6-2006

Evaluation of Gyp7 Protein Ability to Coordinate and Regulate Mitochondrial Genomes Stability

Louis DiDone
The College at Brockport

Evaluation of Gyp7 Protein Ability to Coordinate and Regulate Mitochondrial Genome Stability

To the faculty of the Department of Biological Sciences of the State University of New York College at Brockport a Thesis presentation in fulfillment for the degree of:
Masters of Science.

Louis P. DiDone

THESIS DEFENSE

LOUIS DIDONÉ

APPROVED	NOT APPROVED	MASTER'S DEGREE ADVISORY COMMITTEE	
✓		Major Advisor	6/6/06 Date
✓		Committee Member	6/6/06 Date
✓		Committee Member	6/6/06 Date

Chairman, Graduate Committee

Chairman, Dept. of Biological Sciences 6/27/06

Acknowledgements

First, I would like to acknowledge and thank my research mentor and Masters Degree advisor Dr. Rey Sia. Dr. Sia was very generous in allowing me to be apart of his research while at the same time teaching me the needed skills that I have newly acquired, and by allowing me to gain critical research experience. His patience and guidance through this process was greatly appreciated through all the incidentals that were generated by my participation. Secondly, I would also like to thank him for the guidance in generating this thesis. Without him this endeavor would have never happened or been completed. I would also like to thank Dr. Tracey Householder, Dr. Paul Roberts, and Dr. David Brannigan for being on my graduate committee, and for volunteering of their time with the comprehensive oral exam and thesis defense.

My sincerest respect and regard to the Department of Biological Sciences at SUNY Brockport for providing me the opportunity to extend my education to a higher level in acquiring a Masters Degree in Biological Sciences. This advancement will provide me with the needed education and confidence to succeed in a rewarding and successful career in science, research, or any endeavor this education has opened to me. Also, I would like to thank them for allowing me the opportunity to work as a teaching assistant in the department by allowing me to gain additional experience working in the department.

Most importantly I would like to acknowledge my parents. I would like to thank them for teaching me the value of an education and the endless possibilities that develop and occur in the process of acquiring one. They have always taught me to be grateful for what has been given to me and what I have earned. They have always told me the opportunities I have been given in life can not to be taken for granted, because there are people less fortunate then me and under different circumstance would benefit just as much as me. Without their constant support and reassurance this would have been a winless battle, and I would not be where I am right now without them. Thank you mom and dad. Finally, I would like to thank my aunt and uncle who I have lived with for the last two years in this pursuit. They have provided me an excellent environment to study in with minimal responsibilities in return.

Sincerely,

Louis P. DiDone

Table of Contents

Background and significance:

Introduction:

Animals, plants, fungi, and protists are made up of eukaryotic cells. These cells are larger in size, and structurally more complex in comparison to prokaryotic cells. This is because they contain internal cytoskeletons and many different membranous compartments that carry out individual and interconnected biochemical functions, while prokaryotic cell types do not have these membranous compartments. However, prokaryotic cells are very unique in their own right. They are very diverse in their metabolic capabilities. They can survive on a greater diversity of energy sources than any other living creatures utilizing, for example, sulfur based substances compared to carbon based. One main difference between eukaryotic and prokaryotic cells is the method by which these two types of cells generate their energy requirements. Eukaryotic cells use enzymes within an organelle called the mitochondria to generate energy in the form of a high-energy compound called Adenosine triphosphate (ATP) while prokaryotic cells use their enzymes anchored to the plasma membrane to generate this same compound.

The ability to generate energy is critical for the survival of both cells types. When this function has been lost the cells become inviable and death is the ultimate consequence. Death can be avoided in at least one eukaryotic organism, *Saccharomyces cervisiae*, when total mitochondria activity stops, however it is necessary to have mitochondria present and functional for normal and healthy growth to occur. This organism has developed a method to survive without functional mitochondria by using an alternative energy source. With its ability to use an alternative energy source for the

production of ATP this organism has become the model organism to look at the role of the mitochondria and its function in eukaryotic cells.

Saccharomyces cerevisiae:

The "life cycle" of *Saccharomyces cerevisiae* is unusual in comparison to many other multicellular and unicellular organisms. Traditionally in the life cycle of any organisms you have several steps such as proliferation, mating, and meiosis. *S. cerevisiae* has the ability to be either haploid or diploid while also expressing three distinct and different cellular types. Haploid yeast cells have the ability to alter their cellular structure by a programmed deoxyribonucleic (DNA) rearrangement. This ability allows some of the *S. cerevisiae* haploid yeast strains to express a homothalic life cycle or heterothalic life cycle. A homothalic life cycle is a life cycle by which a single haploid yeast cell can give rise to diploid cells capable of meiosis and spore formation. A heterothalic life cycle differs from a homothalic life cycle since a single haploid yeast cell in a heterothalic life cycle is not capable of producing a diploid cells. Understanding these two attributes is very important since it helps to completely understand other organism's life cycles and raises issues on how cellular diversity is generated (Herskowitz *et al.*, 1988).

S. cerevisiae is a unicellular organism that has the ability to be in three distinctly different and specialized cellular states that are important to the life cycle. All three have the ability to go through mitotic cellular division. The first two specialized states of this yeast are the "a" and alpha "α." When these cell types are placed next to each other they will mate with each other with 100% efficiency. When mating occurs between these two types, cellular and nuclear fusion takes place, resulting in one newly generated diploid cell with only one nucleus. The third specialized cell type is the diploid a/ α type

generated when "a" and alpha "α" mate. This specific cell type can not mate with either "a" or "α", but can under go meiosis. This specific cell type is different from the other based on the fact that when this cell type is grown under starvation conditions it will give rise to four haploid meiotic progeny that are individually coated with a spore coat to protect it. These four spore coated progeny are enclosed in another protective membrane called the ascus.

For mating to occur between "a" and "α" types, each individual cell type needs to communicate with each other. For this communication to take place, pheromones are released by each of the two types. The "a" type releases the pheromone *a*-factor and the "α" releases the pheromone *α*-factor (Duntze et al., 1970 and Wilkinson et al., 1974). The a/α cell type does not mate so they do not produce either of the pheromones produced by the "a" or "α" cell types. These released pheromones signal to the two different cell types that they are mature and in close proximity to each other to allow mating to occur. The *a*-factor is a 12 amino acid peptide and the *α*-factor is 13 amino acid long peptide (Kurjan *et al.,* 1985 and Betz *et al.,* 1987). These factors are important because when secreted they instruct their corresponding partner cells to arrest in the G1 phase just before the initiation of DNA synthesis. This suggests the secreted pheromone factors are negative growth factors used to synchronize the cell cycles of mating partners and allow appropriate fusion events to generate (Bucking-Throm et al., 1973).

The generation of spores or sporulation and meisosis are only generated when two specific environmental stimuli are achieved. The first stimulus is the lack of nitrogen and carbon in their immediate environmental surroundings. The second condition that is required is that the cell needs to be diploid and have the proper genotype MATa/MATα

and have a1- α2 activity. A1- α2 activity is a protein repressor that inhibits haploid-specific genes. Other diploid genotypes such a MATa/MATa or MATα/MATα will not sporulate. Essentially the second requirement is that it has the right genotype rather than being diploid (Kassir *et al.,* 1976)

With the yeast cells having the ability to be undergo homothallic and heterothallic life cycles one must understand how a single haploid cells can give rise to a a/α diploid progeny. One conclusion that has been generated is the idea that homothallic cells types contain a "universal mating type" that allows it to mate with any other haploid cells. Homothallic yeast strains produce the a/α diploid by mating between the usual mating types. Homothallic yeast strains have the ability to produce progeny cells of both mating types during mitotic cell division (Hawthorne *et al.,* 1963 and Hicks *et al.,* 1977).

S. cerevisiae is a facultative anaerobic organism. This characteristic enables this particular organism to satisfy its energy requirements by either ATP production through oxidative phosphorylation or more importantly ATP creation by fermentation. With this ability only a relatively few mitochondrial proteins encoded from the mtDNA are essential for the cell's viability. These proteins are condensed into a few general functions such as importing, processing, and folding of precursor proteins, iron-sulfur cluster assembly, and flavin mononucleotide synthesis. These natural abilities contribute in making this organism the model for analyzing the molecular processes essential for maintenance of respiratory-competent mitochondria. Even with the mitochondria having the ability to transcribe and translate its own proteins, a vast number of nuclear genes are required for total respiratory competence. Mutations developing or induced in genes that

alter the mitochondria respiration functions are identified as nuclear *petite* or *pet* mutants (MIPS, Mewes et al., 2000).

S. cerevisiae has a doubling/replication rate ranging from 90-100 minutes where the 17 chromosomes are replicated and then packaged into the daughter cells (Pringle and Hartwell *et al.,* 1981). Daughter cells are generated by a process called budding. Budding is a process by which the mother yeast cell generates an elliptical daughter cell attached to the mother's cell membrane. The new daughter cell is completely made from new cellular membrane components while containing no cellular membrane from the mother yeast cell. However there are other yeast strains that undergo fission, by which the daughter cells are generated by pinching in the cellular membrane of the mother's forming two smaller daughters. In these cases the daughter cells do contain cellular membranes generated by the mother (Herskowitz *et al.,* 1988).

Yeast Carbon Catabolite Repression:

Saccharomyces cerevisiae along with many other yeast species such as *Schizosaccharomyces pombe* and *Candida albicans* posses the ability to thrive on a variety of carbon sources. However, glucose and fructose are the preferred ones. With the presence of either sugar the enzymes needed for the utilization of the alternative carbon source are synthesized at low rates or not at all. This process is known as carbon catabolite repression. This occurs because no catabolite, an intermediate molecule derived from glucose is involved in this repression of the other alternative enzymes needed for alternative carbon sources besides glucose and fructose has been identified. The direct opposite occurs when the yeast cells lose mitochondria function. The

enzymatic activity increases to compensate for the lost ATP production that would have occurred through oxidative phosphorylation.

