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The Role of Ilv5p Interacting Factors in Mitochondrial DNA Stability

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by

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Abstract

The ease of manipulating yeast allows for advanced studies on the factors affecting the mitochondrial DNA mutation rates. The control mechanisms of the mitochondrial DNA mutation rate has been determined to involve the dual function protein, Ilv5p. The Ilv5p plays an integral role in the proper segregation of newly replicated mitochondrial DNA into daughter cells during cell division. The focus of this study is to find unknown factors involved in mitochondrial DNA stability. This study uses the Ilv5p to pull unknown factors out of the many genes that comprise the yeast genome. The identification of interacting factors of the Ilv5p is the focus of this study. We have found proteins likely to physically interact with the Ilv5p and play a role in the stabilization of mtDNA. These proteins have been identified as the Vma8p and the YOL057W gene product. These proteins have been tested and shown to affect the stability of mitochondrial DNA.

The Vma8p is the D subunit that comprises the V_1 complex. The V-ATPase that is responsible for ATP synthesis possesses two complexes, the V_1 and the V_0 complex. The V_1 and V_0 complexes readily dissociate and reassociate in response to environmental conditions optimizing acidification of vacuole components for regulation of ATPase activity in other cellular components. The loss of this particular protein increases the occurrence of ρ^- petite or non-functional mitochondria. A microsatellite instability assay showed that the mutation rate increased 50 times with the loss of the Vma8p.

The respiration loss assay showed the loss of the *YOL057W* gene product caused a significant increase in the rate of ρ^- petite formations. This assay is the first to show that the putative open reading frame (ORF) encodes a protein whose loss of function leads to a mitochondrial defect. The exact role of this particular protein in the mitochondria has yet to be determined.

Background and Significance

Almost all eukaryotic cells except those called anaerobic protozoans possess mitochondria, essential organelles required for respiration and producing the majority of cellular ATP (Mueller, 1988). The production of energy by the cell is required for viability in higher eukaryotes. Mitochondria also are involved in the production of heme and amino acids (reviewed by Schatz, 1995) along with a crucial role in cellular apoptosis (reviewed by Green and Reed, 1998). The budding yeast, *Saccharomyces cerevisiae*, when provided with a fermentable carbon source can grow in the absence of oxidative respiration. The function of the mitochondria in oxidative respiration is therefore dispensable, but yeast require mitochondria for viability. Yeast are a model organism for mitochondrial gene transcription and translation, study of respiration genes and protein import into the mitochondria.

Mitochondrial genome and nucleoid

The mitochondrial genome encodes many proteins required for respiration, but many other proteins are encoded in the nucleus and imported to the mitochondria. The mitochondrial genome of the budding yeast is 85kb (Foury *et al.* 1998), divided into AT and GC rich regions along with highly repetitive AT-rich sequences. Haploid cells have about 50 copies of the mitochondrial genome and diploid cells have about 100 copies. Yeast mitochondrial DNA may replicate by the "rolling circle" method (Maleszka *et al.* 1991) generating linear molecules found by pulse-field gel electrophoresis (Bendich, 1996).

The DNA of the mitochondria is organized into compartments called nucleoids (Miyakawa *et al.* 1995, Miyakawa *et al.* 1987, Newman *et al.* 1996, Williamson, 1976). Abf2p is one such protein that scaffolds mitochondrial DNA into nucleoids. Abf2p has homologous domains to HMG (high mobility group) proteins. Mitochondrial DNA is bent by HMG proteins allowing transcription and chromatin packaging. Abf2p can be co-purified with mitochondrial nucleoids as Abf2p binds mitochondrial DNA non-specifically (Diffley and Stillman, 1991).

Twenty additional proteins have been discovered to be bound to mitochondrial DNA by formaldehyde crosslinking studies (Kaufman *et al.* 2000). Mass spectroscopy has identified eleven of the twenty proteins, including known mitochondrial DNA interactors and seven new interactors. The seven new interactors are identified as Lpd1p, Atp1p, Ald4p, Aco1p, Hsp10p, Kgd2p, and Hsp60p. Kgd2p and Hsp60p were both further characterized and have a role in mitochondrial DNA stabilization. Kgd2p is a TCA cycle protein and Hsp60p is a DNA binding protein that binds a putative mitochondrial DNA origin of replication.

Yeast mitochondrial DNA compared to its human counterpart is much larger. Human mitochondrial DNA is a circular 16.5 kb molecule (Anderson *et al.* 1981) and 100-10,000 copies are in each cell. The human mitochondrial genome encodes genes required for oxidative phosphorylation as do those present in yeast (reviewed by Shadel and Clayton, 1997).

Mitochondrial DNA replication

DNA polymerase gamma (Pol gamma) is the polymerase that replicates the mitochondrial genome. *MIP1* is the catalytic subunit of Pol gamma in yeast (Foury, 1989). Pol gamma is a high fidelity polymerase *in vitro* and has a 3' to 5' "proofreading" exonuclease domain (reviewed in Roberts and Kunkel, 1996). Point mutations in the mitochondrial DNA are increased by mutations in the proofreading exonuclease domain (Foury and Vanderstraeten, 1992). A human beta-subunit has been co-purified with the human polymerase gamma. In vitro, the beta-subunit increases the affinity of the polymerase gamma for DNA templates allowing for more efficient replication (Lim *et al.* 1999). No yeast homolog to the beta-subunit has been discovered.

Depending on the strain, yeast have three or four mitochondrial genome origins for the initiation of replication (Baldacci *et al.* 1984, de Zamaroczy *et al.* 1984). Yeast mitochondrial DNA is thought to be replicated bidirectionally from the origins (Baldacci *et al.* 1984). Humans have two unidirectional origins of replication. Initiation of replication is similar to the ColE1 plasmid with a transcription-primed intermediate (reviewed by Shadel and Clayton, 1997). The many other factors including topoisomerases and helicases required for initiation have yet to be elucidated (reviewed by Shadel and Clayton, 1997).

Maintenance of the mitochondrial genome involves the action of the *RIM1* gene product. The *RIM1* gene product is a single-stranded DNA binding protein found in yeast and may be involved in mitochondrial DNA replication (Van Dyck *et*

al. 1992). The *h-mtSSB* gene is the human homolog to the *RIM1* gene product (Tiranti *et al.* 1993). In addition, the DNA ligase required for mitochondrial DNA replication may be encoded by an alternative gene product of the nuclear ligase Cdc9p (Willer *et al.* 1999).

Rho^- and Rho^0 petites

Yeast can tolerate deletions or rearrangements of the mitochondrial genome that lead to lethality in higher eukaryotes. Spontaneous mutants that have lost part of the mitochondrial genome are called *rho*$^-$ (ρ^-) mutants. These cells have lost the ability to respire on fermentable carbon sources such as glucose (reviewed in Dujon, 1981). Rho^+ and *rho*$^-$ mutants have the same amount of mitochondrial DNA content, so fragments in *rho*$^-$ mutants are amplified accordingly (Dujon, 1981). Rho^- mutants can arise spontaneously or be induced by treatment with acriflavine or ethidium bromide (Dujon, 1981). Replication in *rho*$^-$ mutants is not completely understood, but the mechanism does not seem to involve the RNA polymerase as in wild-type cells (Fangman *et al.* 1990). Recombination may mediate the replication in *rho*$^-$ mutants as opposed to transcription-primed in wild-type cells (Zelenaya-Troitskaya *et al.* 1998). Rho^0 petites, cells with the complete loss of mitochondrial genome, can arise by the treatment with high concentrations of ethidium bromide (Goldring *et al.* 1970). Rho^0 mutants can be transformed by microprojectile bombardment (Fox *et al.* 1991) that integrates specific mitochondrial sequences. These sequences can then be further integrated into the wild-type genome by recombination during mating.

When specific genes were mutated many mitochondrial-specific processes were elucidated by the mutation of genes important in those processes. Many of the mutations lead to the generation of a *rho*$^{-}$ or *rho*^{0} petite phenotype (reviewed by Contamine and Picard, 2000). Mitochondrial DNA metabolism, transcription and translation, ATP synthase subunits, mitochondrial proteases and chaperones, mitochondrial carriers or transporters, fatty acid and phospholipids metabolism, mitochondrial morphology and inheritance were all determined by the mutation of genes involved in these processes.

There are at least five postulated ways that yeast cells can lose mitochondrial function giving rise to ρ^{-} petites. The lack of proper DNA replication or the improper segregation of the replicated DNA into daughter cells are two mechanisms that can lead to the loss of mitochondrial function. Mitochondrial DNA mutations can lead to the expression of truncated proteins or altered proteins rendering the mitochondria non-functional. The two other ways mitochondria can lose function are by microsatellite instability and through improper homologous recombination. Microsatellites, segments of repeated DNA sequences (...GTACCGTACCGTACC...), can lead to DNA polymerase slippage. This can lead to improper replication causing the loss or addition of nucleotide bases in the DNA. DNA can also be lost through homologous recombination that compromises the genome in important loci.

Mitochondrial segregation

Initially after two strains of yeast with different mitochondrial DNA molecules mate, zygotes contain heteroplasmic mitochondrial genomes. However, after a few generations the zygotes will then contain homoplasmic genomes. The unequal segregation of mitochondrial genomes between the mother and daughter cells during cell division and many rounds of recombination may lead to the generation of the homoplasmic mitochondrial genomes (Dujon, 1981).

The management of the heteroplasmic genomes is under the control of two mitochondrial proteins, Mgt1p and Pif1p. The mutation of the two genes *MGT1* and *PIF1* lead to the disruption of the biased inheritance of hypersuppressive *rho*$^-$ genomes. These two proteins also are involved in mitochondrial DNA recombination. Recombination may at least play a role in the transmission of *rho*$^-$ genomes.

Mitochondrial DNA repair

Proofreading exonuclease activity in the polymerase gamma corrects mutations during mitochondrial DNA replication, but this mechanism cannot repair mutations caused by oxidative damage after replication occurs. Two particular mechanisms are present to correct these mistakes, the base excision repair (BER) and nucleotide excision repair (NER) pathways.

The base excision repair pathway repairs lesions in the DNA. Lesions can be caused by aberrant incorporation of bases during replication and that are not replaced by the polymerase gamma exonuclease or oxidative damage to nuclear DNA. Glycosylases cleave the bond between the base and the sugar leaving an abasic site.

After an abasic site is recognized, an AP endonuclease cleaves the site and the correct sequence is then put in place.

Two mitochondrial glycosylases have been identified in yeast. The Ntg1p and the Ogg1p are both involved in the repair mechanisms of mitochondrial DNA. Ntg1p is a N-glycosylase homolog that directly repairs oxidatively damaged pyrimidines (You *et al.* 1999). When the *NTG1* gene is deleted the frequency of *rho*$^-$ petite formation or number of point mutations does not increase (You *et al.* 1999). This is most likely caused by the presence of another protein with a redundant function. The Ogg1p repairs oxidized guanines (Singh *et al.* 2001). Spontaneous mitochondrial mutants increase 2-fold when that protein is inactivated. Evidence supporting this particular finding is shown in the studies by Singh et al. 2001.

Another protein shown to repair oxidative damage is the Mgm101p (Chen *et al.* 1993). Mgm101p binds to mitochondrial DNA and is associated with nucleoids. A *mgm101* mutant showed a temperature sensitivity and the inability to repair mitochondrial DNA after treatment with oxidizing agents. The exact role of the protein in the maintenance of mitochondrial DNA is not clear. It is unknown if the protein directly repairs mitochondrial DNA lesions or indirectly by protecting the DNA from the oxidizing agents.

The nucleotide excision repair pathway repairs bulky lesions like photoproducts caused by UV irradiation in nuclear DNA. The *RAD1*, *RAD2*, *RAD14*, and *RAD10* genes are required for the functioning of this pathway. The NER pathway excises an oligionucleotide with the bulky lesion leaving a stretch of single stranded

DNA. The single stranded DNA acts as a template for re-synthesis by a DNA polymerase. No evidence supports the existence of a mitochondrial NER pathway.

