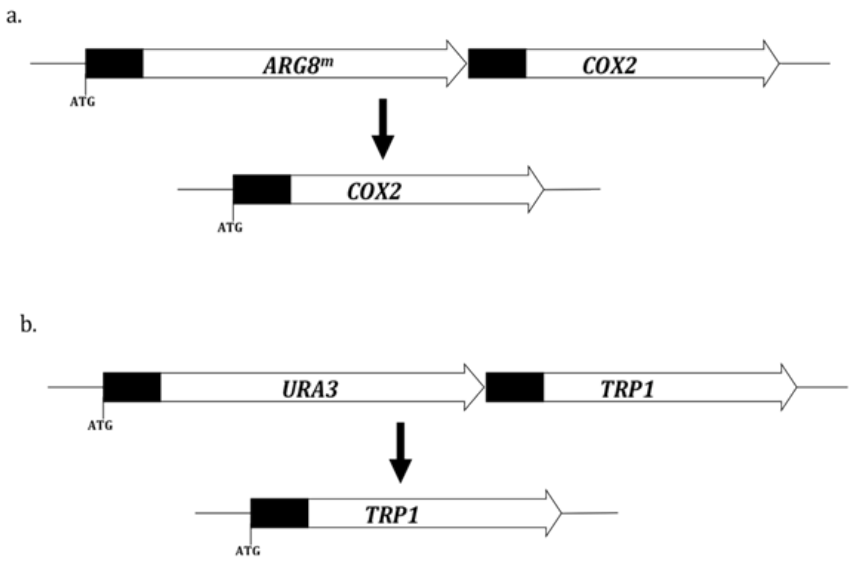

Figure 9. The direct repeat-mediated deletion (DRMD) reporter constructs. Both wild type and *clu1Δ* deletion strains (LKY196) contain both reporter constructs. **(a)** The mitochondrial reporter has an *ARG8^{m}* gene inserted between two 96 bp repeats of the *COX2* gene in the mtDNA. The product of the *COX2* gene is required for respiration. In the absence of a DRMD event, the yeast strain will be unable to respire. Subsequent to a DRMD event, the strain will be respiration proficient. **(b)** The nuclear reporter has the *URA3* gene inserted between two 96 bp repeats of *TRP1* gene. In the absence of a DRMD event, the strain is unable to grow on a media lacking tryptophan. Subsequent to a DRMD event, the strains are prototrophic for tryptophan.

Similar to the method of measuring DRMD events in the mtDNA, the rate of occurrence of these events was also determined in the nuclear genome. As shown in Figure 9b, the nuclear reporter construct is very similar to the mitochondrial reporter construct.

The difference in the nuclear DNA is that the gene *URA3* interrupts the *TRP1*gene, which are genes that codes for a protein necessary in the synthesis of tryptophan and uracil, respectively. Therefore, cells possessing the construct are phenotypically Ura^+ and Trp^- because *URA3* is fused in between two direct repeats of the *TRP1*gene, preventing *TRP1* transcription. If a recombination event mediated by the direct repeats occurs, however, then the *URA3* will be excised and *TRP1* functionality will be regained.

The results of the DRMD assay are presented in Figure 10. The average rate of DRMD events in mtDNA in wild-type LKY196 strains was 2064×10^{-7} deletion events were cell division. The average rate in the *clu1Δ* strains of 2662×10^{-7} per cell division, which is an insignificant difference compared to the wild-type rate.