Mitochondrial Genomes and Their Differences:

Mitochondria are essential organelles of eukaryotic cells. Their enzymes perform a variety of biochemical reactions important in the over all metabolism of the cells. They are involved in the reactions of the tricarboxylic acid cycle, iron/sulfur cluster assembly, and biosynthesis of many cellular metabolites (Epstein. C. *et al.*, 2001). The most paramount of functions is the supply of and generation of energy through oxidative phosphorylation. The cellular role of the mitochondria is directly related to their structure. The mitochondria are a double membrane-bounded organelle forming a branched tubular network below the cell cortex. This network is maintained by active transport along with a balance of membrane fusion and fission events of the double membrane. Inheritance and morphology are dependent on this activity as long as the cytoskeleton is present and membrane-membrane fission and fusion events occur.

To understand the processes that contribute to mitochondrial function and inheritance requires the identification and characterization of some of the molecular components involved in these processes. Many of the proteins required for respiratory growth and maintenance of mitochondria structure have been identified in yeast. Today's technology and the advent of the post-genomic era allows us to conduct systematic genome-wide screens to define whole complements of genes associated with particular functions.

Yeast can be found in nature with either diploid or haploid genomes. This refers to the number of chromosomal copies each cell contains. Diploid refers to having two

copies of the same chromosome while haploid refers to only having one copy. With these facts in relationship to mitochondrial genomes within the two different cell types the number of mitochondrial genome numbers also greatly differ in number. Haploid cells are identified as to contain roughly ~50 copies while diploid cells roughly contain ~100 copies of the mtDNA. The general size of the mitochondria in both cells types are said to range from 78-85 kb in size which is roughly five times that of human mtDNA.

Mitochondrial inheritance in yeast is biparental. During fusion of haploid cells, both parents contribute equally to the diploid zygote. In mammals and other animals this mitochondria genome inheritance into the daughter cell is regulated and controlled so the mother contributes 99% of the mtDNA being inherited, while the other 1% is inherited from the father.

The genetic code used in animal and fungal mitochondria are different compared to the standard code used in all prokaryotic and eukaryotic nuclear genes. These alterations in the genetic code are not understood in why these changes developed (Figure 9) (Osawa. S. *et al* 1992).

Genetic control of the mitochondria genome is not well documented except in *Saccharomyces cerevisiae,* where 100 nuclear genes are involved either directly or indirectly with the rate of production of either rho^+ mtDNA (containing functional mitochondrial DNA) or rho^- mtDNA (nonfunctional mitochondrial DNA). Any alteration in genes involved in either replication, repair, or recombination can cause the complete loss of mtDNA or truncated mtDNAs (Contamine *et al.*, 2000). Most commonly, mutations arise in genes controlling such diverse functions as mitochondrial translation, ATP synthase, iron homeostasis, fatty acid metabolism, and mitochondrial morphology.

When mutant and wild-type mtDNA molecules are present in the same cell it is defined as heteroplasmy. This ratio changes dramatically over the course of that individual's life span (Contamine *et al.*, 2000).

S. cerevisiae exhibits some intrinsic weaknesses as a eukaryotic model even though it is considered the model organism for these studies. First, it is a facultative anaerobe. With this ability it does not need any actively working mitochondria to survive such as higher eukaryotes do, however it does help viability. This causes problems in clearly correlating how alterations in eukaryotic mitochondria will affect its cellular activities. Secondly, being unicellular mitochondria activity in multicellular systems may dramatically differ based on the complexity of the system they are incorporated in. Lastly, *S. cerevisiae* do not stably maintain a heteroplasmic state, and their mtDNA structure differs significantly from other higher eukaryotes such as in size. These complex issues along with it being very accessible to classical, and modern genomic techniques is what truly makes it a model organism even with these differences (Contamine *et al.*, 2000 and Mason *et al.*, 2003).

Mitochondrial Nucleoid Organization:

Inheritance of the mitochondria DNA (mtDNA) is done in a protein-DNA complex referred to as the nucleoid. Wild-type haploid yeast strains have been estimated to contain 25-50 mtDNA molecules that are stably maintained during mitotic growth. In sexual crosses, mtDNA from each parent is faithfully transmitted to the progeny and segregates rapidly to yield homoplasmic cells. It is generally believed that the nucleoid is the unit of mtDNA segregation (Newman *et al.*, 1995). Recently a mutation of the *MGT1* gene will render the yeast cells defective in resolving yeast mtDNA recombination

junctions. It was also found to have alterations in the number of mtDNA nucleoids and in the pattern of mtDNA transmission (Newman *et al.,* 1995).

Mitochondria Petites: *Rho*$^-$ and *Rho*$^+$

Respiratory-deficient mutants in yeast are classified by their inability to use non-fermentable carbon sources such as glycerol for development. Induction of these petites *rho*$^-$ (ρ^0) yeast cells can be induced by several chemicals along with spontaneous mutations. *Rho*$^-$ indicates that mutations in the mitochondrial genomes have developed in genes that are involved in respiration (Goldring *et al.,* 1971). These alteration are serious issues based on the fact mitochondria of higher eukaryotes do not posses the ability to generate ATP by fermentation and these kinds of mutations would be lethal to these eukaryotic animals.

Ethidium bromide, acriflavine, phenanthrene dye, and sodium dodecyl sulfate (SDS) are all known to generate *rho*$^-$ mitochondria. Prolonged exposure will induce mutations involved in genes responsible in mtDNA metabolism (replication, repair, and recombination) can cause a complete loss of mtDNA (rho^0 petites) and/or lead to truncated forms (rho$^-$) of this genome (Cantamine and Picard *et al.*, 2000).

Mitochondrial DNA Replication, Recombination and Repair:

Mitochondrial DNA is subject to higher levels of oxidative damage and mutational occurrences compared to nuclear DNA. The presences of reactive oxygen species (ROS), which are continuously formed in the mitochondria by electron leakage from the respiratory chains in the process of oxidative phosphorylation, induce mutations within the mtDNA genome. To repair these types of mutations, base excision repair pathway (BER) is utilized (Nilsen *et al.,* 2001). BER is regulated by mtDNA proteins

that are encoded and imported from the nuclear DNA. Mitochondrial DNA repair proteins are either identical or isoforms of their counter nuclear DNA repair proteins. The BER pathway has the ability to repair mismatched bp(s), by filling in gaps between the DNA strands, with DNA ligases. It is thought that a glycosylase/AP lyase recognizes and removes the damaged bases from the mtDNA. It is believed that endonuclease III (EndoIII) and endonuclease VIII (EndoVIII) are these glycosylase/ AP lyases that are responsible for this activity. These two enzymes work in a bi-directional manner cleaving the glycosidic bonds removing the base pairs and then allowing DNA polymerase gamma (Pol gamma) to repair the extracted regions within the mtDNA. Pol gamma is important because it posses an exonuclease domain with high fidelity activity decreasing the possibility of DNA slippage to occur in the replication process that could result in frameshift mutations, incorporate microsatellite repeats, or completely falling off the DNA strands leaving truncated segments of DNA (Lyudmila *et al.,* 2004 and Davis *et al.,* 1996)

Mitochondrial genome replication occurs through the rolling circle method of replication in a bi-directional manner. The outer layer of the double stranded circular genome is designated as the heavy strand or (H-strand) while the inner strand is assigned as the light strand or (L-strand). With this model for replication the H-strand transcription occurs at the origin of replication in a clockwise manner, while the L-strand is transcribed in a counterclockwise manner. As transcription occurs, linear pieces of DNA are generated and can be viewed by electron microscope and separated by pulse-field electrophoresis. The number of origins of replication varies in number from 1-4 origins based on the yeast organism of interest (Clayton *et al.,* 1991).

Replication, recombination, and repair are all mechanisms utilized in a cooperative manner in the maintenance of the mtDNA genome. Each system works independently and cooperatively with the next system. Protein *Abf2p* has been identified to be essential for mitochondrial chromosomal function. The function of Abf2p in yeast is essential for mtDNA maintenance by providing a mtDNA-packaging function.

Yhm2p is localized in the mitochondrial inner membrane and is also associated with mitochondrial DNA *in vivo*. Yhm2p appears to be a membrane-bound DNA-binding protein. Any alteration in the *Yhm2* gene will cause growth defects in the presence of nonfermentable carbon sources such as glycerol and ethanol. 2,3,5,-triphenyltetrazolium chloride staining will show structural alterations in phenotype of the inner membrane. It appears that Yhm2p may be functioning as a member of the protein machinery for the mitochondrial inner membrane attachment sites of mitochondrial DNA during replication and segregation of mitochondrial genomes (Hyoung *et al.,* 1998).

Nuclear genes of *S. cerevisiae* have been known to be involved in mitochondrial genome stability. These genes play several roles in this regulation. They have been identified to be involved in mitochondrial translation, transcription, encoding mitochondrial enzymes for nucleotide biosynthesis, pyrophosphatase, GTP-binding proteins, and a matrix protease. With proteins identified in each of these mechanisms this shows several proteins are essential for mitochondrial function and maintenance of the mitochondrial chromosome in direct or indirect ways (Hyoung *et al.,* 1998).