Post-replication repair pathways correct those errors that are introduced into the genome during DNA synthesis and not corrected by DNA polymerase proofreading. The *E.coli* protein homodimer MutS is one such repair mechanism that recognizes mismatched DNA substrates. The nuclear gene *MSH1* encodes the yeast homolog to the *mutS* gene. Those yeast strains that have the *MSH1* gene deleted result in a rapid loss of mitochondrial function whereas diploid strains heterozygous for the *msh1Δ* mutation have a 7-fold increase in the rate of point mutations in mitochondrial DNA (Chi and Kolodner, 1994). The Msh1p has been shown to bind to mismatched DNA substrates *in vitro* (Chi and Kolodner, 1994). The rate of *rho*$^-$ petite formation increased in those haploid yeast strains that had a partial function of the Msh1p. These *msh1* mutants also were lethal for respiration when mutations in the Pol gamma proofreading domain were introduced. This suggests that these cells accumulate mitochondrial mutations at a very high rate (Vanderstraeten *et al.* 1998). The model for the *MSH1* gene further states that the gene is the recognition subunit in an undefined post-replication repair pathway.

Another protein in post-replication repair is the Din7p. This mitochondrial protein has strong homology to the yeast exonuclease Exo1p. The Exo1p functions in the Msh2-dependent mismatch repair pathway as a double-strand DNA-specific exonuclease. The overproduction of Din7p leads to the increase in petite production and frequency of mitochondrial DNA point mutations to Ery^r (Fikus *et al.* 2000).

Mitochondrial DNA recombination

Homologous recombination has shown to increase the amount of mitochondrial DNA deletions leading to the *rho*$^-$ petite phenotype. The *E.coli* proteins involved in homologous recombination are well known and the yeast nuclear counterparts are well defined. The proteins in the actions of initiating a double strand break, homologous pairing, Holliday junction migration and resolution have all been identified through extensive research. However, only a small number of proteins in homologous recombination of mitochondrial DNA are identified.

The mitochondrial protein Abf2p is related to the high mobility group (HMG) class of DNA-binding proteins (Newman *et al.* 1996). The overproduction of the Abf2p leads to the increase of *rho*^{0} petites. The Abf2p is required for the efficient recombination of mitochondrial DNA markers in crosses and can promote or stabilize Holliday junction intermediates in *rho*$^+$ mitochondrial DNA.

The *ABF2* gene is encoded in the nucleus and its protein is transported to the mitochondria. Yeast *abf2Δ* mutants can be maintained indefinitely on glycerol media, but lose mitochondrial DNA quickly when grown on a fermentable carbon source such as glucose (Diffley and Stillman, 1991). In protein profiles comparing the mitochondrial nucleoids from *ABF2* and *abf2Δ* mutants, two proteins other than the Abf2p are missing (Newman *et al.* 1996) suggesting that the Abf2p is important in localizing other nucleoid proteins. When the *ABF2* gene is deleted, replication and recombination of mitochondrial DNA is altered (Zelenaya-Troitskaya *et al.* 1998).

Parisi et al. show that mitochondrial DNA instability can be suppressed in *abf2Δ* mutants by the human homolog, *h-mtTFA* (Parisi *et al.* 1993).

Cce1p, a cruciform cutting enzyme resolves Holliday junctions formed during recombination (Kleff et al.) 1. *CCE1* is allelic to *MGT1*, the gene involved in mitochondrial genome transmission. Cce1p is a mitochondrial protein and is located at the inner membrane. The loss of the Cce1p leads to the increase of mitochondrial DNA molecules linked by recombination junctions (Lockshon et al.). In contrast, the increased activity of the Cce1p decreased the number of branched mitochondrial DNA structures. The loss of the *CCE1* gene causes a slight increase in *rho*$^-$ petite production.

Pif1p is a helicase associated with the mitochondria and is involved in mitochondrial DNA recombination. Those cells with a *pif1* mutation are defective in recombination between tandemly organized *rho*$^+$ and *rho*$^-$ genomes. The *rho*$^-$ genomes in an inverted manner were not sensitive to the mutations.

Cells possessing the mutated form of the Mhr1p (*mhr1-1*) show an increase in vegetative petite induction by UV irradiation at 30°C or when they are grown at an elevated temperature such as 37°C (Ling *et al.* 1995, 2000). These cells are also defective in mitochondrial DNA recombination (Ling *et al.* 2002). The Mhr1p resides in the mitochondrial matrix and pairs single-stranded DNA and homologous double-stranded DNA to form heteroduplex joints *in vitro*. The *mhr1-1* mutants lose this ability showing a role of the Mhr1p in homologous mitochondrial DNA recombination.

Microsatellite instability provides a means to identify genes involved in replication and repair

Microsatellites are regions in the genome where a small number of bases are repeated multiple times. The failure of cells to repair these segments of DNA is indicated by microsatellite sequence instability. Microsatellite instability is associated with predisposition to human diseases such as cancer and tumors (See Mitochondrial mutation and human disease section below). Being able to study the factors involved in microsatellite instability of yeast has lead to the identity of numerous proteins responsible for repairing human DNA and further elucidation of ways to intervene in the progression of cancer.

Eukaryotic genomes possess numerous repetitive sequences and are inherently unstable making microsatellite sequences polymorphic among individuals in a species. Microsatellite instability is caused by DNA polymerase slippage (Streisinger *et al.* 1966). The model of DNA polymerase slippage has the nascent and template strands of DNA synthesis transiently dissociating through a segment of repeated nucleotide bases of a microsatellite. The reassociation of the strands leads to the formation of unpaired loops. These structures are stabilized by the addition of bases to either side of the unpaired loop as the sequences are repetitive. If these single stranded loops are still present through the next round of replication, the length of the repetitive sequence can increase or decrease. If the unpaired loop is on the nascent strand, the length of the sequence will increase whereas the sequence will shorten if the unpaired loop is on the template strand.

The rate of frameshift mutations in yeast nuclear repetitive tracts is increased when mutations are present in the proofreading exonuclease domains of the replicative DNA polymerases, delta and epsilon (Strand *et al.* 1995, Tran *et al.* 1997). Those mispaired strands resulting from polymerase slippage form substrates for DNA mismatch repair. When yeast homologs to the prokaryotic post-replication mismatch repair mechanism were mutated, repetitive DNA segments were further destabilized (Johnson *et al.* 1996, Marsischky *et al.* 1996, Sia *et al.* 1997, Strand *et al.* 1995, Strand *et al.* 1993). The identification of human nuclear counterparts to the yeast mismatch repair components was accomplished through those studies. The studies also showed that destabilization occurred in similar repetitive sequences of human cells when the genes were mutated indicative of the presence of satellite DNA (Bronner *et al.* 1994, Leach *et al.* 1993, Lindblom *et al.* 1993, Nicolaides *et al.* 1998, Nicolaides *et al.* 1994).

Mitochondrial mutation and human disease

Many delayed-onset progressive diseases result from germ-line mutations in the human mitochondrial genome. These diseases include deafness, blindness, dementia, neuromuscular disorders, heart disease, and diabetes. Human mitochondrial DNA is maintained in a homoplasmic state. The penetrance of mitochondrial disease is correlated to the amount of mutant mitochondrial genomes in the person's cells. The random partitioning of the mutant mitochondrial DNA to daughter cells can lead to various symptoms of diseases. This is due to the variety of mutant to wild-type genome ratios each person inherits (reviewed in Wallace, 2000).

The increase of mitochondrial mutations over time has been related to the aging process and age-related disorders like diabetes. The increase in mitochondrial DNA damage and specific deletions accumulate in an age-dependent manner (reviewed in Luft, 1995). Many other age-related disorders are attributed to the decrease in oxidative phosphorylation activity. Diabetes mellitus (Type II) and Alzheimer's disease are two diseases that may result from mutations in the mitochondria which lead to the decreased activity of oxidative phosphorylation (reviewed in Luft, 1995; Wallace, 1999). Many inherited mitochondrial diseases show a delayed onset and progression with age.

Many human genetic disorders and cancers are caused by mutations in the mitochondrial genome. The most common type of mutations leading to these diseases are large deletions, point mutations, and mono and dinucleotide repeat additions or deletions. Breast, colorectal, gastric, and kidney cancers show mono or dinucleotide insertions and deletions (Bianchi *et al*. 2001). Mitochondrial mutations are shown to be homoplasmic or heteroplasmic when comparing normal and tumoral tissue from the same individual. Homoplasmic tumors are caused by the clonal expansion of the mutated mitochondrial DNA within the tumor and heteroplasmic tumors are caused by a mixture of inherited or acquired mutated mitochondrial DNA. It is clear that mitochondrial genomes are inherited from maternal lineages. However, the rates of diseases related to mitochondrial mutations are evenly distributed as there is no bias segregation of mitochondria amongst females and males.

Other genetic disorders arise from large deletions of the mitochondrial DNA content. Homologous recombination or slippage during replication may give rise to the large deletions. Diabetes mellitus, Kearns-Sayre syndrome, Pearson marrow-pancreas syndrome and skeletal muscle, ocular, and brain myopathies are some of the many disorders that have mitochondrial DNA mutations. These diseases showed that the deletions ranged in size from 1.5 Kb to 5.8 Kb in direct repeated sequences of 7 to 13 bp in length of the mitochondrial genome.

The conserved mismatch repair system monitors and repairs point mutations, mono and dinucleotide tract instabilities, and insertions and deletions of mono and dinucleotides in the nucleus. The Rad52 pathway is required for nuclear homologous recombination. There is similarity in the DNA mutations found in the nucleus and the mitochondria. This points to the existence of a mismatch repair and a homologous recombination pathway in the mitochondria. The evidence for a mismatch repair mechanism in the mitochondria has been shown using a biochemical assay (Mason *et al.* 2003). If mutations block these monitoring or repair pathways mutant phenotypes arise.

The general amino acid control pathway and nucleoid organization

The factors regulating copy number of individual mitochondrial DNA molecules and nucleoids are still being discovered. The signal pathways that control these processes are more elusive. MacAlphine et al. (2000) show that the general amino acid control (GAC) regulates the nucleoid number in mitochondrial DNA molecules. They show that the GAC pathway in *rho*$^-$ and *rho*$^+$ cells increases the

nucleoid number, but does not affect the mitochondrial DNA copy number. The increase in nucleoids in *rho*$^-$ cells was attributed to an increase in homologous recombination between tandem repeats within the mitochondrial DNA leading to the production of smaller, circular oligiomers identified by 2-D gel electrophoresis. The amount of the circular oligiomers increased 5-fold in response to the GAC pathway correlated to a 10-fold increase in nucleoid number. MacAlphine speculate that the recombination rate in the mitochondria is decreased due to the general amino acid control pathway. The GAC pathway involves the Abf2p and the Mgt1p/Cce1p for nucleoid reorganization and increase in nucleoid number. The Ilv5p was found to play an integral role in the GAC pathway in addition to Abf2p and Mgt1p/Cce1p.

The expression of more than 1000 genes has been shown to be affected by the induction of the GAC pathway by 3-amino triazole which mimics histidine starvation. Under starvation conditions yeast initiate the general amino acid control pathway that allows for the synthesis of essential amino acids. This pathway also controls the expression of the transcriptional activator, Gcn4p, of the *ILV5* gene. The constitutive expression of the *GCN4* gene activates the GAC pathway. When cells are subjected to starvation conditions the *GCN4* gene is de-repressed which allows for the expression of the *ILV5* gene. The *ILV5* gene encodes the acetohydroxy acid reductoisomerase enzyme, a matrix enzyme of the mitochondria, which allows for the synthesis of isoleucine, leucine, and valine and other branched chain amino acids. The Ilv5p is also required in the Gcn4p-dependent reorganization of mitochondrial nucleoids in haploid yeast cells making it a dual function protein. The reorganization of the

mitochondrial nucleoids improves the segregation of the mitochondrial genomes into daughter cells during cell division (MacAlphine *et al.* 2000). Those cells with the *ilv5Δ* accumulated *rho*$^-$ petites at a higher frequency compared to wild-type cells (Zelenaya-Troitskaya *et al.* 1995). The lack of Ilv5p may affect the segregation of the mitochondrial DNA during cell division leading to the observed increase in *rho*$^-$ petites. This shows that Ilv5p has a distinct role in mitochondrial DNA nucleoid reorganization and number in response to the activation of the GAC pathway. This is independent of its other function in branched chain amino acid synthesis.