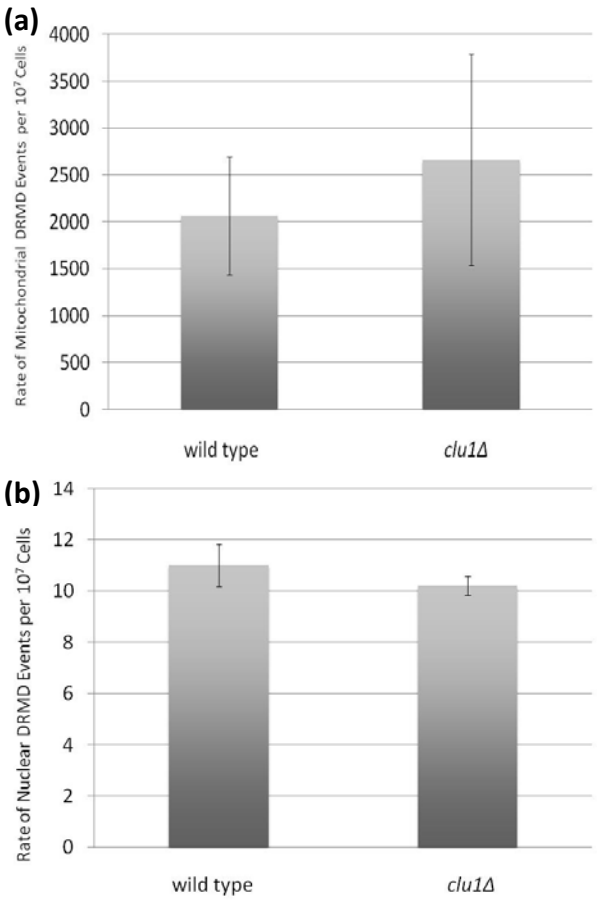

Figure 10. Direct-Repeat mediated deletion events data. (a) Shows the rate of mitochondrial DRMD events. (b) Shows the rate of nuclear DRMD events. DRMD events are slightly increased in the mtDNA of *clu1Δ* compared to wild-type. Comparison of nuclear DRMD events between wild-type and *clu1Δ* appear negligible.

The difference in the rates of nuclear DRMD events is similarly negligible (Figure 9B). These results support the conclusion that Clu1p does not influence DRMD events during mitotic division.

clu1Δ strains have altered mitochondrial morphology

It was previously observed by Fields *et al.* (1998) that *clu1Δ* strains have altered mitochondrial morphology. These results were confirmed in our own microscopy study (Figure 11).

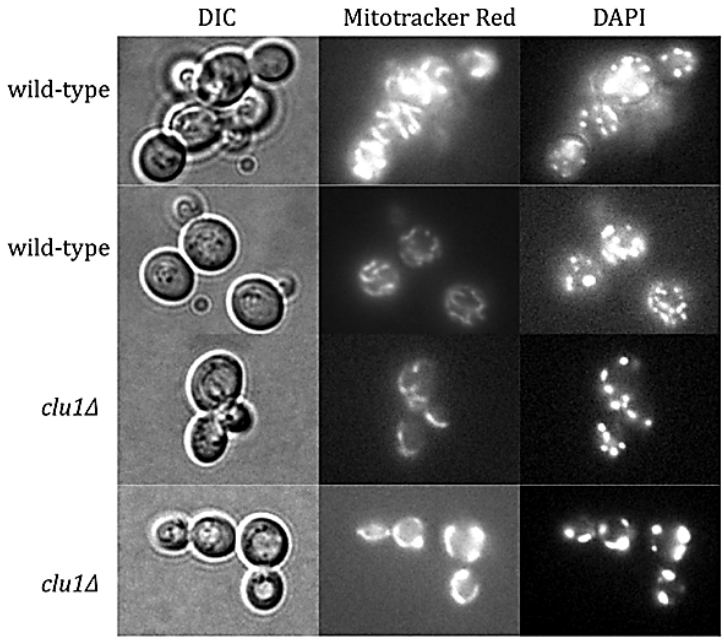

Figure 11. Fluorescent Microscopy. The first column of the figure depicts differential inference contrast (DIC) microscopy. Each row is an overlay of the respective cells; two for wild-type, two for *clu1Δ*.

Mitotracker was used to fluorescently tag the mitochondria. As seen in Figure 11, the wild-type strains seem to have the mitochondria evenly distributed throughout the cell.

Similarly, the mtDNA, fluorescently tagged by the DAPI, seem to form the distinct nucleoid structure predicted. In the clu1Δ strains, however, the mitochondria seems to aggregate to the periphery of the cell. Likewise, the mtDNA appear to coalesce and find the same aggregated position in the cellular cortex as the mitochondria in which they are contained.

DISCUSSION

The goal of this study was to determine the role of the Clu1p in the maintenance of mitochondrial morphology and mitochondrial genome stability. The identification of proteins that influence mitochondrial morphology and/or mitochondrial genome stability may give us a better understanding of the diseases that arise when these features are altered. Given that cellular respiration is an essential metabolic pathway in countless organisms, maintaining the integrity of the mitochondria and its genome is essential.

Through our genetic respiration loss assay using null mutants, the status of the mtDNA can indirectly be measured. If the loss of the Clu1p protein causes an abnormal instability of the mtDNA to where mutations can accumulate rendering the DNA unable to transcribe or the mutated transcript unable to translate a functional protein, then a distinct loss of respiration would be observed.

Our mitochondrial DNA digest study indicated that there were significant deletion events happening in the non-respiring cells of *clu1Δ* strains. Such large deletion events can best be described by the direct-repeat medicated deletion (DRMD) events that apparently happen at a slightly increased frequency compared to wild type. Additionally, the yeast mitochondrial genome is plagued with direct repeats that could potentially encourage these deletions. But the question remains: how does Clu1p contribute to these alterations?