Microsatellite Generation and Instability in DNA Repair and Replication:

Microsatellites are simple sequence repeats (SSRs) that are tandemly repeated within tracts of the DNA and can be any where from 1-6 base-pairs in length. These

SSRs can be found in both prokaryotes and eukaryotes, and even the smallest of bacterial genomes (Field and Wills *et al.*, 1996). Microsatellites can be used as indicators to determine DNA repair mechanisms in base pair mismatches. Screening mutated yeast strains with increased microsatellite instability several genes were identified to be possibly involved in mismatch repair mechanisms. Point mutations in genes *MSH2*, *MSH3*, *MLH1*, and *PMS1* created phenotypes different in comparison to the creation of null mutations within these same genes (E.A. Sia *et al.*, 1997 and 2001). Microsatellites can be found in the noncoding and protein-coding regions with in the genome. Microsatellites and their high probability for mutation are thought to be involved in creating and maintaining quantitative genetic variation (Tautz *et al.*, 1986 and 1994). This may occur through the altering of the promoter regions by changing the lengths of the SSRs influence on the transcriptional activity of that promoter. When this occurs it can change the polyglutamine or polyproline tracts that are encoded by the SSRs affect the protein-protein interactions that are involved in transcription factors (Kashi *et al.*, 1997).

Yeast mitochondrial genomes contain many microsatellites. To examine these regions a reporter gene (arg8(m)) containing an out-of-frame insertions of either the poly(AT) or poly(GT) tracts was used. With this reporter gene generated, yeast strains with this reporter gene were constructed. Using this newly created yeast strain it was identified that poly(AT) regions within the genome were considerably less stable than poly(GT) tracts. Alterations that were caused by insertion of the reporter gene normally involved deletions rather than additions within the repeat units. To see if these data were isolated only to mitochondrial genomes it was compared to poly(AT) and poly(GT) tracts

within the nuclear genome of the yeast. This time in the nuclear genome it was shown that the rate of stability was similar to the mitochondria rate however, the derived alterations this time in the nuclear genome involved additions rather than deletions. Also, comparing poly(GT) tracts in haploid and diploid yeast cells revealed that the poly(GT) tracts were more stable. Correspondingly, a mutation in the *MSH1* gene helps to destabilize the poly(GT) tracts within the mitochondrial genomes (Sia EA, *et al.,* 2000).

SSRs have been found to be less abundant in exons than in noncoding regions (introns) (Hancock *et al.*, 1995). The overall ratio of SSRs within the genome is directly correlated to the overall size of the genome with that particular organism (Hancock *et al.*, 1995, 1996a, 1996b).

SSRs are unstable, and two models have been proposed to explain microsatellite generation and instability. These two models involve DNA polymerase slippage and unequal recombination. The DNA polymerase slippage model involves the transient dissociation of the replicating DNA strands followed by the improper base pair alignment of the newly dissociated strands when they reanneal. The newly annealed strands can become destabilized by hairpin arrangements within the DNA. Repeats that are able to form such alternative DNA conformations would be generated more frequently than others. To stabilize this structures addition or deletion of bases are needed to remove the hairpin loops. If the hairpin loop forms on the daughter strand the length of the base pairs will increase due to the addition of a repeat caused by DNA polymerase slippage. If the hairpin loop forms on the template strand the sequence will become shorten. If the hairpin loops are not removed before the next round of replication occurs the newly added or deleted base pairs will become incorporated into new generated DNA and

repetitive sequence lengths can either increase or decrease. Mitochondria mutations are acquired by the mitocondrial genome ~10 faster than nuclear genome (Brown *et al.*, 1979)

Frameshift mutations develop when the coding region of a gene is changed by the addition and deletion of a base pair(s) that are not a multiple of three. The presence of a deletion versus an insertion frameshift is directly related to the intermediate structure formed during DNA synthesis. Extrahelical bases on the strand being synthesized produce insertions, while extrahelical bases on the template strand give rise to deletions. This was solved using a +1 frameshift mutation to analyze the role of mismatch repair (MMR) in correcting –1 frameshift intermediates within a defined region of the yeast *LYS2* gene.

Possible triplet repeat structures in human diseases have been analyzed extensively particularly these repeats $(CTG)_n$, $(CAG)_n$, $(CCG)_n$, $(GAA)_n$, $(TTC)_n$, $(AGC)_n$, $(CCT)_n$, and $(TGG)_n$ (Gacy *et al.*, 1995, Bidichandani *et al.*, 1998, Usdin *et al.*, 1998). Homologous recombination may also result in genetic instability of certain SSRs (Jakupciak and Wells *et al.*, 1999).

Saccharomyces cerevisiae DNA helicase Srs2 has been identified as a potent and selective inhibitor to SSRs. Yeast cells with mutant Srs2 helicases had up to 40x fold increase expansion rates of $(CTG)_n$, $(CAG)_n$, and $(CGG)_n$ repeats. The mutation rates of dinucleotide repeats in unique sequences in Srs2 mutants were not altered. Srs2 is also known to suppress inappropriate genetic recombination. For this to occur it was shown that Srs2 functioned with DNA polymerase delta to block the expansion of the SSRs (Krejci *et al.*, 2003).

Human Diseases Linked To Mitochondria Dysfunctions:

Mitochondria dysfunction has been linked to several disease types such neurological disorders, aging, deafness dystonia and diabetes. The more profound are the neurological disorders such as Kearns-Sayre syndrome, Huntington disease (HD), Friedreich ataxia, hereditary spastic paraplegia, and rare familial forms of Parkinson disease (PD), Alzheimer disease (AD), and Amyotrophic lateral sclerosis (ALS). The genetic links for the development of these neurological disorders are believed to be linked to the dysfunction of the mitochondria within the neurons of patients with these diseases. Since neurons are highly dependent on energy derived from oxidative phosphorylation, a suggestive unified pathogenetic mechanism of neurodegeneration based on the underlying dysfunction in the mitochondrial energy metabolism has been established (Manfredi and Schon *et al.,* 2003). This connection was generated based on that fact the mitochondria operate under several unique systems of controls. First, mitochondria have multiple copies of the mtDNA (approximately five mtDNAs per organelle) while there are only two copies of nuclear DNA in the nucleus. Secondly, organelle replication and division operate independently from the cell cycle in dividing cells. Third, once cellular division has begun mitochondria and their mtDNA are partitioned randomly between daughter cells. Lastly, the overall number of mitochondrial organelles varies among the particular cell types. This happens specifically due to the cell culture's metabolic requirements. Skin fibroblasts may house a few hundred mitochondria, whereas neurons may contain thousands, and cardiomyocytes tens of thousands. The significance here is the fact mitochondria do not follow mendelian genetics, but rather laws of population genetics.

Mutations in the mitochondria usually arise within one mtDNA and when replication occurs copies are then generated expanding in an exponential manner. The ratio of mutated mtDNAs compared to wild type mtDNAs determines along with spatial and temporal distribution when phenotypical consequences are shown. With mitochondria maternally inherited the disease are normally recessive and the mutated mtDNA must comprise at least 70% of the total population of mtDNAs in order for dysfunction to be noticed. With alterations in the nuclear DNA that encode proteins that target mitochondria or other organelles indirectly or directly may alter any aspect of cellular function. In the mutated mtDNA the mutations vary from simple to complex sequences of additions, deletions, point mutations, and repeat additions and deletions with in the mtDNA (Koehler *et al.,* 1996).

Mutations in the $tRNA^{Leu(UUR)}$ gene causes mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes. Kearns-Sayre syndrome is brought on by a large spontaneous deletion in the tRNA. Human deafness dystonia syndrome is caused by a mutation in the DDP protein, which has no known function described for this protein. However, DDP is a mitochondrial encoded protein that is similar to several proteins (Tim8p, Tim9p, Tim10p, Tim12p and Tim13p) found in yeast mitochondrial inner membrane space. Tim9p, Tim10p, and Tim12p are involved in mediating metabolite transporters from the cytoplasm into the mitochondrial inner membrane and directly interact with Tim8p and Tim13p. Tim8p of the five proteins is the closest in relation to DDP. Tim8p is a 70-kDa complex. Deafness dystonia syndrome is a standard mitochondrial disease linked to deficient mitochondrial protein-import system (Koehler *et al.,* 1996).

Amyotrophic lateral sclerosis has been linked to mitochondrial vacuolation in the early phases of this disease. Mitochondrial Vacuolation by Intermembrane Space Expansion (MVISE) is not related to autophagic vaculation mechanisms caused by a mutation in SOD1 ^{G93A} and it is an uncharacterized mechanism for mitochondrial degeneration in mammalian cells (Rognoni *et al.*, 1996). Mitochondrial vacuolation occurs by the expansion of the intermembrane space and extension of the outer membrane. This induces mitochondrial degeneration by inducing extension and leakage of the outer mitochondrial membrane, and increasing the intermembrane space between the inner and outer membrane of the mitochondria. Increasing the intermembrane space could stimulate the release of molecules normally residing in the intermembrane space, which then initiate motor neuron degeneration (Higgins *et al.*, 2003).

Preliminary studies:

The following studies were conducted to characterize the role, mechanism and interactions of the nuclear gene *GYP7* within the budding yeast *Saccharomyces cerevisiae*. *GYP7* is also synonymous with the names *GAP*, and *YDL234C*. *Gyp7* is a member of the *Ras Superfamily of monomeric GTPases* that are involved in signal transduction and motility within the cell.