McAlphine et al. show that the number of mitochondrial DNA nucleoids increased in response to activation of the general amino acid pathway. In their study, they show that *ilv5* mutants had a lower number of nucleoids, but essentially the same amount of mitochondrial DNA content leading to an increase of ρ^- petites or non-functional mitochondria. Wild-type cells (ρ^+) contain 25-50 mitochondrial DNA nucleoids per cell. In ρ^- petites, those cells that have lost respiratory function, large segments of the mitochondrial genome is lost and the remaining segments of mitochondrial DNA is amplified as tandem repeats. ρ^- petites contain the same amount of mitochondrial DNA as wild-type genomes, but there are less nucleoids present in the cells. These nucleoids are larger and stain brighter with DAPI than wild-type nucleoids. The contrast in nucleoid numbers and size is correlated to the larger molecules of mitochondrial DNA in the ρ^- petites and recombination between the tandem repeats in the DNA (MacAlphine *et al.* 2000, Bateman *et al.* 2002, Bateman *et al.* 2002).

This study investigates the loss of specific proteins, direct interactors with the Ilv5p, on mitochondrial function. Initially a respiration loss assay will determine if loss of the proteins alter the rate of mitochondrial DNA ρ^- petites formation. The use of a non-fermentable carbon source such as glycerol with a limited amount of dextrose will allow for the differentiation of functional and non-functional mitochondria. Further analysis of the molecular mechanisms affecting the rate of ρ^- petites formation will include homologous recombination and microsatellite stability reporters. These assays will determine how the losses of the proteins lead to the increase or decrease of ρ^- petites formation. The homologous recombination reporter tests the ability of segments of mitochondrial DNA to be excised through a mutational event causing dysfunction of the mitochondria. The microsatellite reporter assesses the occurrence of DNA polymerase slippage events leading to the addition or deletion of DNA.

Preliminary Studies

The following studies use a mitochondrial frameshift (Sia et al., 2000) to estimate the rate of mitochondrial microsatellite alteration. The reporter is a translational fusion between the *COX3* gene in the mitochondrial genome and the recoded *ARG8* gene, *ARG8*^{m}. The *ARG8* gene product is imported to the mitochondrial matrix where the biosynthesis of arginine occurs. Steele et al. (1996) showed that a derivative of the *ARG8* gene, with a codon preference for a mitochondrial gene, could complement an *ARG8* deletion as a translational fusion with the *COX3* gene. A microsatellite sequence of 16 poly(GT)s was inserted to the 5' end of the translational fusion putting the *ARG8* gene out-of-frame and read it in the +2 reading frame. Those strains containing this construct are phenotypically Arg-. The insertion of this construct also inactivates the *COX3* gene responsible for oxidative phosphorylation. These cells are unable to respire providing no selection for the mitochondrial genome. Once mutations occur, repeats in the poly(GT) tract are lost or gained bringing the *ARG8* gene back in frame. These cells are phenotypically Arg+. Estimation of the mutation rate in the tract can be done by selecting cells that can grow on medium lacking arginine by fluctuation analysis. Sia et al. (2003) have shown that the Arg+ cells arise exclusively from alterations in the microsatellite sequence. The rate of mutations is determined by the rate the mutations arise, are repaired, and are segregated into the daughter cell in cell division.

In order to determine the rate of point mutations, the reporter of resistance to erythromycin (ery^{r}) was used. Mutations leading to the resistance to erythromycin

map to the mitochondrial gene encoding the 21S ribosomal RNA (reviewed in Dujon 1981). The rate of ery^r mutations was measured comparing those cells able to grow in the presence of erythromycin and in its absence. Stationary cells from individual colonies were grown in the presence of erythromycin and viable cells were measured by plating in the absence of erythromycin. The rates of erythromycin resistance were calculated from frequency data in the fluctuation analysis.

Sia et al. have shown that increasing ploidy and inducing general amino acid control affect the rate of both frameshift and point mutations in the mitochondrial genome. Increasing ploidy and inducing general amino acid control both lead to an increase in mitochondrial DNA content. Diploid cells showed a 100-fold decrease in the rate of microsatellite alterations compared to isogenic haploid cells (Sia et al. 2000). Stabilization of nuclear microsatellites was not observed in these cells. The diploid cells were found to have approximately twice the mitochondrial DNA content of haploid cells. Sia et al. (2003) studied three models where increased mitochondrial genome copy number could affect the rate of mitochondrial DNA mutations between diploid cells and isogenic haploid cells.

The first model states that ploidy alone reduces mitochondrial DNA mutation rate. It states that increasing the copy number of the mitochondrial genome decreases the probability cells achieve a ratio of mutant to wild-type genomes generating the mutant phenotype. The second model states the *MAT* locus information can affect the rate that mitochondrial DNA mutations are observed. Those studies showed that diploid cells had a 47 to 74-fold reduction compared to isogenic haploid cells

regardless of information at the *MAT* loci. The third model states that ploidy-specific gene expression leads to the stability of the mitochondrial genome in diploid cells as opposed to mating type-specific gene expression. Galitski et al. (1999) used microarrays to study genes from the genome that respond to increased ploidy independent to mating type of the cells.

They found that ten genes were induced by increased ploidy and seven genes were repressed by ploidy. The total number of genes found to respond to increased ploidy was seventeen. Sia et al. (2003) tested the hypothesis that alteration of one of the genes expression can lead to the disparity in the mutation rate between diploid and haploid cells. Homozygous diploid deletion strains for fourteen of the seventeen genes were obtained from the systematic deletion series. The rates of mutation to Ery^R for the isogenic wild-type and haploid and diploid strains was determined for use in constructing the systematic deletion series. There was a 2.1-fold decrease in the mutation rate in the diploid strain compared to the haploid strain in the strain background. The rates of mitochondrial point mutation accumulation in the isogenic haploid and diploid strains carrying the fourteen deletions showed that the rate of point mutation accumulation was lower in the diploid strain than the haploid strain.

In contrast, mutation of some of the genes mentioned above affect the rate of point mutation accumulation in yeast mitochondria. In changing the expression of any one of the genes, no specific gene was identified that affects the mutation rate differences found between haploid and diploid strains. Sia et al. (2003) state that a change in expression of the genes or an unidentified pathway in response to ploidy is

the more likely factor for the difference in mutation rates observed in isogenic haploid and diploid cells. Specifically they postulate that either an increase in the mitochondrial DNA copy number or ploidy-specific gene expression results in the decrease in mitochondrial DNA mutation rates observed in diploid yeast cells. This is independent to information at the *MAT* loci.

Growth conditions, in addition to ploidy, can affect the mutation rates of microsatellite alterations and point mutations of the mitochondrial genome. MacAlphine et al. (2000) showed that constitutive expression of an allele of *GCN4*, *gcn4*^{c}, display an increase in number of mitochondrial nucleoids as observed from DAPI staining and altered mitochondrial DNA segregation. De-repression of the general amino acid pathway is accommodated by the *gcn4*^{c} allele. In a cross between ρ^{+}, cells with mitochondrial function and ρ^{-} cells, those cells with no mitochondrial function, diploid progeny inherit either ρ^{+} or ρ^{-} mitochondrial genomes. In the presence of the *gcn4*^{c} allele progeny have an increased transmission of ρ^{-} mitochondrial genomes in a cross between ρ^{+} and ρ^{-} non-respiring ρ^{-} strains (MacAlphine et al. 2000). Sia et al. (2003) postulate that if cells spend an increased amount of time in the heteroplasmic state, the mitochondrial DNA mutation rate should be affected by the change in segregation of mitochondrial DNA into daughter cells. The mutation rates and mitochondrial DNA content have been determined under conditions where the general amino acid pathway is partially de-repressed. Sia et al. (2003) state that the mitochondrial DNA content in diploid cells increased 1.4-fold and haploid cells increased 1.8-fold in conditions that activate the general amino

acid pathway. There was also a 7-fold decrease in microsatellite instability rate and 13-fold decrease in the rate of mitochondrial point mutations in haploid cells. There was no significant change in the rate of microsatellite instability and a 3-fold decrease in the rate of mitochondrial point mutations in diploid cells.

Previous studies showed that there was no increase in mitochondrial DNA content, but the number of individual DAPI-stained nucleoids increased under activation of the general amino acid pathway (MacAlphine et al. 2000). The explanations for this discrepancy include strain specific differences, different method of quantification of mitochondrial DNA, or different measurements of the mitochondrial DNA content in ρ^- petite strains.

Sia et al. (2003) have formed a model in which mitochondrial DNA mutation rates and copy numbers are regulated by amino acid starvation. They have found the Ilv5p to be an important regulator in mitochondrial DNA mutation rate and copy number in response to the amino acid pathway.

The Ilv5p is a protein associated with mitochondrial DNA. The Ilv5p is required in the Gcn4p-dependent reorganization of nucleoids in haploid cells. The Gcn4p is a transcriptional regulator of the general amino acid pathway. The segregation of mitochondrial genomes into daughter cells may be improved by the reorganization function of the Ilv5p. When the *ILV5* gene is deleted the mitochondrial DNA mutation rate increases leading to more ρ^- petites. The loss of the Ilv5p may affect the organization of the mitochondrial genome leading to aberrant segregation into daughter cells increasing the mitochondrial DNA mutation rate. In order to test

this hypothesis, Sia et al. (2003) analyzed point mutation rates and mitochondrial DNA copy number in wild-type cells and *ilv5Δ* strains. The point mutation rates were used to test the hypothesis as opposed to using microsatellite instability rates because the microsatellite reporter is lost due to the lack of its selection in these strains.

Wild-type and *ilv5Δ* strains were grown in rich or amino acid limiting media for activation of the general amino acid pathway under regulation of the Gcn4p. The rate of point mutation to Ery^R of the wild-type and *ilv5Δ* strains was not significantly different. However, the *ilv5Δ* strain had a lower rate of point mutation compared to the wild-type strain when both are pre-grown in rich media. The *ilv5Δ* strain pre-grown in rich media had a 1.5-fold reduction in the point mutation rate compared to those *ilv5Δ* strains pre-grown in amino acid limiting media.

Southern blot analysis was performed to find if the mitochondrial DNA copy number is altered between the two strains and under the different growth conditions. Sia et al. (2003) found that there was a significant increase in mitochondrial DNA content in the *ilv5Δ* strains comparing the wild-type strains and *ilv5Δ* strains grown in rich media. There was no difference in the mitochondrial DNA content in those strains grown in amino acid limiting media.

Through the use of DAPI staining, nucleoid organization was observed in strains pre-grown in rich or amino acid limiting medias. As MacAlphine et al. (2000) show, there was an increased number of DAPI nucleoids in wild-type cells grown in amino acid limiting media. This particular finding is dependent upon the presence of

the Ilv5p. The *ilv5Δ* strains contained fewer DAPI-stained nucleoids independent of growth conditions.

The decrease in the copy number of mitochondrial DNA in amino acid abundance is dependent on the presence of the Ilv5p. These strains also show a decrease in accumulation of mitochondrial DNA point mutations. As a reduction in the rate of point mutations in *ilv5Δ* yeast cells may appear inconsistent with the increase in ρ^- petites in the strains the mechanism leading to the mutant molecules is likely different. The ρ^- petites arise by recombination between repetitive intergenic regions leading to genomic sequences being deleted between the repeats whereas the point mutations arise from replication errors or a result of DNA damage. The *ilv5Δ* mutation results in the increase of petite colonies in the strain background. The assay for Ery^R done by Sia et al. (2003) only takes into account yeast cells that contain a wild-type mitochondrial genome. In the assay only those mitochondrial genomes that have not experienced an intramolecular event are scored in point mutation accumulation. The increase in mitochondrial DNA copy number may lead to the decrease in point mutation. The increase in mitochondrial DNA copy number can affect mutation rates by either increasing the amount of mitochondrial DNA available for replication or segregation into daughter cells or making it harder for cells to reach the ratio of mutant to wild-type mitochondrial genomes required for expression of the mutant phenotype. The change in the organization of the mitochondrial nucleoids in *ilv5Δ* cells may lead to the aberrant mitochondrial DNA copy number regulation and an increase in intramolecular recombination that generate the ρ^- petites.