There have been several proteins that have been known to play a role in regulating mitochondrial morphology and genome stability. Some of these proteins have been associated with nucleoid segregation and mitochondrial morphology in the budding process. The nucleoid segregation process during budding is depicted in Figure 12.

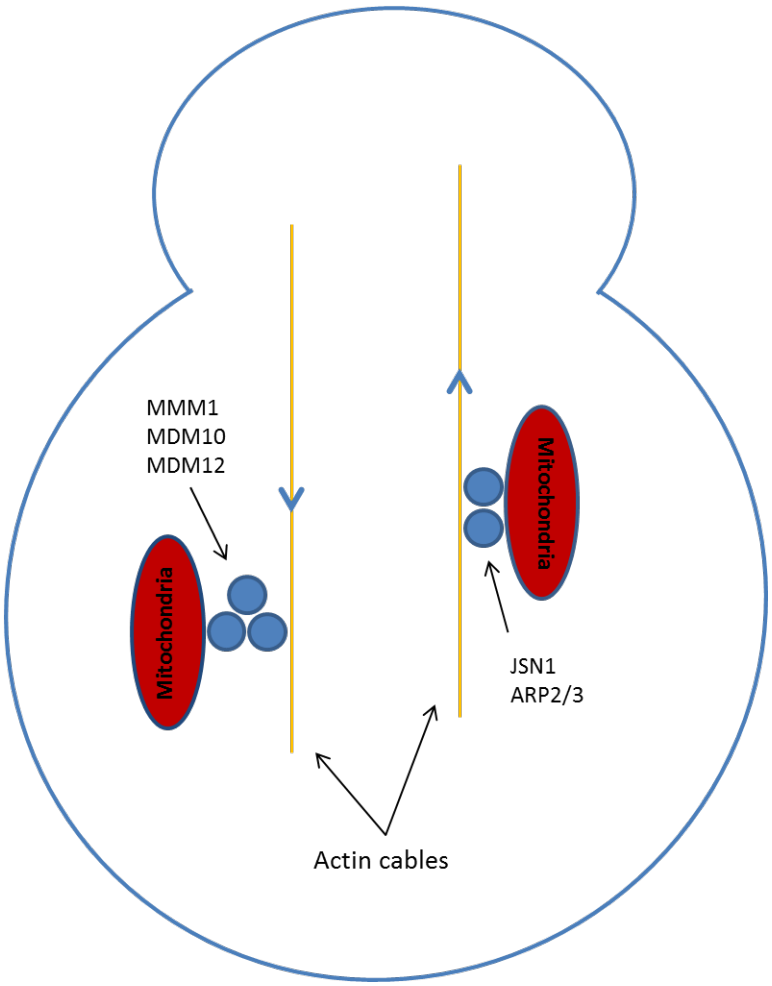

Figure 12. Budding yeast. This figure depicts the tethering of the mitochondria to actin cables during the yeast budding process.

The protein products of MMM1, MDM10, and MDM 12 have been shown to tether the outer mitochondrial membrane to the actin cable with a specific directionality during the budding process (Boldogh, et al., 2003) (Hobbs, Srinivasan, McCaffery, & Jensen, 2001) (Meeusen & Nunnari, 2003). A simplified magnification of the tethering 'bridge' is shown in Figure 13.