GYP7 gene encodes a GTPase-activator protein (GAP) that is hydrophilic and its gene length is 2.2 Kb in length located on chromosome IV. This gene is encoded on the Crick strand starting at the chromosomal coordinate 34238-bp and continuing to the 36478-bp. The Gyp7p protein encoded by this gene has a molecular mass of 87 kDA. The protein is comprised of 746 amino acids. The N-terminal sequence is MSKILFC and the C-terminal is RSKNSVK. Comparing the primary amino acid sequence of other

known GAPs for other transport GTPases Gyp6 and Gyp1 proteins were able to show similarity with *Gyp7*. *GYP7p* interacts with six known proteins. These proteins are *Ydr131cp*, *Gdi1p*, *Cdc23p*, *Imp4p*, *Sog2p*, and newly identified *Ilv5p*. This identification was through the two-hybrid analysis. The first five have been identified by TR. Hazbun in 2003. However, *Ilv5p* was found in R. A. Sia's lab at SUNY Brockport by Anthony Mirando in his unpublished thesis. *Gyp7p* GAP primarily acts on *Ypt7p*, but also interacts to a lesser degree on *Ypt31p*, *Ypt32p*, *Ypt1p*, *Ypt6p*, and *Sec4p*.

GTPases are classified as Ypt's in yeast and Rab's in mammals. GTPases serve as molecular switches to regulate growth, morphogenesis, cell mobility, cytokinesis, and transport vesicle targeting to and/or fusion with specific acceptor compartment membranes (Vollmer et al 1999).

The GTPases mechanisms that regulates the switching from the active to inactive state of the signal transduction of G- proteins is controlled by a common mechanism for all GTPases. This controlling mechanism is performed in an unidirectional manner when the GTPase in its active state has guanine triphosphate (GTP) bound to it. The inactive conformation has a phosphate group hydrolyzed removing it and the newly generated guanine diphosphate (GDP) bound form. This process occurs through its own intrinsic GTPase-activity. This hydrolysis reaction is stimulated by GTPase-activating proteins (GAPs). This is how *GYP7* works by encoding a GAP protein that interacts with *Ypt7p* primarily. The reactivation of GTPase back to the active conformation is performed by guanine nucleotide exchange factors (GEFs) and with help from nucleotide dissociation inhibitors (GDI). GEFs cause the GDP to dissociate from the GTPase enzyme. The newly freed GTPase can now interact and reassociate with free GTP generating the active state

of the GTPase. Since, hydrolysis of GTP to GDP is irreversible, this mechanism is unidirectional. Of the active and inactive conformations only the active state of the GTPase can transduce a signal reaction. Ras and Rho GTPases interact with a variety of effector proteins to promote cellular responses when a signal transduction is generated by a GTPase.

Signal transduction via GTPase is directly related to the ratio of active to inactive GTPase. The mathematical equation (GTPase * GTP) / (GTPase * GDP) = (^{k}diss. GDP) / (^{k}cat. GTP) can be used to describe this relationship. The ^{k}*diss*. GDP being used to describe the dissociation constant of GDP, and ^{k}*cat.GTP* being used as the hydrolysis constant of GTP being used to describe the specific activity of the GTPase(s) being used. Both constants can be modified by special regulatory proteins. Several modifications can be used to change the amount and rate of activation of the GTPase. To increase the amount of active GTPase present you can increase the concentration of GEFs to accelerate GDP dissociation. Guanine nucleotide dissociation inhibitors (GDIs) can be used to inhibit GDP dissociation slowing down the building of active GTPase. To decrease the amount of GTPase activity you can use GAPs to increase the acceleration of GTP hydrolysis, and finally you can use GTP analogues such as *γ-S-GTP*, *β,γ-methylene-GTP*, and *β,γ-imino-GTP, and β,γ-imino-GTP* (http://en.wikipedia.org/wiki/GTPase.)

With the general mechanisms of how GTPases work and how you can alter the reaction rates we need to directly see what roles the protein *Gyp7p* encoded by *GYP7* is involved in. It has been determined that *Gyp7p* is involved in homotypic vacuole fusion through sequential priming, docking and fusion events (Alberts *et* al., 1999). With *Gdi1p* and *Ypt7p* identifed as known protein interactors with *Gyp7p,* and with *Gyp7p* acting as a

GAP protein it was looked at how the GDP and GTP states of *Ypt7p* would be affected using *Gdi1p, Gyp7p, GTPγS, GppNHp* (non-hydrolyzable nucleotides), and mutant forms of *Ypt7p* that favor either GTP or GDP states. *Ypt7p* bound with GDP-bound on isolated vacuoles can be identified and extracted by using *Gdi1p*, however only GTP-bound state will allow for docking to occur. Homotypic fusion and vacuole protein sorting complex when bound to *Ypt7p* are converted to the GTP-bound state. *Gyp7p* acting as a GAP then causes *Ypt7p* to hydrolyze bound GTP to GDP, driving homotypic fusion events while releasing and accelerating Gdi1p-mediated release of *Ypt7p. Ypt7p* extraction does not inhibit the Ca^{2+}-triggered cascade that leads to fusion. In the absence of *Ypt7p* fusion vacuole are sensitive to GTPγS and GppNHp. This shows that there is a second specific GTPase that regulates the calcium flux and hence fusion events that occur meaning there are two GTPases consecutively and independently governing vacuole docking and fusion (Eitzen et al 2000.)

Ypt7p is an essential protein required for vacuole docking (Mayer and Wickner.) Once docking is complete, the final stage of membrane fusion is not affected by the extraction of *Ypt7p* yet remains sensitive to GTPγS (Eitzen *et al.*, 2000).

The catalytic domains of *Gyp7* and *Gyp1p* have been identified. Comparing *Gyp7p* and *Gyp1p* another known GTPase located in *Saccharomyces cerevisiae* it has been determined that the catalytic domains are isolated in the C-terminals in these two particular GAPs. To determine this, segmental deletions were done on both the N- and C- terminal ends in varying lengths of *Gyp1p* and *Gyp7p* while looking for the GAP activities of the newly created truncated forms of the proteins (Alberts *et al.*, 1999). Glutathiome-S-transferace was fused to *Gyp1p* and *Gyp7p* and their corresponding

truncated form to produce *GST-Gyp1p* and *GST-Gyp7p* fusion proteins with deleting segments of varying lengths from the N- and C-terminal ends of *Gyp1p* and *Gyp7p* and determined the GAP activities of the truncated proteins after expression in *Saccharomyces cerevisiae* (Alberts *et al.,* 1999). The N-terminal 248 amino acids of *Gyp1p* were dispensable for GAP activity, whereas the deletion of the N-terminal 300 or the C-terminal 104 amino acids rendered it inactive. *GST-Gyp1p* and *GST-Gyp1-46p* were affinity-purified from *S. cerevisiae* (Alberts *et al.,* 1999). The yield and the purity of full-length *GST-Gyp1p* were significantly lower than those of *GST-Gyp1-46p* and varied from preparation to preparation (Alberts *et al.,* 1999). When the GAP activities were determined in crude preparations with comparable amounts of full-length *Gyp1p* and its active fragment (tested by Western blot analysis with anti-GST antibodies), it was noted that full-length *GST-Gyp1p* was always less active than the N-terminally truncated, active fragment *GST-Gyp1-46p* (Alberts *et al.,* 1999). The same observations were made with *Gyp7p* and its truncated forms.

The above conclusions and preliminary research identified and determined the molecular role of the *Gyp7* gene by mapping out its chemical characteristics such as protein size, protein weight, primary amino acid sequence, and its chromosomal location. It was determined that the *Gyp7p* molecular function is to act as a GTPase activator protein. This particular protein and its ability to function properly can be altered by changing the concentration of GTP and GDP present within the cell. Also, the above data provided us with a substrate that *Gyp7p* interacts with such as *Ypt7p.* The establishment of *Ypt7p* being involved in vacuole fusion and docking mechanisms, while at the same time being affected by the Gyp7 gene provides us with a better understanding of how

complex the regulation of GTPase activator proteins are and the identification of *Gyp7p* as a GTPase.

Abstract:

Evaluation of Gyp7 Protein Ability to Coordinate and Regulate Mitochondrial Genomes Stability:

Cellular creation of adenosine triphosphate, ATP, is essential for eukaryotic cells to function properly. The ATP molecule drives most of the biochemical and metabolic pathways of the cell. The cell's ATP is produced in the mitochondria. Mutations within the genome of the mitochondria will alter the cell's ability to generate ATP. Preliminary work has shown that loss of the *Gyp7p* in *Saccharomyces cerevisiae* blocks the ability of mitochondria to properly function. The *Gyp7* gene was isolated using a technique called two-hybrid analysis with a known mitochondrial protein called Ilv5p, which was used as 'bait'. We have shown that a deletion of the *Gyp7* gene is not essential for cellular viability in *S. cerevisiae.* We observed that loss of *Gyp7* decreases both the occurrence of point mutations at microsatellite sequences as well as decreasing the rate at which recombination between direct-repeat DNA sequences occurs. This contributes to the effective that cellular respiration mutation rate increase when Gyp7p is removed. *Gyp7* encodes for the production of the GTPase-activating protein (GAP) Gyp7p within the Ypt/Rab transport GTPase pathway. This pathway is involved in protein trafficking within the cell.

Materials and Methods:

PCR Construction of *gyp7 Δ::ura3* knockout cassette:

The PCR construct of the *gyp7 Δ::ura3* knockout cassette was generated by using yeast cells containing plasmid pRS406. This plasmid houses the *Ura3* reporter gene. This plasmid will act as the template DNA sequence that will be amplified. To amplify the *ura3* reporter gene a downstream primer (GYP7 pRS dn) with the DNA sequence of 5'- ACG AAT TAT ATC TCC GGT ATT CAA TAT GTA AAG TTC CGT TTC TAT TTA CCA *CTG TGC GGT ATT TCA CAC CGC* and up the stream primer GYP7 pRS with the DNA sequence of 5'- TAG GTT GCA CCA AAT TGG CAA AAT TGG CAA AGT TCT ACA AGA GTC ATT CAT ACA TCC CCT G*CA GAT TGT ACT GAG AGT GCA CC*- 3' were used. The italic regions of the primers indicate the annealing regions of these primers to the *Ura3* reporter gene. The remaining non-italic regions of the primer are the homologous sequences of *Gyp7* gene that will be used and needed later for the homologous recombination events to occur that will knockout the *Gyp7* gene with the *ura3* reporter gene. The knockout was generated using a 50 ul total volume PCR reaction. (Refer to Polymerase Chain Reaction Below).