The Ilv5p may regulate mitochondrial DNA copy number in cells grown in rich nutrient conditions. Cells are given a growth advantage when the replication conditions are tightly regulated. These cells can generate more offspring in the nutrient rich conditions giving the cells a genotypic advantage in the population. When the growth conditions are poor the cells with a strong genotype are given a selective advantage. The occurrence of less point mutations in the mitochondrial DNA allows for more fit offspring. Increasing the copy number of mitochondrial DNA in poor growth conditions help daughter cells receive a greater pool of non-mutated mitochondrial genomes.

Results

Two-Hybrid Screen

The two-hybrid assay used in this study allows for identifying interacting factors with the Ilv5p. The assay uses the GAL4 protein and its ability to be separated into DNA binding and activation domains. The Ilv5p is fused in frame to the DNA binding domain of the GAL4 protein and the yeast genomic library is fused in frame to the activation domain of the GAL4 protein. The alcohol dehydrogenase promoter (ADH1) drives the fusion of the GAL4 protein DNA binding and activation domains to the Ilv5p and the genomic library. The positive interaction between the Ilv5p and an interacting protein from the genomic library conjoin allow for transcription of the reporter gene. The reporter for this assay is the *HIS3* gene encoding the production of the amino acid histidine (Figure 1).

The plasmid pGAL4$_{DB}$-Ilv5p was transformed into a yeast strain bearing the *HIS3* reporter gene. Selection of this plasmid was done on synthetic media lacking tryptophan. This strain was then transformed with the pGAL4$_{AD}$-genomic library plasmid selected onto THULL media plates. The use of the THULL media plates allows for the selection of all components that activate the transcription of the HIS3 reporter, the tryptophan plasmid bearing the Ilv5p, the genomic library on a leucine plasmid, and the *HIS3* gene. Over 10,000 transformants were screened for interactors with the Ilv5p using the THULL media plates.

Number of positive interactions

After 4 days of incubation at 30°C, 16 colonies grew on a total of nine THULL media plates. On each plate, 3-5 colonies were observed after the four days of incubation. Specificity of the interactions was assessed with the ability of the cells to complement a histidine auxotroph. The two-hybrid reporter gene *HIS3* is activated with the specific interaction between the Ilv5p and a protein from the genomic library.

In order to identify the genes of interest the pGAL4$_{DB}$-Ilv5p bait plasmid was removed from the strain by curing on synthetic media lacking leucine and subsequent replica-plating onto synthetic media lacking tryptophan. After curing of the pGAL4$_{DB}$-Ilv5p bait plasmid only those colonies (~70) that possess plasmids that express proteins that interact with the Ilv5p would grow on the media lacking leucine, but would not be able to grow on the media lacking tryptophan. The ability to grow on media lacking leucine is due to the presence of the *LEU2* gene on the genomic library plasmid. The inability to grow on the media lacking tryptophan is caused by successive rounds of replication which loses the tryptophan plasmid over time. Subsequent plating onto media lacking tryptophan selects for those cells that have lost the tryptophan plasmid bearing the Ilv5p.

Identity of genes

After removing the bait plasmid, library plasmids were recovered using standard procedures in Materials and Methods. Genes were sent for DNA sequencing. Sequences were matched against yeast genome sequences to identify possible

interactors. Four genes were analyzed. The identities of the four genes were *ILV5*, *VMA8*, *YOL057W,* and *GYP7*. Genes were digested with *EcoR1* or *Xho1* restriction enzymes and run on a 1% agarose gel to confirm expected restriction size fragments.

The Ilv5p is a dual function protein present in the nucleus and mitochondria of yeast. The nuclear function is involved in branched chain amino acid synthesis of isoleucine, leucine, and valine conferring chemical properties to essential proteins in the cell. This study focuses on the mitochondrial function of Ilv5p in maintaining mitochondrial DNA stability. Under starvation conditions yeast undergo the general amino acid control pathway that allows for the synthesis of essential amino acids. This pathway also controls the expression of the transcriptional activator, Gcn4p, of the *ILV5* gene. When cells are subjected to starvation conditions the *GCN4* gene is de-repressed which allows for the expression of the *ILV5* gene. The *ILV5* gene encodes the acetohydroxy acid reductoisomerase enzyme, a matrix enzyme of the mitochondria, which allows for the synthesis of isoleucine, leucine, and valine and other branched chain amino acids. The expression of the *ILV5* gene also increases the packaging of mitochondrial DNA into nucleoids allowing for more rapid transmission of the mitochondrial DNA into daughter cells. MacAlphine *et al.* show that the number of mitochondrial DNA nucleoids increased in response to activation of the general amino acid pathway. In their study, they show that *ilv5* mutants had a lower number of nucleoids, but essentially the same amount of mitochondrial DNA content. This mutation also leads to an increase of ρ^- petites or non-functional mitochondria (MacAlphine *et al.* 2000, Bateman *et al.* 2002, Bateman *et al.* 2002).

The Vma8p is the D subunit in the V_1 complex of the vacuolar-ATPase in yeast. Vacuolar-ATPases are proton pumps that couple the hydrolysis of cytoplasmic ATP to proton transport from the cytosol into organelles or cell membranes. The *VMA8* gene product is homologous to the F_1F_0-ATPases in mitochondria, cholorplasts, and bacteria. V-ATPases in higher eukaryotes play an integral role in acidification of cellular organelles that allow for cellular processes including bone resorption by osteoclasts and tumor metastasis by tumor cells. V-ATPases also play a role in apoptosis. The V_1 complex resides in the peripheral membrane of the vacuole possessing ATP-binding sites. The V_1 and V_0 complexes dissociate and reassociate readily in response to environmental conditions optimizing acidification of vacuole components or regulation of ATPase activity in other cellular components. Xu and Forgac (2000) show that the dissociation of the complex regulates the proton transfer and ATP hydrolysis activity of the vacuolar H+-ATPase in yeast. The dissociation and reassociation of the ATPase complex also affect the cell's response to limiting conditions of glucose correlating the function of mitochondria. Cells displayed sensitivity to pH growth media, slowed growth, and inability to grow on non-fermentable carbon sources when the *VMA8* gene was disrupted (Kane *et al.* 1999, Graham *et al.* 1995, Xu and Forgac. 2000, Kane. 1995). However, this study has shown that *vma8* mutants can grow on a non-fermentable carbon source such as glycerol. This is the first documented finding of growth on a non-fermentable carbon source. There is no previous correlation between the functions of Vma8p and the

mitochondrial DNA mutation rate. This study has found the Vma8p to play a direct novel role in the mitochondria.

The YOL057W putative opening reading frame is homologous to the mammalian dipeptidyl peptidase III proteolytic enzyme. Proteolytic enzymes are important in mammalian cellular processes including growth, cell cycle regulation, apoptosis, and malignancy of cancer cells. Dipeptidyl peptidase III (DPP III) is an exopeptidase, proteins not extensively studied in cell growth or cancer. These proteins have been found to be directly involved in metastasis of tumor cells and cell cycle control. DPP III has been used as a marker for malignancy in cells. DPP III is known to cleave biological peptides such as oligiopeptides, enkephalins, and angiotensins. Simaga *et al.* (1998) state that the levels of DPP III activity and content increase in malignant gynecological tissue. The results show that the activity increased due to the increase in DPP III content. In further studies by Simaga *et al.* (2003) show that the DPP III activity increased in malignant ovarian tissue tumors. The activity of DPP III in benign tissue was equal to normal tissue (Simaga *et al.* 1998, Simaga *et al.* 2003).

The *GYP7* gene encodes the GTPase-activating protein (GAP), conferring GTPase activity to the Ypt7p in yeast. Ypt7p, a member of the Rab family of proteins, is involved in protein trafficking in eukaryotic cells. Many steps in endocytosis and exocytosis involve these Rab proteins (Ypt). The protein can be in two different states, a GDP-bound present in the membrane of the cell or in a GTP-bound state present in the cytosol. This protein is only functional in its GTP-bound

state. The loss of Gyp7p does not affect the transporting function of Ypt7p. The role of Gyp7p may be to recycle the GTPases (Albert *et al.* 1999, Eitzen *et al.* 2000).

Gene homology

In order to measure the homology to human counterparts of the genes identified in the two-hybrid assay, the gene sequences were submitted into the Clustalw gene sequence homology database (http://www.ebi.ac.uk/cgi-bin/clustalw). The submitted sequences and homology results were analyzed for exact matches of amino acids and those amino acids that share comparable substitutions. In the sequence homology search for the Vma8p from yeast and the homolog from *H. sapiens*, 129 amino acids were exact matches between the two homologs and 54 amino acids were comparable substitutions. The yeast Vma8p gene is a strong homolog of the human D subunit of the vacuolar ATPase sharing 129 out of 256 amino acids. In the remaining amino acids, 54 were suitable substitutions conferring similar chemical properties to the two proteins. In the homology search of the yeast and human dipeptidyl peptidase III enzymes, the yeast enzyme shares 252 exact amino acids with the human dipeptidyl peptidase III enzyme of its 711 total amino acids. In comparing the two enzymes, 177 amino acids are comparable substitutions giving similar chemical properties to the yeast and human enzymes. In sharing 429 exact or similar amino acids with the human enzyme, the yeast dipeptidyl peptidase III enzyme is a strong homolog. The results of the homology analysis for the Vma8p are presented in Figure 2. The results of the homology analysis for the YOL057W gene product are presented in Figure 3.

Gene disruption constructs

Genes recovered from the yeast two-hybrid assay were used to create gene disruption plasmids. Restriction enzyme digestion of the *VMA8* and *YOL057W* genes from the pGAL4$_{AD}$-library plasmid were ligated into the integrating vector pRS406. The pRS406 plasmid contains the URA3 selectable marker. The pRS406-vma8 or pRS406-yol057w disruption plasmids were linearized and transformed into DFS188, EAS748, and CAB193-1. Homologous recombination gave rise to genomic disruption of *vma8* and *yol057w* in the three reporter strains (Figure 4).

Respiration Loss Assay

The respiration loss assay was performed using strain DFS188 containing a vma8Δ or a yol057wΔ. The respiration loss assay shows the effect of a gene knockout on the function of mitochondria. The 2% glycerol, 0.1% dextrose media used in the respiration loss assay assesses the formation of cells with non-functional mitochondria, or ρ^- petites. Yeast cells can utilize fermentable carbon sources in the absence of mitochondrial activity. However, mitochondrial activity is required for yeast to grow on non-fermentable carbon sources. The non-fermentable carbon source glycerol would render the cells inviable due to the lack of functional mitochondria. The limited supply of dextrose allows the ρ^- petites to form colonies until the dextrose is exhausted. The cells then stop dividing. Cells with functional mitochondria form large colonies (Figure 5).

Previous studies done by Sia *et al.* (2003) have determined that the lack of *ILV5* increases the rate of ρ^- petite formation by 11 fold as compared to the wild-type

strain. The frequency of ρ^- petite formation in wild-type cells was calculated to be 0.2%. The frequency of ρ^- petite formation in *ilv5* mutants was 2.2%. This evidence supports the reasoning that the Ilv5p has a distinct role in mitochondrial function. The occurrence of ρ^- petites in the mutant *vma8* strain was calculated to be 2.33%, an almost 12 fold increase compared to the wild-type rate. The rate of ρ^- petite formation for the strain containing the knockout of the *YOL057W* gene was 4.7%, a 23.5 fold increase in the frequency of ρ^- petite formation. As this study has found this gene plays an integral role in the mitochondria of yeast. The respiration loss assay on the *GYP7* gene product determined the rate of ρ^- petite formation to be 0.15%. This slight decrease in the rate was assayed as not being significant for extensive further tests (Table 1).