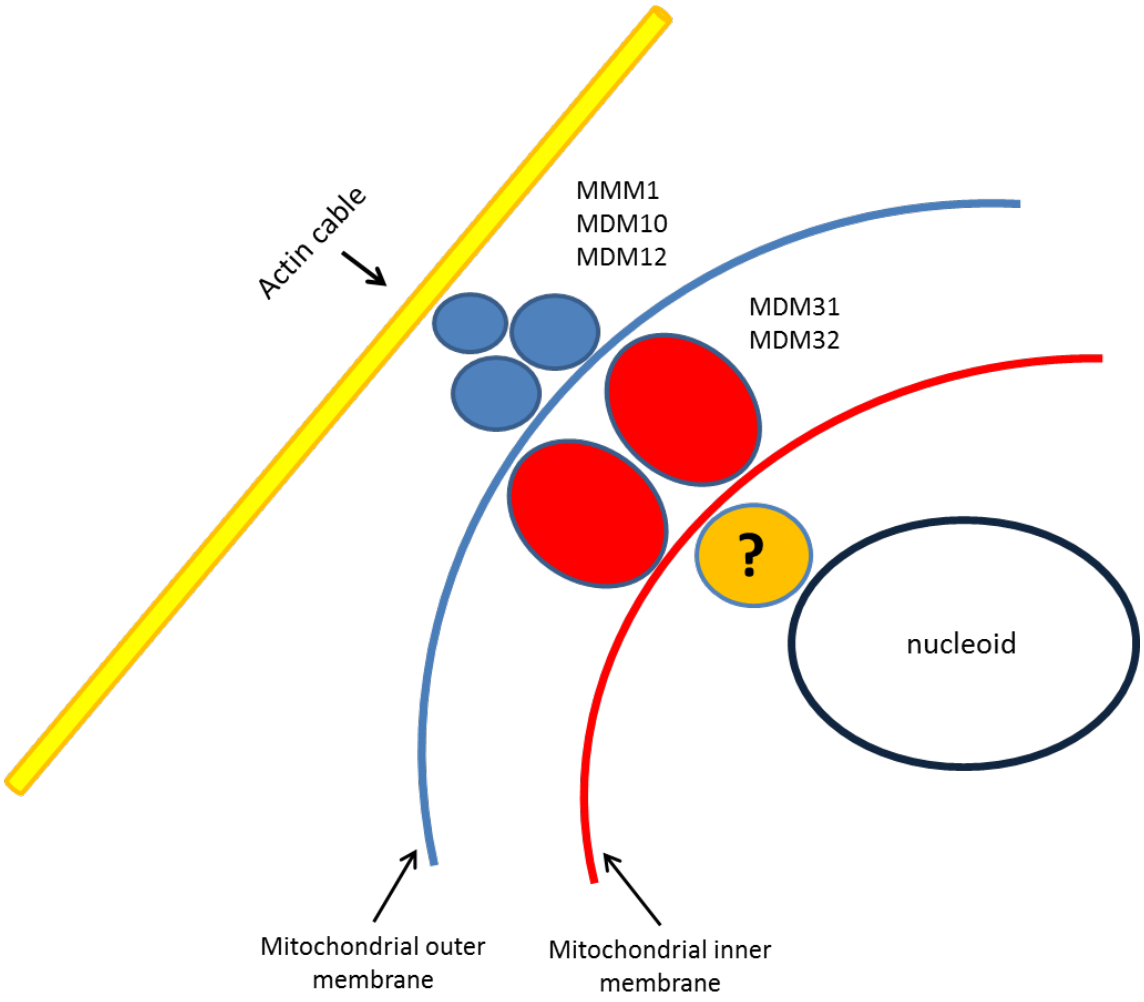

Figure 13. Mitochondrial tethering model. Nucleoid is tether to the inner mitochondrial membrane and the mitochondria is tether to the actin cable via proteins MMM1, MDM10, and MDM 12.

The gene products of MMM1, MDM10, and MDM12 tether the outer mitochondrial membrane to the actin cable responsible for the segregation of the mitochondria during the budding process (Boldogh, et al., 2003). Additionally, gene products MDM31 and MDM32 located between the outer and inner mitochondrial membranes seem to contribute to the stability of the stable structure (Boldogh, et al., 2003). All of these gene products have been

identified contributing to the stable structure that also – through a protein of unknown identity – bind the nucleoid structure providing a thermodynamically stable construct. All these products together form a 'bridge' that allow the actin cable to leash the mitochondria and direct its migration.

It is our theory that Clu1p somehow contributes to this cascade of tethering proteins in a way that when it is not present, this 'bridge' structure cannot form. When the nucleoid is not bound to the inner mitochondrial membrane its instability is increased and is more susceptible to the oxidative damage that plagues the mitochondrial interior. This instability could explain the increased rate of DRMD events and point mutations described by the data discussed earlier. Additionally, this lack of stability could also make the mtDNA more vulnerable to induced DNA damage as seen in the UV light study.

CONCLUSIONS

The mitochondrion is a complex and dynamic organelle. Its role in cellular respiration is vital to nearly all aerobic organisms. The mitochondrion's genome proves essential in supplying the mitochondrion with the necessary proteins to accomplish oxidative phosphorylation. Mutations within the mitochondrial genome have been affiliated with many debilitating and incurable diseases, as well as, the aging process. With a better understanding of the mitochondria and its propensity to rearrange and mutate, perhaps we draw closer to combatting mitochondrial genetic disorders.

FUTURE WORK

Nearly identical to the Clu1p study, another protein of interest also seems to play a role in mitochondrial morphology and mitochondrial genome stability. DNM1 is another nuclear encoded protein that has also previously shown to alter mitochondrial morphology upon its nullification. Thus far *dmn1Δ* strains have been created and verified using similar processes as *clu1Δ*. Additionally, d*nm1Δ* strains preliminarily display a 12.4 (±3.7) % respiration loss compared to the wild-type loss of nearly 4 %.

Similar to the Clu1p study, research plans to be done to verify the observed respiration loss and to determine the cause of the near 3-fold increase in respiration loss.

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