Yeast Transformation of *gyp7 Δ::ura3* Knockout Cassette:

With the generation of the *gyp7 Δ::ura3* knockout cassette completed transformation of this cassette was done in the yeast strains DFS -188, CAB –193, and EAS. This transformation was done using a high efficiency yeast transformation protocol. The three different yeast strains were grown in 5mls of YPD (Yeast Peptone Dextrose) overnight at 30°C. The following day the 5mls of cells were diluted into 200 mls of YPD and grown for three hours to mid-log phase (OD600=0.3) From there they were pippetted into 50ml conical tubes and centrifuged for 5 minutes at 3000 revolutions/minute. The yeast cells were then resuspended in 1ml of dH_2O and transferred to an Eppendorf tube. Following this, the cells were then centrifuged again for one minute and then resuspended in 500ul 0.1 M LiOAc, TE. Next, 100ul of the cells were then transferred to a new eppendorf tube. To each tube 15ul of carrier DNA (10mg/ml salmon sperm DNA) and 2ul of the *gyp7 Δ::ura3* at (2 mg/ml) was added to the cells and incubated at 30°C on a shaker for thirty minutes. Upon the completion of the 30 minutes on the shaker 1ml of

40% PEG, O.1 M LiOAc, TE was added and mixed vigorously by pipetting. From here the cells were incubated on a shaker again at 30°C for 30 minutes. Upon completion of the incubation on the shaker the cells were heat shocked at 42°C for 15 minutes. The cells were then spun in a microcentrifuge for a minute at 13,000 revolutions per minute while having the supernatant removed by aspiration upon the completion of the centrifugation. The cells were then resuspended in 100ul sterile dH_2O and then plated on SD-ura plates and placed at 30°C for three days. The numbers of transformants on the plates were then counted after the completion of the third day.

The transformation of the *gyp7 Δ::ura3* into the three different yeast strains DFS, CAB and EAS is to allow the different reporter genes that are incorporated into these strains to be looked at while under the absence of the Gyp7p within each of these strains. The DFS strain looks at how the spontaneous loss of mitochondrial function affects the overall respiration rates of the yeast with the lost of the Gyp7p. This rate is then compared to the wild-type yeast strain rates. The CAB yeast strain houses a microsatellite reporter sequence in a respiration required COX3 gene that brings the $ARG8^m$ reporter gene out of frame. This yeast strain is important since it will be used to evaluate how microsatellites are generated during replication and repair mechanisms when again Gyp7p is no longer present. The EAS yeast strain is important here because it evaluates how the loss of the Gyp7p will affect the homologous recombination events that may take place within the mitochondria genome. This will show whether or not Gyp7p protein is involved or not in the mechanisms that are involved during homologous recombination events.

Whole Cell PCR Verification of *gyp7::ura3* Transformations Through Polymerase Chain Reaction:

With the construction of the *gyp7 Δ::ura3* knockout cassette and the transformation of the cassette into the three different yeast strains (DFS, CAB, and EAS) verification that transformation actually took place within each of the three different yeast strains is needed. This verification is done through whole cell PCR.

These reactions were conducted under the following conditions: the first cycle of this continuous 31 cycle reaction was at 94°C for 2 minutes, 55°C for 15 seconds,

followed by 72°C for 1 minute. The next 10 cycles where performed at 94°C for 15 seconds, 55°C for 15 seconds, followed by 72°C for 1 minute. The next 20 cycles of this reaction where at 94°C for 15 seconds, 55°C for 15 seconds, followed 72°C at 1 minute plus an additional 5 seconds added to this stage for every reaction there after until reaction set 31. This means cycle set 12 of this reaction had an elongation period of 1 minute while cycle set 13 had an elongation period of 1 minute and 5 seconds, and cycle set 14 had an elongation period of 1 minute and 10 sections etc. This program is called Expand PCR reaction. The components that comprised this reaction were the following: 1.0 µL Taq DNA Polymerase (Bioline, Randolph, MA), 2.5 µL 50 mM $MgCl_2$ (Bioline, Randolph, MA), 1.5 µL at 100 mM dNTP mix (Bioline, Randolph, MA), 1.0 µL pRS-406 1:100 dilution, 36.0 µL dH_20, 1.5 µL 20 µM Gyp7 F-83 up-stream primer, 1.5 µL 20 µM Gyp7 R207 down-stream primer, 5.0 µL 10x NH_4 buffer, and a micropipetter tip gently dipped into sample yeast colonies to the point where a pin tip size amount of cell had been collected on the micropipette tip This reaction generated a total volume of 50 µL in each reaction tube. Results were then viewed on 1.0 % Bioline agarose gel (Bioline, Randolph, MA) using 5.0 µL of 0.5 µg/ml Ethidium Bromide (EtBr) staining.

Respiration Loss Assay:

Respiration loss assay is a technique used to determine the relative function of a single gene knockout construct in helping to identify if this gene has any effect on mitochondria function and performance in relationship to the mitochondria's ability to perform oxidative phosphorylation. The respiration loss assay was conducted using strain *DFS 188 gyp7Δ::ura3*. To conduct this experiment YPG + 0.1% dextrose (Yeast Peptone 2% glycerol +0.1% dextrose) media were used. To perform this assay strain *DFS 188 gyp7Δ::ura3* was streaked out on SD-ura (Drop out or Omission plates) plates and allowed to grow at 30°C for 1-2 nights. 20 individual colonies are picked and a dilution series were generated. Each individually picked colony is first placed separately in an Eppendorf tube with 100 µL of dH_2O and mixed vigorously until no cell pellets exist.

Next, 5 µL is removed and placed into another Eppendorf tube with 500 µL dH_2O followed by vortexing. This generated a $10^{-}2$ dilution set. From here 5 µL from the 10^{-2} dilution set is removed and placed into another Eppendorf with 500 µL dH_2O. This now generated a $10^{-}4$ dilution set. From the 10^{-4} dilution set 100 µL is removed and then plated on YPG + 0.1% dextrose. The 100 µL sample that is applied to the YPG + 0.1% dextrose plates are then spread uniformly by 3-5 mm diameter glass breads being shook over the surface of the plates. The shaking is done by gently rocking the plates from side to side in your hands until the 100 µL has fully been absorbed into the media. The glass beads are then removed and the media is then allowed to incubate at 30°C for three nights. Following, the incubation period the plates were removed and the ρ^{-} petites (experimental colonies or cells that have lost mitochondria function) and ρ^{+} cells (cells with functional mitochondria) are counted. The rate of respiration loss per cell division was calculated using the method of the median (Lea and Coulson et al., 1949). Refer to Appendix A. The experiment was repeated twice.

Directed-Repeat Recombination Assay:

The directed-repeat recombination assay is involved at looking at how homologous recombination rates are affected when the lost of *Gyp7* has occurred. To perform this assay, strain *EAS gyp7Δ::ura3* is used with media plates YPG (Yeast Peptone 2% Glycerol) and YPD (Yeast Peptone 2% Dextrose) media. To perform this assay the strain is streaked out on SD-arg plates and allowed to grow at 30°C for 1-2 nights. From these plates 20 separate colonies were picked and the identical dilution set as previously mentioned in the Respiration Loss Assay was generated. For this dilution set the YPD are the control plates and the YPG are the experimental plates. The YPD

have 50 μL from the undiluted mixture plated onto them, and the YPG have 100 μL from the 10^{-4} dilution set. Again, glass beads are used to uniformly spread the suspended cells over the media. The media is then allowed to incubate at 30°C for three nights. Following, the incubation period the plates were removed and the cells were counted and analyzed. The rate of recombination per cell division was calculated using the method of the median (Lea and Coulson et al., 1949). Refer to Appendix A. The experiment was repeated twice.

Microsatellite Stability Assay:

Microsatellite stability assays were conducted to identify the effects that *Gyp7* has towards regulating microsatellite stability. To perform this assay yeast strain CAB gyp7Δ is used. For this assay the media used is YPD and SD-arg. To perform this assay, the strain is streaked on SD-arg plates and incubated at 30°C for 1-2 nights. From these plates 20 separate colonies are picked and the identical dilution construct mentioned in the respiration loss assay is then generated. The YPD are the control plates and the SD-arg are the experimental plates. The YPD plates have 100 μL of the 10^{-4} dilution set plated on them and the SD-arg have 95 μL of the 10^{-2} dilution set plated on them. Again, glass beads are used to separate the suspended cells uniformly on the media. The plates are then allowed to incubate at 30°C for 3 nights. Following the incubation periods the plates are removed and the cells are counted on both plates and analyzed. The rate of microsatellite mutations per cell division was calculated using the method of the median (Lea and Coulson et al., 1949). Refer to Appendix A. The experiment was repeated twice.