Homologous Recombination

This assay is used to determine if loss of an Ilv5p interacting factor affects homologous recombination rates of mitochondrial DNA. Intragenic recombination has previously been associated with spontaneous loss of mitochondrial function. Mitochondrial DNA is spontaneously deleted between regions of homology leading to a ρ^- petite phenotype. The construct used in the assay uses the *COX2* gene, a mitochondrial gene necessary for respiration, fused to the *ARG8* gene integrated in the mitochondrial genome. The *ARG8* gene is a nuclear gene coding for the production of the amino acid arginine. The *ARG8* gene is flanked by identical 100bp sequences of *COX2*. The presence of the *ARG8* gene blocks the transcription of the *COX2* gene. Those cells possessing this construct are phenotypically Arg+, but lack

the ability to respire on a non-fermentable carbon source such as glycerol due to the lack of the *COX2* gene transcript. *COX2* is required for mitochondrial function. When a homologous recombination event occurs the *ARG8* gene is excised and a functional *COX2* gene is transcribed. Those cells that undergo this recombination event are phenotypically Arg-, but gain the ability to respire on glycerol. The assay uses media lacking arginine to quantify the amount of cells that contain the *ARG8* gene before recombination and cells are plated onto glycerol media to assay the occurrence of a recombination event (Figure 6).

The rate of recombinations per generation for the wild-type strain of EAS748 was calculated to be 9.8×10^{-5}. The rate of recombination in the *vma8* mutant was 9.7×10^{-5} recombinations per generation. The rate in the *vma8* mutant was slightly lower compared to the wild-type. Statistically the decrease in the rate is not significant. The *yol057w* mutant strain generated 8.8×10^{-5} recombinations per generation which is not statistically significant from the wild-type. The *yol057w* mutant like the *vma8* mutant did not affect the rate of recombination events significantly to be considered a strong factor in homologous recombination (Table 2).

Microsatellite Stability

This assay was performed to determine the effects of loss of the Ilv5p interacting factors on microsatellite stability. The assay uses the presence of microsatellites, repetitive segments of DNA, to test DNA polymerase slippage events that lead to the addition or loss of bases in the microsatellites. These polymerase slippage events can lead to spontaneous loss of gene function. If DNA slippage events

occur in the replication of mitochondrial DNA, spontaneous loss of mitochondrial function can arise. The microsatellite stability assay uses a reporter of a translational fusion between the mitochondrial *COX3* gene and the nuclear *ARG8* gene. The microsatellite sequence possesses 16 repeats of poly(GT) fused to the 5' end of the reporter. The ARG8 gene is at the 3' end of the microsatellite sequence. The reporter construct assays cells that undergo mutational events to bring the coding sequence of the *ARG8* gene back in frame to allow its transcription. The construct is made so the *ARG8* gene is out of frame and read at the +2 site. The cells that are out of frame are phenotypically Arg-. When a mutational event occurs base pairs of the microsatellite sequence are added or lost due to polymerase slippage. These occurrences bring the *ARG8* gene coding sequence back in frame. The cells that possess these mutations are phenotypically Arg+. This process allows for simple assessment of cells that undergo mutational events that bring the coding sequence back in frame by plating on media lacking arginine (Figure 7).

The mutation rate of the wild-type strain of CAB193-1 in the microsatellite reporter assay was determined to be 3.4×10^{-7} slippage events per generation. The vma8 strain mutation rate was found to be 2.7×10^{-6} slippage events per generation, a 36 fold increase in the mutation rate compared to the wild-type strain. The Vma8p was found to have an integral role in mitochondrial DNA microsatellite stability. The mutation rate of the mutant *yol057w* strain was 4.5×10^{-7} slippage events per generation, a 1.3 fold increase in the mutation rate compared the wild-type strain.

However, the loss of the *YOL057W* gene product does not affect microsatellite stability in mitochondrial DNA (Table 3).

Discussion

This study investigates the loss of specific proteins, direct interactors with the Ilv5p, on mitochondrial function. The respiration loss assay determines if loss of the proteins alter the rate of mitochondrial DNA ρ^- petite formation, cells where mitochondrial DNA is mutated preventing oxidative phosphorylation. The loss of respiration causes inhibited growth and subsequent cessation of cellular processes. The use of a non-fermentable carbon source such as glycerol with a limited amount of dextrose allow for the differentiation of functional and non-functional mitochondria. Molecular mechanisms affecting the rate of ρ^- petite formation are analyzed through homologous recombination and microsatellite stability reporters. These assays determine how the losses of the proteins lead to the increase or decrease of ρ^- petite formation. The homologous recombination reporter tests the ability of segments of mitochondrial DNA to be excised through a mutational event causing dysfunction of the mitochondria. The microsatellite reporter assesses the occurrence of DNA polymerase slippage events leading to the addition or deletion of DNA.

In the Clustalw homology search for the two proteins tested in the study both yeast proteins were determined to be strong homologs to the human counterparts. The Vma8p protein shared 129 amino acids with the human subunit D of the vacuolar ATPase. This is approximately half the amino acids that comprise the protein. Also 54 amino acids were similar in chemical properties to the human protein. In sharing 183 exact or similar amino acids the yeast Vma8p is a strong homolog to the human protein making up the D subunit of the vacuolar ATPase. In the homology search for

the yeast *YOL057W* gene product, 252 amino acids are exact matches to those in the human dipeptidyl peptidase III enzyme. The two proteins also share 177 similar amino acids in chemical composition. In having 429 amino acids either exact matches or similar in chemical properties, the yeast *YOL057W* gene product is a strong homolog to the human dipeptidyl peptidase III enzyme.

In having strong homology with the human counterparts, the yeast Vma8p and the yeast *YOL057W* gene product are good proteins for studying molecular mechanisms that these proteins are involved in and their role in the cell. The Vma8p can be used to study energy processes in the mitochondria and elucidate its function in maintaining the mitochondrial DNA mutation rate. The *YOL057W* gene product can be studied for its function in the mitochondria involving cell cycle control or possible apoptosis signals and the direct connection to the mutation rate of mitochondria.

Sia et al. (2003) have shown that the loss of the Ilv5p dramatically increases the rate of ρ^- formations by approximately 11%. When the Ilv5p is absent, alteration of mitochondrial nucleoids may affect mitochondrial DNA copy number regulation and increase intramolecular recombination generating ρ^- petites. The increase in the formation of ρ^- petites leads to the higher mutation rate of 2.2%. The ρ^- petites arise by recombination between repetitive intergenic regions leading to genomic sequences being deleted. The loss of DNA causes replication errors leading to the formation of point mutations. Ultimately, these point mutation increase the formation of ρ^- petites.

The presence of the Ilv5p may supply a sufficient quantity of DNA for replication or segregation into daughter cells. They postulated when the Ilv5p is present mitochondrial DNA copy number increases providing enough DNA to override mutations in the DNA allowing for the growth of healthy cells. The increase in mitochondrial DNA copy number can affect mutation rates by either increasing the amount of mitochondrial DNA available for replication or segregation into daughter cells or making it harder for cells to reach the ratio of mutant to wild-type mitochondrial genomes required for expression of the mutant phenotype. The increase in the mitochondrial DNA copy number may lead to the decrease in point mutations that cause ρ^- petites.

The Ilv5p may regulate mitochondrial DNA copy number in cells grown in rich nutrient conditions. When replication conditions are tightly regulated cells are given a selective growth advantage. These cells can generate more offspring in the nutrient rich conditions giving the cells a genotypic advantage in the population. The occurrence of less point mutations in the mitochondrial DNA allows for more fit offspring. When cells are grown in an amino acid limiting media, cells with a strong genotype are given a selective advantage. Increasing the copy number of mitochondrial DNA in amino acid limiting growth conditions help daughter cells receive a greater pool of non-mutated mitochondrial genomes.

In response to amino acid limiting conditions, the cell activates the general amino acid control pathway. This pathway de-represses the Gcn4p transcriptional regulator controlling the expression of the *ILV5* gene. The *ILV5* gene is transcribed

and the Ilv5p can respond to the growth conditions leading to an increase in mitochondrial DNA copy number and nucleoid number. The increase in mitochondrial DNA copy number allows for the cell to survive in less than optimal conditions.

The rate of ρ^- formations increased 10 fold in the *vma8* mutants compared to the DFS188 wild-type cells. The yeast Vma8p is homologous to F_1F_0-ATPases in mitochondria sharing identity to bovine and C.elegans V-ATPases. Vacuolar-ATPases are proton pumps that couple the hydrolysis of cytoplasmic ATP to proton transport from the cytosol into organelles or cell membranes. The Vma8p is the D subunit that comprises the V_1 complex. The V-ATPase that is responsible for ATP synthesis possesses two complexes, the V_1 and the V_0 complex. The V_1 and V_0 complexes dissociate and reassociate readily in response to environmental conditions optimizing acidification of vacuole components or regulation of ATPase activity in other cellular components. Xu and Forgac (2000) show that the dissociation of the complex regulates the proton transfer and ATP hydrolysis activity of the vacuolar H+-ATPase in yeast. The dissociation and reassociation of the ATPase complex also affect the cell's response to limiting conditions of glucose correlating the function of mitochondria. When the *VMA8* gene was disrupted cells displayed sensitivity to pH growth media, slowed growth, and inability to grow on non-fermentable carbon sources (Kane *et al.* 1999, Graham *et al.* 1995, Xu and Forgac. 2000, Kane. 1995). However, this study has shown that *vma8* mutants can grow on a non-fermentable carbon source such as glycerol. Possibly, another protein present in the mitochondria

has a similar function of the Vma8p allowing for sufficient ATP synthesis on glycerol media. The cells are able to compensate for the loss of the Vma8p and grow on the glycerol media. The Vma8p may not be necessary for the V-ATPase assembly. This finding also shows that the D subunit is not the catalytic component of the V-ATPase. In the absence of the Vma8p, cells are still able to synthesize ATP and survive on the glycerol media. There is no previous correlation between the functions of the Vma8p and the mitochondria. This study has found the Vma8p to play a direct novel role in the mitochondria.

The respiration loss assay showed the loss of the *YOL057W* gene product caused a significant increase in the rate of ρ^- petite formations. It is the first to show that this putative open reading frame (ORF) encodes a protein whose loss of function leads to a mitochondrial defect. This study determined the rate of respiration loss with the *yol057w* mutant increased by 23.5 times compared to the wild-type strain of DFS188 generating 4.7% ρ^- formations per cell division. This protein being homologous to dipeptidyl peptidase III may be involved in cell cycle regulation or the induction of apoptosis. However, this protein is not a direct homolog as the presence of the protein in humans increased malignancy in cells whereas the loss of the protein increased the formation of mutant ρ^- petites in yeast. The loss of this protein in some manner affects the ability of the cells to properly replicate and propagate mitochondrial DNA generating the ρ^- phenotype and inhibiting survival on a non-fermentable carbon source. In this finding the *YOL057W* gene product may be involved in correcting or signaling errors in the DNA. If the DNA is beyond repair

the *YOL057W* gene product may induce the process of apoptosis to prevent mutations from propagating in the progeny. Possibly, the *YOL057W* gene product activates one of the components in apoptosis to allow for cell death.

The fluctuation analysis conducted on the *gyp7* mutant provided a direct relationship between the ability of yeast to respire in the absence of the Gyp7p. The *gyp7* mutant rate of 0.15% ρ^- formations per cell division found in this study is not significant compared to the control rate of 0.2% ρ^- formations per cell division. Previous studies have shown the absence of *GYP7* does not affect the vacuole morphology in yeast cells or the GTPase function. These studies also state the intrinsic GTPase activity of the cell may be sufficient for cellular transport processes. This does not affect growth of the cells or the ability of cells to undergo transport mechanisms (Albert *et al.* 1999, Eitzen *et al.* 2000). The interaction with Ilv5p is the first known connection between Gyp7p and a role in the mitochondria. In correlation with previous findings, this study showed that the lack of the Gyp7p does not sufficiently alter the yeast cell processes specifically those in the mitochondria to render them mutated or inviable. It is possible that the Gyp7p transports the nuclear Ilv5p to the mitochondria for its distinct role in mitochondrial DNA copy number regulation and segregation into daughter cells.

The homologous recombination assay determines if the loss of an Ilv5p interacting factor affects homologous recombination rates of mitochondrial DNA. Homologous recombination between regions of homology in mitochondrial DNA has previously been associated with spontaneous loss of mitochondrial function. The

deletion of mitochondrial DNA by homologous recombination leads to a ρ^- petite phenotype. The rate of recombination in the *vma8* mutant was 9.7×10^{-5} recombinations per generation, a slight decrease in the rate compared to the wild-type EAS748 strain that generated 9.8×10^{-5} recombinations per generation. Statistically the decrease in the rate is not significant. Likewise, the *yol057w* mutant strain generated 8.8×10^{-5} recombinations per generation that is not statistically significant from the wild-type. The *yol057w* mutant and the *vma8* mutant did not affect the rate of recombination events significantly to be considered strong factors in homologous recombination. This particular process in the mitochondria is not well known. This study focused on identifying components of this process. Components in this process may be elucidated in testing Ilv5p interacting factors in the homologous recombination assay.

In the microsatellite instability assay, after a mutation event occurs the reading frame of the *ARG8* gene is restored and allows for the transcription and translation of the gene. In the *vma8* mutant strain the mutation rate increased by 50 times compared to the wild-type. This finding is possibly due to maintaining the fidelity of the DNA polymerase that replicates the mitochondrial DNA in the cell. The DNA polymerase transiently associates with the mitochondrial DNA strand. The lack of the Vma8p may increase the dissociation of the polymerase with the DNA. This leads to an increase in incorporating additions or deletions of DNA.

With the loss of ATP hydrolysis and proton transport the cell must try to conserve energy and use its available energy in ways to ensure its survival. The cell

might conserve most of its available energy for proofreading the newly replicated mitochondrial DNA and propagate mitochondrial DNA that possesses fewer mutations. If many mutational events occur that require the cell to expend more energy to sustain its viability the cell will ultimately use all its energy and become inviable. This reasoning extends on the fact that having ample energy allows for greater replication fidelity and proper mitochondrial DNA content.

In the presence of the Vma8p, the errors incorporated into the mitochondrial DNA by polymerase slippage events are corrected. The exonuclease domain of the delta and epsilon polymerases or the mismatch repair mechanisms ensures that the mitochondrial DNA is void of replication errors. The lack of available energy prevents these processes from occurring leading to more mutations in the mitochondrial DNA.

The *yol057w* mutant strain did not generate a significant increase compared to the CAB193-1 wild-type strain. The loss of the protein from the putative open reading frame in this assay does not account for the significant increase in ρ^- petite formations in the respiration loss assay. Distinctively the loss of the protein from the putative open reading frame *YOL057W* does not affect microsatellite stability in mitochondria. With the mutation rate increasing in the absence of the protein then the protein must be a positive regulator of the DNA replication mechanism in the mitochondria. The protein might increase the fidelity of the DNA polymerase allowing for fewer mutations in cells. It might also be a protein involved in cell cycle control inducing apoptosis if numerous mutations are present.

Materials and Methods

Yeast library transformation

Yeast cells containing pBTM-ILV5, plasmid expressing the GAL4-activation domain fused to the Ilv5p, were grown overnight in 3ml of SD-trp liquid media at 30°C. The next day the cells were grown for three hours to mid-log phase (OD_{600}=0.3) in YPD at 30°C. Cells were aliquoted into two 50ml conical tubes and spun for two minutes in a centrifuge at 3000 revolutions/minute. The cells were further collected by spinning in a microcentrifuge and then resuspended in 100µl of 0.1M LiOAc,TE. 15µl of carrier DNA (10mg/ml salmon sperm DNA) and 2µl of yeast genomic library DNA (Liskay DNA) was added to the cells and incubated at 30°C on a shaker for thirty minutes. After thirty minutes, 1ml of 40% PEG, 0.1M LiOAc, TE was added to the tube and mixed by pipetting. The cells were incubated at 30°C on a shaker for thirty minutes. After incubation the cells were heat shocked at 42°C for fifteen minutes. Cells were spun in a microcentrifuge for 10 seconds at 14,000 revolutions per minute and resuspended in 1ml of sterile dH_20. Cells were plated onto nine THULL media plates in 200ul aliquots and placed at 30°C for 4-11 days. In order to determine how many yeast library transformants were used in the two-hybrid screen, 200µl of cells were plated on a SD-leu plate and placed at 30°C for 4 days. The numbers of transformants were counted on the plate after the four days of incubation.

Extraction of yeast plasmid

To isolate genomic library plasmids, the yeast colonies from the high efficiency transformation containing both the pBTM-ILV5 and genomic library plasmids were streaked on SD-leu plates to obtain single colonies. Growth on the presence of tryptophan containing plates will allow for the loss of the pBTM-ILV5 plasmid. These colonies were subsequently replica-plated onto SD-trp plates to see which had lost the pBTM-ILV5 plasmid. Samples that grew on SD-leu, but not on SD-trp were used to inoculate glucose-leu liquid. The cells were grown overnight at 30°C while shaking. The overnight cultures were spun at 14,000 revolutions per minute, transferred to clean eppendorf tubes, and resuspended in 200µl Southern lysis solution (2% triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1mM Na_2EDTA) in addition to 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads. The cells were lysed by vortexing the solution for two minutes and placed on ice. The resulting solutions were centrifuged for five minutes and the aqueous layer solution was transferred to clean eppendorf tubes. The DNA in the solutions was then cleansed by adding 500µl of 100% ethanol and 20µl of 3M NaAc and placed at -20°C for 48 hours. After cleansing the DNA was spun for 6 minutes at 14,000 revolutions per minute. The DNA was washed with 200µl of 70% ethanol and briefly dried under a vacuum. The pellet of DNA was resuspended in 50µl of sterile dH_20 and placed at -20°C for storage.

Transformation of yeast plasmid into *E.coli*

In order to amplify yeast genomic library plasmids, they were transformed into electrocompetent *E.coli* cells. 3µl of each plasmid was placed into 40µl aliquots of electrocompetent *E.coli* cells and transferred to an electroporation cuvette. The cells were electroporated at 1.5kV for five milliseconds. The cells were treated with 1ml of Lurania-broth (LB) and transferred to an eppendorf tube. The tubes were placed at 30°C for ten minutes and spun for 15 seconds to pellet the *E.coli* cells. The cells were resuspended in the remaining liquid and plated onto LB-ampicillin agar plates. After 24 hours the plates were observed for any *E.coli* colonies present.

Extraction of yeast genomic library plasmids from *E.coli*

E.coli colonies that grew on the LB-amp agar plates for each recovered genomic library plasmid were used to inoculate LB-ampicillin liquid media and placed overnight at 30°C while shaking. The next day 1.5ml of overnight culture was transferred to corresponding eppendorf tubes, spun for 15 seconds in a microcentrifuge and resuspended in 100µl of GTE buffer (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA). Then 200µl of NaOH/SDS lysis solution (0.2M NaOH, 1% SDS) was added to each tube and the solution was mixed and allowed to incubate at room temperature for five minutes. 150µl of KOAc lysis solution (60 ml 5M KOAc, 11.5ml glacial acetic acid, 28.5ml dH_2O) was added to each tube and the solution was mixed and incubated at room temperature for five minutes. Then 400µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube and the solution was mixed and then spun in a microcentrifuge for five minutes. The

supernatant present in the tubes (800µl) was transferred to new tubes, treated with 1ml of 100% ethanol, and then placed at -20°C for fifteen minutes. The plasmid DNA was spun for five minutes in a microcentrifuge, washed with 70% ethanol, spun again, and dried briefly under a vacuum. The plasmids were resuspended in 50µl 1X RNAse/TE.

Identification of genes, homology

Samples of the genomic library plasmids extracted from *E.coli* were sent to a sequencing facility for sequence determination. The sequence outputs were sent by email to recipients. Further analysis using the NCBI website BLAST software identified the submitted gene sequences by comparing to already identified sequences. The homology of the genes with human counterparts was conducted using the Clustalw database software (http://www.ebi.ac.uk/cgi-bin/clustalw). The gene sequences of the yeast Vma8p and human subunit D protein of the vacuolar ATPase along with the yeast and human dipeptidyl peptidase III enzymes were submitted into the Clustalw software for analysis.

Restriction enzyme digestion of yeast genomic library plasmid DNA

5µl of corresponding *E.coli* plasmids were digested with an *EcoR1* or *Xho1* restriction enzyme solution containing the following components: 1µl of *EcoR1* or *Xho1*, 2µl of buffer H (*EcoR1*) or buffer D (*Xho1*), 0.2µl BSA (bovine serum albumin), and 11.8µl of sterile dH_2O. Each plasmid was incubated for 2 hours at 37°C. Each of the plasmids were run on a 1% agarose gel using 1X TAE as the buffer.

Gene disruptions

YOL057W and VMA8 gene fragments were isolated from the corresponding yeast genomic library plasmids using appropriate restriction enzymes indicated from the gene sequence. These fragments were ligated into pRS406 to yield plasmids pRS406-YOL057W and pRS406-VMA8. The plasmids were linearized within the gene sequences of YOL057W and VMA8, transformed into strains DFS188, EAS748, and CAB193-1 and selected on SD-ura plates. Disruption strains were verified by PCR analysis or phenotype.

Transformation into reporter constructs

Each of the reporter strains DFS188, EAS748, and CAB193-1 were transformed with the corresponding gene disruptions using the yeast transformation protocol. Gene disruptions were verified using PCR and expected phenotypes.

The DFS188 strain shows the effect of a gene knockout on the function of mitochondria. The 2% glycerol, 0.1% dextrose media used in the respiration loss assay assesses the formation of cells with non-functional mitochondria, or ρ^- petites. Yeast require mitochondria to grow on non-fermentable carbon sources such as glycerol. The limited source of dextrose allows cells with functional mitochondria to form large colonies. Those cells with non-functional mitochondria produce small colonies.

The homologous recombination reporter in the EAS748 strain uses the *COX2* gene, a mitochondrial gene necessary for respiration, fused to the *ARG8* nuclear gene integrated in the mitochondrial genome. The microsatellite stability reporter in the

CAB 193-1 strain uses the presence of microsatellites, repetitive segments of DNA, to test DNA polymerase slippage events that lead to the addition or loss of bases in the microsatellites. These polymerase slippage events can lead to spontaneous loss of gene and mitochondrial functions.

Respiration Loss Assay

DFS188 wild type and cells that possess the gene knockouts were selected on synthetic media lacking uracil for single colonies for 3 days at 30°C. Individual colonies were resuspended in 100µl of sterile dH_2O. Two serial dilutions of 1:100 were done using the original 100µl of cells. 100µl of the 1×10^{-4} dilution was plated onto a 2% glycerol media containing 0.1% dextrose. Cells were incubated for 3 days at 30°C. Cells that did not respire formed small colonies and those that respired formed large colonies.

Homologous Recombination

EAS748 wild type and cells that possess the gene knockouts were selected on synthetic media lacking uracil for single colonies for 3 days at 30°C. Individual colonies were resuspended into 100µl of sterile dH_2O. Two serial dilutions of 1:100 were done using the original 100µl of cells. Cells were plated onto synthetic media lacking arginine (50µl 1×10^{-4} dilution) and glycerol rich media (200µl 1×10^{-2} dilution). Cells were incubated at 30°C for 3-4 days for mutation rate analysis. Cells were assayed for the ability to undergo homologous recombination to excise the *ARG8* gene from the reporter construct strain.

Microsatellite stability

CAB 193-1 wild type and cells that possess the gene knockouts were selected on synthetic media lacking uracil for single colonies for 3 days at 30°C. Individual colonies were picked and resuspended in 100µl of sterile dH_2O for two serial dilutions of 1:100. Cells were plated onto synthetic complete media (50µl of 1×10^{-4} dilution) and medium lacking arginine (95µl of reuspended cells) for mutation rate analysis. Cells were incubated for 3-7 days at 30°C. The formation of ARG+ cells on media lacking arginine conferred a mutation event affecting DNA polymerase slippage on microsatellites.