Heteroplasmy Assay:

The purpose of the Heteroplasmy assay was to evaluate how point mutations within the mitochondrial genomes are segregated during mitosis formation of daughter cells. This is important based on the fact that *S. cerevisiae* are homothallic in nature when it comes to their mitochondria. This assay will help to determine whether the parent or daughter cells have the mutated mitochondrial genomes packaged and shipped into them. This assay will help determine if the Gyp7p is involved in this regulation of inheritance of mitochondrial genomes. The Heteroplasmy assays are performed by using yeast strain *EAS gyp7Δ::ura3*. To perform this assay SD+glycerol-arg and SD-arg media are required. *EAS gyp7Δ::ura3* strain is streaked out onto SD-arg and incubated at 30°C for 1-2 nights. From these plates 20 separate colonies are picked and the identical dilution construct mentioned in the respiration loss assay is then generated. The SD-arg plates are the control plates and the SD+glycerol-arg are the experimental plates. 95 μL of the undiluted are plated onto the SD+glycerol-arg plates and 100 μL of the 10^{-4} dilution are plated on the SD-arg plates. Again, glass beads are used to separate the suspended cells uniformly on the media. The plates are then allowed to incubate at 30°C for 7 nights. Following the incubation periods the plates are removed and the cells are counted on both plates and analyzed. The rate of point mutation segregation per cell division was calculated using the method of the median (Lea and Coulson et al., 1949). Refer to Appendix A. The experiment was done only once.

Microscopy of *Saccharomyces cerevisiae* with the loss of *gyp7*:

Microscopy studies of the structure of the mitochondria are useful since we can compare the wild-type structure of the mitochondria, which are a series of branched tubular networks below the cell's cortex to the mutated phenotypes that might be

generated under the absence of the Gyp7p. This will help identify that structure and location of the mitochondria are important in the cells ability to perform the production of ATP through oxidative phosphorylation. Images were taken using *S. cerevisiae* strain *EAS gyp7Δ::ura3*. The culture was grown overnight in 2-3ml of LB rich media. 1 mL of the culture was removed and transferred to an Eppendorf tube and 1mM of Mitotracker stock solution was added. It was then shaken at 30°C for one hour. After, being on the shaker for 55 minutes 1 µL of 10 mM of DAPI stock solution was added and allowed to shake for the last five minutes. Once the full hour has past the cell can be centrifuged for a 1 minute and then washed with ddH_2O, and then resuspended in 200 µL ddH_2O and vortex to ensure no clumping of cells occurs. For slide preparation of the samples the microscope slides need to be coated with 0.1 Poly-L-Lysine coat to help reduce floating and movement of the cells under the microscope. Gently cover the slide to saturation and allow the slides to air dry over night. With mounting the cultures 6 µL of the resuspended cells were applied to the slide and a cover-slip is then placed over the culture sample. Any excess water can be removed by using a Kimwipe. Once the slide were prepared fully they were viewed under 100x oil immersion.

Results:

Yeast Strain Verification:

Once the *gyp7::ura3* knockout cassette was transformed into the three different yeast strains further identification was performed. Twenty individual colonies were picked and tested using the two different primers sets. Primer set one used the GYP7 F-83 and primer GYP7 R207. This primer set would identify if the twenty colonies picked housed the wild-type *Gyp7* gene. If the colonies did have the wild-type *Gyp7* gene the generation of a 290 base-pair band would be seen. This would indicate that the transformation did not work. When the use of this primer resulted in no band production for any colonies another primer set was used to see if the *Ura3* gene had been transformed. This time the GYP7 F-83 primer and the URA3 primer were used. The use of these primers will amplify the ura3 reporter gene generating a 421 base pair band if it is present within the strains indicating that the knockout transformations had worked. The GYP7 F-83 primer has a DNA sequence of 5' – CGT GCT CAA ATA TAC TAT TCA G

– 3' and the GYP7 R207 primer has a DNA sequence of 5' – GGA CAT GTG CCC ACT TTA GC – 3'. The ura3 primer's DNA sequence is 5' – CCG TGT GCA TTC GTA ATG TC – 3'. Depending on what primer set you decide to use either one will identify whether of not the transformations of knockout work based on the results they provide, however the use of two primer sets helps to accurately identify that the transformation of the knockout cassette worked properly.

Respiration Loss Assay:

The respiration loss assay (Figure 10) uses the yeast strain *DFS 188 gyp7Δ::ura3* (Refer to figure 1). This particular assay is used to determine if *Gyp7* gene knockout has any affect on the function of mitochondria. This assay will show if the removal of the *Gyp7* gene will be involved in generating yeast cells with functional or non-functional mitochondria. This selection of yeast cells with functional or non-functional mitochondria is coordinated by the 0.1% dextrose and 2.0% glycerol that are incorporated into the media that this strain is grown on. This selective and differential media is important because it generates either rho^- petites or rho^+ phenotypes. When yeast loses mitochondrial function they resort to fermenting carbon sources for energy production. This assay limits the amount of fermentable carbon by only allowing 0.1% dextrose in the media and then supplying the cells with glycerol as the only other carbon source in the media, which is not fermentable. When the dextrose is used to exhaustion the glycerol induces the cells lacking functional mitochondria to become inviable because glycerol is non-fermentable and can't perform anerobic respiration anymore preventing cellular division, and keeping the colonies smaller in size (rho^- petites) to there healthy siblings with functional mitochondrial (rho^+.) This assay generated experimental average value of $2.3x10^{-3}$ cell mutational rate on this media when *Gyp7* was removed. The wild-type value

was $3.9x10^{-4}$ for when *Gyp7* was present. This generated a 5.89 fold increase in the cell mutational rate. Refer to figure 1.

Directed-Repeat Recombination:

The Direct-Repeat assay uses (Figure 11) the yeast strain *EAS gyp7 Δ::ura3* (Refer to figure 1) and we are looking at how the lost of *Gyp7* is involved in altering homologous recombination rates within mitochondrial DNA. When homologous regions of DNA come into contact with each other intragenic recombination has the possibility to take place within the genome. If this takes place there is a possibility for spontaneous loss of function in mitochondrial function by the deleting of DNA between the regions of DNA containing homology. If the removal of DNA takes place it can lead to rho$^-$ petite phenotype. This particular assay uses the yeast strain *EAS gyp7Δ::ura3*. In this particular yeast strain the *Cox2* gene is used. This gene is a mitochondrial gene involved in the mitochondrial electron transport chain by regulating electron movement from cytochrome C to oxygen. In this strain the *Cox2* gene is linked to the *ARG8* gene, which is integrated into the mitochondrial genome. What is important here is that fact that the *ARG8* gene is a nuclear gene that encodes for the amino acid arginine, and is now connected to a mitochondrial gene. In this construct the *Arg8* becomes flanked on both sides by identical 100bp sequences of the *Cox2* gene. By inserting the *Arg8* into the *Cox2* gene this disrupts the *Cox2* gene from being properly transcribed and translated. With the newly inserted *Arg8* gene this creates an Arg+ phenotype, however at the same time generating the inability to respire on non-fermentable carbon sources by disrupting the *Cox2* gene which is needed for respiration to occur. With this fact the use of glycerol is used as the sole carbon source in this media since it is non-fermentable. With this construct created any

homologous recombination events that occur in the region of homology of the *Cox2* gene regions will expunge the *Arg8* gene and generate a functional *Cox2* gene that can be transcribed and translated. When the homologous recombination events do occur this now generates a new phenotype since it removed the *Arg8* gene resulting in an Arg- phenotype while also generating and acquiring the ability to respire on glycerol. To show that homologous recombination is actually taking place the cells are plated out on media lacking arginine to allow for a numerical value of cells that contain the *Arg8* gene to be generated and counted, while also plating cells onto glycerol media to show that recombination events have occurred, and that the *Arg8* has been removed by homologous recombination resulting in an active *Cox2* transcript.

The calculated rate of recombination for the wild-type was 8.15×10^{-5}. The rate of the mutant EAS gyp7Δ::ura3 was 1.80×10^{-5}. This generated a 4.5 fold decrease in the cell mutational rate. (Refer to figure 1.)

Microsatellite Reporter:

The microsatellite reporter assay (Figure 12) was conducted to determine if the lost of the gene *Gyp7* is involved in coordinating and regulating the frequency of how often additions or deletions of repetitive DNA sequences (microsatellite regions) are acquired during replication of the mitochondrial genome due to DNA polymerase slippage events. This particular assay used the yeast strain *CAB-193 gyp7Δ::ura3*. The addition or deletion of microsatellites caused by DNA polymerase slippage events can be responsible for the spontaneous loss of gene function, which can then generate loss of mitochondrial function by altering the frame-shift within the genome altering the codon reading frames. Just like the Directed-Repeat assay the microsatellite also uses the nuclear gene *Arg8* and

the mitochondrial gene *Cox3*. Again, the *Cox3* gene is fused to the *Arg8* gene, but the *Cox3* also has 16 repeats of poly(GT or AT) attached to the 5' end of the *Cox3* reporter gene. The addition of the poly(GT or AT) tracks throws the natural reading frame of the *Arg8* gene out of frame generating typically Arg- phenotypes. What this reporter construct assays for are spontaneous mutational events that bring the reading frame for the *Arg8* gene back into frame and newly generating Arg+ phenotype. By bring the *Arg8* back into frame this allows for the transcription of the *Arg8* gene to occur allowing for the synthesis of arginine to begin since the media lacks arginine. This construct is created so the *Arg8* gene is out of frame and read at the +2 site. If any spontaneous mutations arise base pairs are either added or deleted due to the DNA polymerase slippage events. DNA polymerase slippage causes a frame shift event to occur which can either bring the *Arg8* back into frame or continue to keep it out of frame. This experiment is a simple technique used to measure the rate at which the coding sequence is brought back into its natural reading frame.

For the microsatellite reporter assay the wild-type mutational rate was found to be 2.25x10^-6 while the experimental value using *CAB gyp7Δ::ura3* was found to be 1.05x10^-6. This generated a 2.14 fold decrease in the cell mutational rate. (Refer to figure 1).