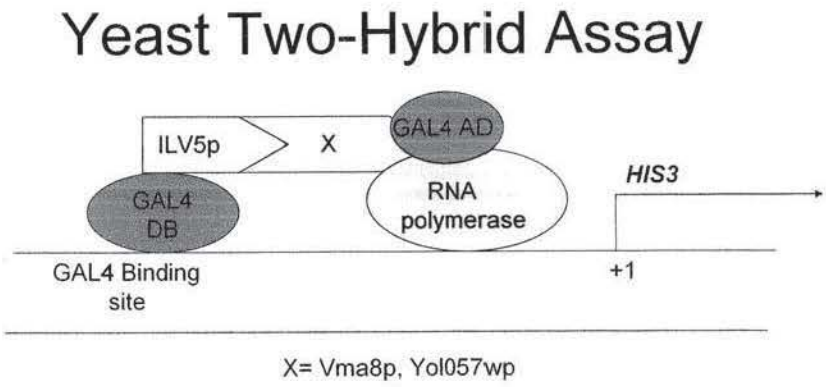

Figure 1. Yeast Two-Hybrid Assay. A pGAL4$_{DB}$-Ilv5p fusion was used as the bait protein in the assay. A genomic library (X) was fused to the GAL4$_{AD}$. The positive interaction between the Ilv5p and an interacting protein from the genomic library conjoin allowing for transcription of the reporter gene. The reporter for this assay is the *HIS3* gene encoding the production of the amino acid histidine.

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CLUSTAL W (1.82) multiple sequence alignment

sp|P32610|VATD_YEAST   MSG-NREQVFPTRMTLGLMKTKLKGANQGYSLLKRKSEALTKRFRDITKRIDDAKQKMGR 59
sp|VATD HUMAN          MSGKDRIEIFPSRMAQTIMKARLKGAQTGRNLLKKKSDALTLRFRQILKKIIETKMLMGE 60
                       *** :* ::**:**:  :**::****: * .***:**:*** ***:* *:* ::*  **.

sp|P32610|VATD_YEAST   VMQTAAFSLAEVSYATGENIGYQVQESVSTARFKVRARQENVSGVYLSQFESYIDPEIND 119
sp|VATD HUMAN          VMREAAFSLAEAKFTAGD-FSTTVIQNVNKAQVKIRAKKDNVAGVTLPVFEHYHEG-TDS 118
                       **: *******..::::*: :.  * :.*..*:.*:**:::**:** *. ** * :   :.

sp|P32610|VATD_YEAST   FRLTGLGRGGQQVQRAKEIYSRAVETLVELASLQTAFIILDEVIKVTNRRVNAIEHVIIP 179
sp|VATD HUMAN          YELTGLARGGEQLAKLKRNYAKAVELLVELASLQTSFVTLDEAIKITNRRVNAIEHVIIP 178
                       :.****.***:*: : *. *::*** *********:*: ***.**:***************

sp|P32610|VATD_YEAST   RTENTIAYINSELDELDREEFYRLKKVQEKKQNETAKLDAEMKLKRDRAEQDASEVAADE 239
sp|VATD HUMAN          RIERTLAYIITELDEREREEFYRLKKIQEKK--ILKEKSEKDLEQRRAAGRCWSLLI-- 234
                       * *.*:*** :**** :*********:****:    * .:* .*:: **   . .:

sp|P32610|VATD_YEAST   EPQGETLVADQEDDVIF- 256
sp|VATD HUMAN          -----FLAEEKDEDLLFE 247
                             *. ::::*::*
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"*" denotes amino acids are identical in sequence alignment.

":" denotes conserved substitutions used in sequence alignment.

"." denotes semi-conserved substitutions used in sequence alignment.

Figure 2: Vma8p homology sequence alignment. The amino acid sequence of the yeast Vma8p is denoted by the VATD YEAST sequence. The amino acid sequence of the human D subunit of the vacuolar ATPase is denoted by the VATD HUMAN sequence. Sequence alignment was conducted using the Clustalw software database (http://www.ebi.ac.uk/cgi-bin/clustalw).

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CLUSTAL W (1.82) multiple sequence alignment

sp|Q08225|DPP3_YEAST      --MSHFFADHDAPLSMLSVKTEYFPQLTDKEQKYAHFMSKASHAGSRVVMRQVSHESEPI 58
sp|DPP3_HUMAN             MADTQYILPNDIGVSSLDCR-EAFRLLSPTERLYAYHLSRAAWYGGLAVLLQTSPEAPYI 59
                             ::::  :*  :* *. : * *  *: .*: **:.:*:*:  *. .*: *.* *:  *

sp|Q08225|DPP3_YEAST      FDLILAIHSKLN----GKYPEDDITQKQQTGLYLEYVSQFLSNLCNFKSFGDTKFIPRCE 114
sp|DPP3_HUMAN             YALLSRLFRAQDPDQLRQHALAEGLTEEEYQAFLVYAAGVYSNMCNYKSFGDTKFVP--- 116
                          : *:  :.   :     ::.  :   :::   :* *.: . **:**:*******:*

sp|Q08225|DPP3_YEAST      VKFFKQLLELAKINPCSSPLTLSPVDVNHEFTSHHLFSTINELIDIGIYHVEEKAALLGF 174
sp|DPP3_HUMAN             -NLPKEKLERVILG--------SEAAQQHPEEVRGLWHTCGELMFS----LEPRLRHLGL 163
                           :: *: ** . :.        * .  :*    : *: * .**:      :* :   **:

sp|Q08225|DPP3_YEAST      PSQGYTSAYYLGLPVTPEDMALLKEQLFAELAILPEN---TRINEVGENSFQIWVASEN- 230
sp|DPP3_HUMAN             GKEGITTYFSG--NCTMEDAKLAQDFLDSQNLSAYNTRLFKEVDGEGKPYYEVRLASVLG 221
                           .:* *: :      * **  * :: * ::     :.   ..::  *:  ::: :**

sp|Q08225|DPP3_YEAST      VKNQITETYPSGQITLSNAVTKVEFIFGDHSREMRLVASYLKEAQKFAANDTQKAMLQEY 290
sp|DPP3_HUMAN             SEPSLDSEVTSKLKSYEFRGSPFQVTRGDYAPILQKVVEQLEKAKAYAANSHQGQMLAQY 281
                           : .: .  .*   : .   : .:.  **::  :: *.. *::*: :***. *  ** :*

sp|Q08225|DPP3_YEAST      INHFVTGSSQAHKEAQKLWVKDISPVIETNIGFIETYREPSGIIGEFESLVAIQNKERTA 350
sp|DPP3_HUMAN             IESFTQGSIEAHKRGSRFWIQDKGPIVESYIGFIESYRDPPGSRGEFEGFVAVVNKAMSA 341
                          *: *. ** :***...::*::* .*::*: *****:**:* *  ****.:**: **  :*

sp|Q08225|DPP3_YEAST      KFSSLVNNAEEFISLLPWSKDYEKPIFNPPDFTSLEVLTFTGSGIPAGINIPNYDDVRLK 410
sp|DPP3_HUMAN             KFERLVASAEQLLKELPWPPTFEKDKFLTPDFTSLDVLTFAGSGIPAGINIPNYDDLRQT 401
                          **. ** .**:::. ***.  :**  * .******:****:**************:* .

sp|Q08225|DPP3_YEAST      IGFKNVSLGNILSAAAKSSSKHPPSFISQEDRPIFEKYQSDSFEVQVGIHELLGHGSGKL 470
sp|DPP3_HUMAN             EGFKNVSLGNVLAVAYATQ-REKLTFLEEDDKDLYILWKGPSFDVQVGLHELLGHGSGKL 460
                           *********:*:.*  :. :.  :*:.::*: ::  ::. **:****:**********

sp|Q08225|DPP3_YEAST      LTEFTDG-FNFDKENPPLGLDGKPVSTYYKVGETWGSKFGQLAGPFEECRAEVIAMFLLT 529
sp|DPP3_HUMAN             FVQDEKGAFNFDQETVINPETGEQIQSWYRSGETWDSKFSTIASSYEECRAESVGLYLCL 520
                          :.:  .* ****:*.      *: :.::*: ****.***. :*..:****** :.::*

sp|Q08225|DPP3_YEAST      NKKILDIFGFHDVESQDKVIYAGYLQMARAGLLALEYWNPKTGKWGQPHMQARFSIMKTF 589
sp|DPP3_HUMAN             HPQVLEIFGFEGADARD-VIYVNWLNMVRAGLLALEFYTPEAFNWRQAHMQARFVILRVL 579
                          : ::*:****...:::* ***..:*:*.********::.*:: :* *.****** *::.:

sp|Q08225|DPP3_YEAST      MKHSTDKNFLKLEMNSTN-DDFAIKLDKSLIKTAGHECVKDYLKILHVYKCSGDVEQGSK 648
sp|DPP3_HUMAN             LEAGEGLVTITPTTGSDGRPDARVRLDRSKIRSVGKPALERFLRLQVLKSTGDVAGGRA 639
                          :: . .   :.   .* .  *  ::**:* *::.*: .:: :*::*:* *.:***  *

sp|Q08225|DPP3_YEAST      YFIDRSTVTPD----LASLRDIVLSKRLPRRQFIQSNSYIDDNNKVTLKEYDETPQGMLQ 704
sp|DPP3_HUMAN             LYEGYATVTDAPPECFLTLRDTVLLRKESRKLIVQPNTHLEGSD-VQLLEYEASAAGLIR 698
                           : . :***      : :*** ** :: .*: ::*.*::::..: * * **: :. *:::

sp|Q08225|DPP3_YEAST      SFLDREL---------------------------------- 711
sp|DPP3_HUMAN             SFSERFPEDGPELEEILTQLATADARFWKGPSEAPSGQA 737
                          ** :*
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"*" denotes amino acids are identical in sequence alignment.

":" denotes conserved substitutions used in sequence alignment.

"." denotes semi-conserved substitutions used in sequence alignment.

Figure 3: Dipeptidyl peptidase III homology sequence alignment. The amino acid sequence of the yeast dipeptidyl peptidase III is denoted by the DPP3_YEAST sequence. The amino acid sequence of the human dipeptidyl peptidase III is denoted by the DPP3_HUMAN sequence. Sequence alignment was conducted using the Clustalw software database (http://www.ebi.ac.uk/cgi-bin/clustalw).

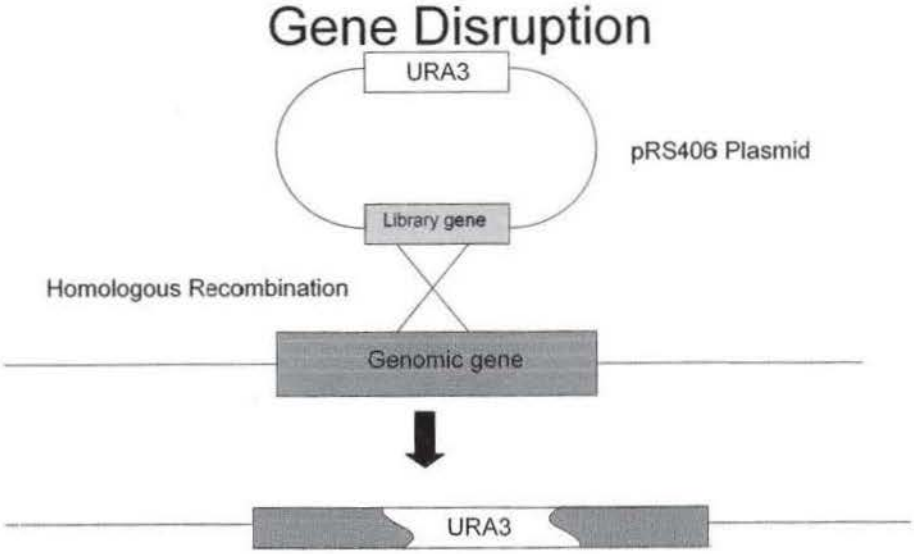

Figure 4. Gene Disruption. Restriction enzyme digestion of the VMA8 and YOL057W genes from the pGAL4$_{AD}$-library plasmid were ligated into the integrating vector pRS406. The pRS406 plasmid contains the URA3 selectable marker. The pRS406-vma8 or pRS406-yol057w disruption plasmids were linearized and transformed into DFS188, EAS748, and CAB193-1. Homologous recombination gives rise to genomic disruption of vma8 and yol057w in the three reporter strains.

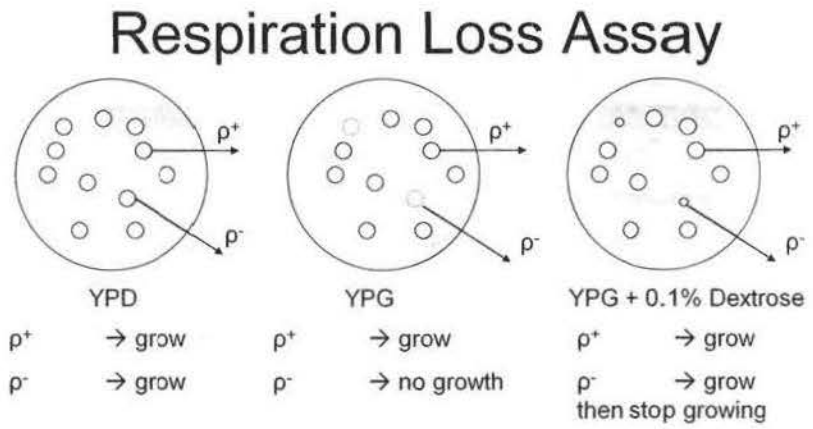

Figure 5. Respiration Loss Assay. The assay tests the spontaneous loss of mitochondrial function. The selection on a non-fermentable carbon source can distinguish cells with functional (ρ^+) or non-functional (ρ^-) mitochondria.

Table 1 Respiration Loss Assay	
Strain	**Frequency Percentage**
DFS188	**0.2% respiration loss frequency**
DFS188ilv5Δ::ura3	**2.2% respiration loss frequency**
DFS188vma8Δ::ura3	**2.3% respiration loss frequency**
DFS188yol057wΔ::ura3	**4.7% respiration loss frequency**
DFS188gyp7Δ::ura3	**0.15% respiration loss frequency**

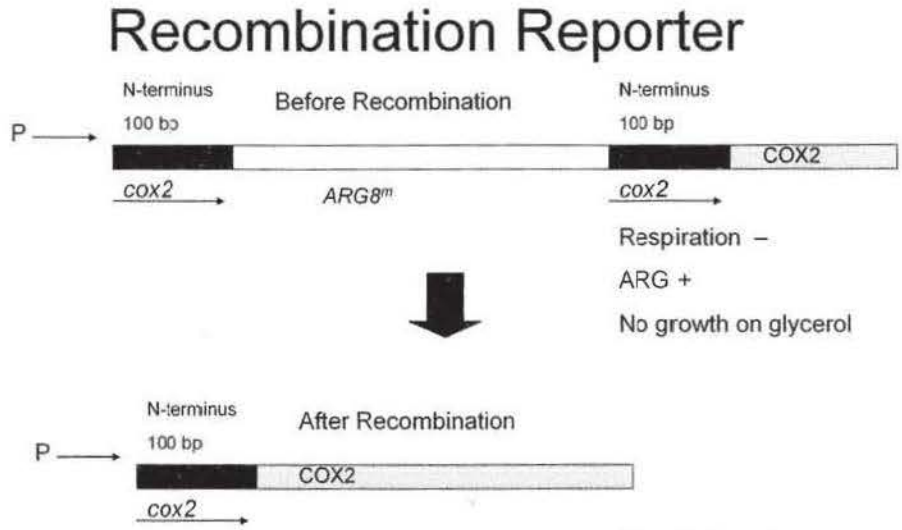

Figure 6. Recombination Reporter. The assay tests the loss of an Ilv5p interacting factor on homologous recombination. It uses the *ARG8* gene and the mitochondrial gene *COX2*. The presence of the *ARG8* gene blocks transcription of the *COX2* gene. The *ARG8* gene is flanked by 100bp segments of the *cox2* gene for regions of homology. After a recombination event the *COX2* gene is transcribed and respiration allows for growth on glycerol.

Table 2 Recombination Reporter Assay	
Strain	**Mutation Rate**
EAS748	**9.8×10^{-5} recombination rate/generation**
EAS748vma8Δ::ura3	**9.7×10^{-5} recombination rate/generation**
EAS748yol057wΔ::ura3	**8.8×10^{-5} recombination rate/generation**

Microsatellite Reporter

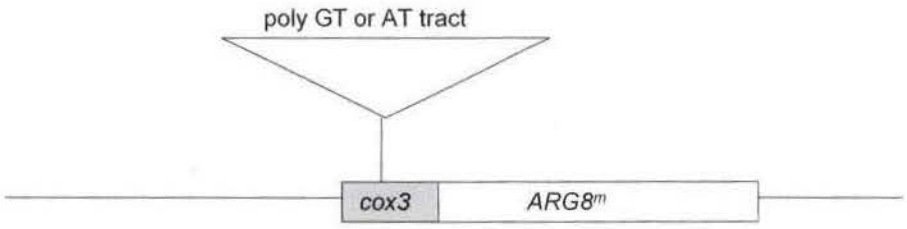

Figure 7. Microsatellite Reporter. A poly GT or AT tract is inserted into the *cox3* gene bringing the *ARG8* gene out of frame where cells are phenotypically ARG-. After a mutation event the *ARG8* gene is brought back in frame and cells are phenotypically ARG+.

Table 3 Microsatellite Reporter Assay	
Strain	**Mutation Rate**
CAB193-1	**3.4×10^{-7} slippage rate/generation**
CAB193vma8Δ::ura3	**2.7×10^{-6} slippage rate/generation**
CAB193yol057wΔ::ura3	**4.5×10^{-7} slippage rate/generation**

Appendix I: Media

THULL media agar plates
1000 ml dH_2O
1.7 g Yeast Nitrogen Base (YNB)
5.0 g Ammonium sulfate
10 g succinic acid
6 g NaOH
20 g glucose (dextrose)
20 g Bacto-agar

Autoclave and cool to 65°C.
Add 0.8 g THULL media mix.

THULL media mix
0.1 g ade
0.1 g arg
0.1 g cys
0.1 g thr
0.05 g asp
0.05 g ile
0.05 g met
0.05 g phe
0.05 g pro
0.05 g ser
0.05 g tyr
0.05 g val

YPD liquid media (1L)
1000 ml dH_2O
10 g Yeast Extract
20 g Bacteriological peptone
20 g Dextrose

Autoclave.

YPD agar plates (1L)
1000 ml dH_2O
10 g Yeast Extract
20 g Bacto-peptone
20 g Dextrose
30 g Bacto-agar

Autoclave.

YPG agar plates
900 ml dH_2O
10 g Yeast Extract
20 g Bacto-peptone
25 g Bacto-agar

Autoclave.
Add 100 ml 20% filter sterilized glycerol.

2% Glycerol, 0.1% Dextrose agar plates
900 ml dH_2O
10 g Yeast Extract
20 g Bacto-peptone
25 g Bacto-agar

Autoclave.
Add 100 ml 2% filter sterilized glycerol.
Add 1 g dextrose.

SD drop out mix

1 g	L-adenine
1 g	L-uracil
2 g	L-tryptophan
1 g	L-histidine
1 g	L-arginine
1 g	L-methionine
3 g	L-tyrosine
4 g	L-leucine
4 g	L-isoleucine
3 g	L-lysine
2.5 g	L-phenylalanine
5 g	L-glutamic acid
5 g	L-aspartic acid
7.5 g	L-valine
10 g	L-threonine
20 g	L-serine

Omit appropriate amino acids to make corresponding drop out mixes.
Add 1.4 g of mixture per liter of media.

SD-trp
1000 ml dH_2O
1.7 g Yeast Nitrogen Base (YNB)
5.0 g Ammonium sulfate
20 g Dextrose
25 g Bacto-agar

Autoclave and cool to 65°C.
Add 1.4 g -trp, -ura drop-out mix. Stir.
Add 10 ml uracil stock solution (2mg/ml).

SD-trp liquid (100 ml)
10 ml 20% glucose
10 ml 10x YNB with ammonium sulfate
10 ml 10x amino acid -trp, -ura
1 ml uracil stock (2mg/ml)
69 ml sterile dH_2O

SD-leu agar plates
1000 ml dH_2O
1.7 g Yeast Nitrogen Base (YNB)
5.0 g Ammonium sulfate
20 g Dextrose
25 g Bacto-agar

Autoclave and cool to 65°C.
Add 1.4 g -leu, -ura, -his drop-out mix. Stir.
Add 10 ml uracil stock solution (2mg/ml).
Add 2 ml histidine stock solution (10mg/ml).

SD-leu liquid media (100 ml)
10 ml 20% glucose
10 ml 10x amino acid -leu, -ura, -his
1 ml uracil stock solution (2mg/ml)
0.2 ml histidine stock solution (10mg/ml)
10 ml 10x YNB with ammonium sulfate
68.8 ml sterile dH_2O

SD-ura agar plates
1000 ml dH_2O
1.7 g Yeast Nitrogen Base (YNB)
5.0 g Ammonium sulfate
20 g Dextrose
25 g Bacto-agar

Autoclave and cool to 65°C.
Add 1.4 g -trp, -ura drop-out mix. Stir.
Add 2 ml tryptophan stock solution (10mg/ml).

SD-ura liquid media (100 ml)
10 ml 20% glucose
10 ml 10x YNB with ammonium sulfate
10 ml 10x amino acid -trp, -ura
0.2 ml tryptophan stock solution (10mg/ml)
69.8 ml sterile dH_2O

SD-arg agar plates
1000 ml dH_2O
1.7 g Yeast Nitrogen Base (YNB)
5.0 g Ammonium sulfate
20 g Dextrose
25 g Bacto-agar

Autoclave and cool to 65°C.
Add 1.4 g -arg, -trp, -leu, -ura, -his drop-out mix. Stir.
Add 2 ml tryptophan stock solution (10mg/ml).
Add 10 ml leucine stock solution (10mg/ml).
Add 10 ml uracil stock solution (2mg/ml).
Add 2 ml histidine stock solution (10mg/ml).

SD-complete agar plates
1000 ml dH_2O
1.7 g Yeast Nitrogen Base (YNB)
5.0 g Ammonium sulfate
20 g Dextrose
25 g Bacto-agar

Autoclave and cool to 65°C
Add 1.4 g -trp, -ura drop-out mix. Stir.
Add 2 ml tryptophan stock solution (10mg/ml).
Add 10 ml uracil stock solution (2mg/ml).

SD-complete liquid media (100 ml)
10 ml 20% glucose
10 ml 10x YNB with ammonium sulfate
10 ml 10x amino acid -trp, -ura
0.2 ml tryptophan stock solution (10mg/ml)
1 ml uracil stock solution (2mg/ml)
68.8 ml sterile dH_2O

LB-amp agar plates
1000 ml dH_2O
10 g Tryptone
5 g Yeast Extract
10 g Sodium Chloride
15 g Bacto-agar

Autoclave and cool to 65°C.
Add 1 ml 1000x ampicillin stock solution.

LB-amp liquid media (1 L)
1000 ml dH_2O
10 g Tryptone
5 g Yeast Extract
10 g Sodium Chloride

Autoclave and cool to 65°C.
Add 1000x ampicillin to a final concentration of 1x (1µl/ml).

References:

1. David M. MacAlpine, Philip S. Perlman, and Ronald A. Butow. 2000. **The numbers of individual mitochondrial DNA molecules and mitochondrial DNA nucleoids in yeast are co-regulated by the general amino acid control pathway.**
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