Heteroplasmy Assay:

This assay was conducted to determine how the segregation of the mitochondrial genomes occurred during replication. This assay uses the yeast strain *EAS gyp7Δ::ura3* (Refer to figure 1) The mitochondrial genomes replicates themselves using the "rolling circle model" method of replication. This method generates linear strands of DNA. The

progeny DNA strands being newly synthesized can reach several mitochondria genomes in length. These genomes are interconnected to one another and are called concatemers. The individual mitochondrial genomes comprising these concatemers have to be separated from one another through enzymatic activities. If separation of the genomes happens to takes place at inappropriate places, incomplete genomes will be generated. This could lead to mitochondria that have lost the ability to respire due to the lost of these specific genome regions that were left out when they were separated and packaged into the nucleoids. This assay works by using the *Arg8* gene the *Cox3* reporter gene. If segregation of the mitochondria takes place properly the mitochondria will posses the *Arg8* gene and a functional *Cox3* reporter gene involved in respiration. When these cells are grown on SD+glycerol-arg plates only cells that have had the proper segregation of the genome that incorporated the *Arg8* gene and a functional *Cox3* gene will it be able to grown on media lacking arginine, while also only having glycerol as the only carbon source which is not fermentable.

This assay is still in development and determining the exact quantities to plate to generate statistically viable plates that are countable has not been identified yet. At this time we are looking at plating 50 ul of the 10^{-4} dilution and 100 ul of the 10-^{4} on the experimental plates (SD+glycerol-arg), and 95 ul of the undiluted being plated on the control plates (SD-arg.) The 50 µl of the 10^{-4} dilution generated a value of 2.00×10^{-6} and the 100 µl of the 10-^{4} generated numbers of 7.28×10^{-7} for the *EAS gyp7Δ::ura3* strain.

Microscopy: The effects *Gyp7* On Mitochondria Cellular Structure

Microscopy was used to determine if the lost of *Gyp7* would have any effects on the overall structure of the mitochondria within the cells. The fluorescence staining

markers of Mitotracker and DAPI were used to stain the mitochondria and the mitochondrial genomes. Mitotracker fluorescence dyes work by covalently bind free sulfhydryls within the mitochondrial plasma membranes. DAPI staining works by the nuclear stain emitting blue fluorescence upon binding to AT regions of DNA.

The three images seen for wild-type and mutant can be seen in Figure 2 are all under 100x oil immersion. The first picture is captured only using transmitted light only. The second picture shows the mitochondria stained with mitotracker (red), and finally the third picture shows the nuclear and mitochondrial genomes stain with DAPI (blue.) The mitotracker shows that *Gyp7* does alter the physical structure of the mitochondria. The mitochondria are separated from one another and are shaped in an oval manner instead of being tubular and interconnected with the other mitochondria that are also present within the cells. The DAPI staining shows the locations of the nucleoids that house the mitochondrial genomes as well as some of the nuclear DNA within some of the cells. (Refer to figure 2).

Discussion:

This investigation into the function of the *Gyp7p* on mitochondrial welfare helped show how the loss of the *Gyp7* gene in the genome would directly and/or indirectly affect the respiration rates of mitochondria DNA ρ^- petite formation rates. The respiration loss assay, directed-repeat homologous recombination reporter, microsatellite stability reporters, heteroplasmy reporter, and microscopy assays were used as the molecular protocols used to evaluate and analyze the rate of ρ^- petite formations generated by *Gyp7's* involvement.

The respiration loss assay (Refer to Figure 10) is used to identify whether the removal of nuclear gene and the protein by-product of that gene alters the respiration rate within the yeast cells by either altering oxidative phosphorylation natural rate or by completely rendering it non-functional by completely stopping oxidative phosphorylation. The directed-repeat homologous recombination reporter assay (Refer to Figure 11) involving the use of homologous recombination can be used to remove mutational occurrences and determine how often this occurrence happens within the mitochondria genome. The microsatellite reporter assay (Refer to Figure 12) focuses on the frequency of DNA polymerase slippage events that are incorporated in new additional DNA segments or deletions of inherit DNA segments. The heteroplasmy assay is used to determine how the mitochondria genomes are being separated from one another during the replication process of the rolling circle method. Microscopy of the yeast cells was also used to see if any structural changes occurred due to the absence of the *gyp7p* (Refer to Figure 2). These techniques will be important in showing whether altering the genotype also altered the phenotype of the mitochondria and this alteration in phenotype could also be contributing to the alteration in the mitochondria DNA ρ^- petite formation rates.

With the yeast *Gyp7* protein being identified as a GTPase-activator protein, and with it already known that GTPase-activator proteins having the functions of acting as molecular switches to regulate growth, morphogenesis, cell mobility, cyotkinesis, and transport vesicle targeting, and fusion with specific acceptor compartments membranes this makes it a good model to see how these molecular mechanisms are involved in the

overall homeostasis of the yeast cells, and directly the function of the mitochondria within these yeast cells.

The wrongful addition of base-pairs into the genome is regulated and controlled by two proofreading mechanisms that correspondingly work together to ensure the high efficiency of proper base-pair, pairing during DNA replication. The first activity is the activity of the DNA polymerase. In *S. cerevisiae* there are three DNA polymerases that work together during DNA replication. The three polymerases have been identified as either to be α, δ, and ε polymerases. Of these three polymerases δ and ε are extremely important since it is believed that they contain 3'-5' exonuclease proofreading activity. This activity is essential for proper homologous recombination to take place by removing the 3' ends. The second suggested proof reading role resides in the enzymatic activities involved in DNA mismatch repair. These enzymes identify mismatched base-pairs that were newly generated in the newly synthesized daughter DNA strand compared to the template DNA strand. This recognition between template and newly generated DNA strands is done by the identifying the parent DNA strand since it has become methylated and the newly synthesized has not become methylated yet.

Previous studies have shown that recombination resulting with repeated sequences within the genome can be performed by several recombination mechanisms. These mechanisms can use either direct or inverted orientations of the sequence within the DNA and can lead to deletions of the intervening non-homologous DNA regions during the recombination process. In yeast two mechanisms have been recognized and established as the major recombination mechanisms in nuclear DNA. One mechanisms is the sister chromatid exchange (SCE) and the other is single-strand annealing (SSA). In both processes the first step in each is the creating of double-strand breaks (DSBs) in the DNA that can occur within the repeated sequences or within intervening non-homologous DNA sequences of the chromatids.

If the DSBs happen to occur within repeated sequences it generates ends that now posses the ability to invade other sister chromatids. If misaligned sequences try to recombine possible deletions can arise. The second and most creditable process for recombination between repeated segments is the SSA. In this mechanism you have

double-stranded DNA breaks occurring within the DNA, but outside the repeated regions within the DNA. These breaks within the DNA now generated a newly formed 5' and 3' ends within the once continuous parent strands of DNA. The next step is for a 5'-3' exonucleases to create 3' single stranded ends and gaps within the DNA. This allows the repeated sequences to come into contact with one another allowing them a chance to anneal with one another. When the repeated regions of DNA start to anneal with one another this displaces their 3' ends and allowing the 5' of the parent DNA to move back into position for ligation to occur sealing the original DNA and the newly created gaps back into one continuous double-stranded DNA . For this ligation to occur between the repeated DNA regions that have newly annealed together and the remaining parent DNA strands a 3' flap endonuclease removes the 3' ends off the repeated DNA sequences. This removal of DNA now allows the 5' ends of the parent DNA to ligate back to the newly annealed repeated DNA sequences sealing the original DNA breaks that occurred. In this process the activities of the exonucleases removes DNA from the parent DNA template resulting in base-pair deletions. In this process several nuclear chaperone proteins such as Msh2p and Msh3p are required to help stabilize and regulate annealing of the homologous DNA repeats (Prado. F. *et al.*, 2003).

In studies involving recombination in mitochondria DNA genomes, no proteins have been fully identified as having any true activity in regulating or coordinating involvement in mitochondrial DNA replication. However, several potential proteins have been identified as having some activities in this event. MHR1 was isolated during screens for mutants with deficiencies in mitochondria recombination during yeast mating (Ling, F. *et al.*, 1995). It also has been identified as being used in helping to align single-stranded DNA with homologous double-stranded DNA (Ling, F. *et al.*, 2002). Another protein suspected of having a role in mitochondria genome recombination is Nuc1p. This protein is believed to be involved in controlling the suggested 5'-3' exonuclease activity that is required for the double-stranded break (DSB) model. It is thought that this protein is used to generate the 3' overhanging ends that are needed to achieve strand invasion and proper alignment of the homologous repeat DNA segments (Zassenhaus, H. P. *et al.*, 1994).

Microsatellites contribute to genome instability in nuclear and mitochondria DNA by altering the mechanisms by which the DNA polymerase reads the template DNA

strands. These alterations are brought on by these repeated DNA base pairs being grouped next to one another. Being in close proximity to one another confuses the DNA polymerase during the replication process. As the polymerase moves along the DNA strand it encounters these repeated groupings of base-pairs during the replication process. As DNA transcription begins these repetitive sequences hinder the fidelity ability of the polymerase to stay bound to the parent DNA template. The repetitive sequences "bewilder" the polymerase. This stimulates the DNA polymerase to separate and try to properly realign itself on the parent DNA strand. By disassociating from the parent DNA strand the replication rate decreases as the polymerase moves slower across the parent DNA strand. Secondly, these repeats can hold the polymerase stagnant on the parent DNA strand while it searches for the proper nucleotides to be matched correctly. Thirdly, the addition or deletion of improperly positioned DNA base-pairs can be generated and incorporated into the DNA. The addition and deletion mechanisms the polymerase disassociates and then re-associates improperly from the point from which it disassociated by either moving downstream or upstream from where it disassociated from generating the deletions or additions within the newly transcribed daughter DNA strand. The addition and deletion of base-pairs can change the natural reading frames of the genes within the DNA. This alteration in the reading generates a domino effect by possible altering the codon sequences used to encode for the amino acids during the translation of proteins from that particular gene. Also, a +1 or –1 in reading frame can also generate null or stop mutations. A stop codon in the middle of the gene can be generated and a truncated protein could be translated. This newly truncated protein now has a different primary, secondary, and tertiary structures and these alteration could potentially cause completely lost in its intended function or significantly reducing its function altogether. This suggested infidelity of the polymerase in regions of microsatellites in nuclear genomes is also the same mechanisms suggested by this data. To remove the +1 frame-shift in the reporter gene (arg8(m)) in this particular case you can either remove the extra added base pair that has been added to the reporter gene that is throwing the reading frame out of frame or you can add two more base-pairs into the reporter sequence to properly orient the DNA polymerase so the proper amino acid codons are now in frame.

The heteroplasmy assay is still in the developmental stage thus far. However the preliminary data generated from it provides us with a general idea of how the mitochondria are being separated and packaged from one another during the replication process. In the *gyp7Δ::ura3* strain there is a 2.74 decrease in heteroplasmy resulting in more mutated mtDNAs being present within the mother cells.

After analyzing the final mutations / cellular division for the respiration, directed-repeat, microsatellite, and heteroplasmy assays the conclusion that can be established contradicts the original hypothesis on the grounds that the removal of the *Gyp7* gene decreases the fold difference between *wild-type* and the *gyp7Δ::ura3* strain in the directed–repeat, microsatellite, and heteroplasmy genetic reporter assays, which were not expected. This indicates that the removal of the *Gyp7* gene is not involved in preventing homologous recombination events from taking place, the incorporation of microsatellite regions that can lead to frameshift mutation in the reading frame of the amino acid codons, increased DNA polymerase infidelity, and the segregation of mutated DNA from mother to daughter cells. The truly interesting conclusion that can be identified from this data is that there is a decrease in occurrence of these mutations when *Gyp7* gene is removed, which then generates an increase in the fold difference for the respiration loss assay. This assay indicates the over viability (how healthy is the cell) of the cell to continue to perform its necessary metabolic activities to survive and mainly the production of ATP through oxidative phosphorylation. Any decrease in this rate shows that the cells are struggling to maintain healthy metabolic activity due to reduced ATP production. The removal of the *Gyp7* gene actually seems to increase the cells ability to handle DNA mutations and increases its ability to repair these problems, however at the same time sacrificing the performance of cellular respiration in the mitochondria, which then reduces the energy output generated by the mitochondria forcing the cell to then revert to alternative energy generating mechanism such as fermentation to maintain a sufficient level of energy being generated within the cell.

Analysis of the mitochondria structure in wild-type vs. experimental yeast cells has shown that there is a considerable difference in the physical structure of the mitochondria. Under 100x oil immersion lens the wild-type mitochondria when stained

with Mitotracker and Dapi can be seen as tubular structures interconnected between each mitochondria equally distributed throughout the cytosol. In yeast cells that have had the *Gyp7p* removed the mitochondria structure has changed. They have taken on a phenotype where the wild-type tubular structures have collapsed onto one another. Also, you can see the mitochondria are starting to congregate on one side of the yeast cells in some cases. The Dapi stains the mitochondria and nuclear DNA within the cell. This is important since the mitochondria DNA should be located near or exactly where the mitochondria are within the cell. When comparing the Mitotracker and Dapi pictures in figure 2 they correspond in locations. The changes in appearance suggest by these images is believed to be responsible by the role *Gyp7p* since no other known mutations were introduced into the yeast cells used for these images. These alterations in appearance can be either directly responsible by the loss of the *Gyp7p* or the secondary effects it would have stimulated by interacting with other unidentified cellular components. These images as a whole support the belief that *Gyp7p* is involved in maintaining the wild-type structure and ensuring uniform dispersal of the mitochondria throughout the cell.

Further research in mitochondria genome stability is needed and is underway as we speak. Several other identified genes associated with mitochondria activity have been targeted for further study. Genes *Fmp35*, *Clu1*, and *Yolo57w* have been identified as having mitochondria activity based on the results of a 2-hybrid yeast screen using Ilv5p, a known mitochondrial protein, by previous and graduated Masters student Anthony J. Mirando. A good direction to continue in with this study would to use the *Gyp7p* as the "bait" protein in the 2-hybrid yeast screen and see what other interactions could be identified through this assay using the *Gyp7p*. Continual research on these genes will provide a better under standing of the exact regulation and coordination of the mitochondria genome by establish plausible mechanism while at the same time eliminating extraneous ones as the research data are generated for each of these individual genes. Also, the next step in this process once each individual gene has been fully researched will be to see how these four genes and any other newly identified genes or proteins interact with each other in controlling this extremely delicate and precise mechanism. Each of these genes is currently under research by either fellow Master

students or undergraduate students here at the State University of New York College at Brockport.

The reason this research is needed and is so beneficial to humanity is because of the many diseases that have been linked and thought to be possible caused by containing mutant mitochondria. With further understanding of what causes this dysfunction of the mitochondria to arise and how to prevent and repair this dysfunction could hopefully help in the process of curing or generating new clinical treatment methods for such heartfelt and emotional diseases such as Amyotrophic lateral sclerosis (Lou Gehrig's Disease), Kearns-Sayre syndrome, Alzheimer disease (AD), Huntington disease (HD), Friedreich ataxia, hereditary spastic paraplegia, and rare familial forms of Parkinson disease (PD).

Literature Cited

1. **Bidichandani, S.I., Ashizawa, T., and Patel, P.I.** 1998. The GAA triplet-repeat expansion in Friedreich ataxia interferes with transcription and may be associated with an unusual DNA structure. *Am. J. Hum. Genet.* **62:** 111-121

2. **Contamine, Véronique., and Marguerite Picard**. 2000. Maintenance and Integrity of the Mitochondrial Genome: a Plethora of Nuclear Genes in the Budding Yeast. Micorbiol *Mol Bio Rev*. June; 64(2):281-315.

3. **Duntze, W., V.L. Mackay, and T.R. Manney.** 1970. *Saccharomyces cerevisiae*: a diffusible sex factor. *Science.* 168: 1472-1473

4. **Epstein, C. B., J. Waddle, W. Hale, V. Dave, J. Thornton, T. Macatee, H. Garner, R. Butow.** 2001. *Mol Cell Bio*. February; 12(**2**): 297–308.

5. **Gacy, A.M., Goellner, G., Juranić, N., Macura, S., and MacMurray, C.T.** 1995. Trinucleotide repeats that expand in human disease form hairpin structures in vitro. *Cell* **81:** 533-540

6. **Hancok, J.M.** 1995. The contribution of slippage-like processes to genome evolution *J. Mol. Evol.* **41**: 1038-1047

7. **Hancock, J.M.** 1996a. Simple sequences in a 'minimal' genome. *Nat. Genet.* **14:** 14-15.

8. **Hancock, J.M.** 1996b. Simple sequences and the expanding genome. *Bio. Essays.***18:** 421-425

9. **Hawthorne, D.C.** 1963. A deletion in Yeast and its bearing on the structure of the mating type locus. *Genetics*. **48**:1727-1729

10. **Herskowitz, I.** 1998. Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Micro. Biol. Rev.* 536-553

11. **Hicks, J.B., and I. Herskowitz.** 1997. Interconversion of yeast mating types. II. Restoration of mating ability to sterile mutants in homothallic and heterothallic strains. *Genetics.* **85**:373-393

12. **Jakupciak, J.P. and Wells, R.D. 1999.** Genetic instabilities in (CTG•CAG) repeats occur by recombination. *J. Biol. Chem.* **274:** 23468-23479

13. **Kashi, Y., King, D., and Soller, M.** 1997. Simple sequence repeats as a source of quantitative genetic variation. *Trends Genet.* **13:** 74-78

14. **Kurjan, J.** 1985. α-factor structural gene mutations in *Saccharomyces cerevisiae*: effects on the α-factor production and mating. *Mol. Cell. Biol.* **5**:787-796

15. **Lea and Couslson.** 1949. *J. Genetics*. **49**:264-285

16. **Ling, F., Makishima, N.Morishima, and T. Shibata**. 1995. A nuclear mutation defective in mitochondria recombination in yeast. *EMBO J.* **14**:4090-4101.

17. **Ling, F., and T. Shibata**. 2002. Recombination-dependent mtDNA partitioning: *in vivo* role of Mhr1p to promote pairing of homologous DNA. *EMBO J.* **21**:4730-4740.

18. **Manfredi, G., and E. Schon.** 2003. Neuronal degeneration and mitochondrial dysfunction. *J. Clin. Invest.* Feb 1; **111**(3): 303-312.

19. **Mason, PA., Matheson EC., Hall AG., Lightowlers RN**. Mismatch repair activity in mammalian mitochondria. *Nucleic Acids Res*. 2003 Feb 1; **31**(3):1052-1058

20. **Mewes HW, et al. MIPS**: A database for genomes and protein sequences. *Nucleic Acids Res.* 2000; **28**:37–40

21. **Newman, S.M., Zelenaya-Troitskaya, O. Perlman, P.S., Butow, R.A.** 1995. Analysis of mitochondria DNA nucleoids in wild type and a mutant strain of Saccharomyces cerevisiae that lacks the mitochondria HMG box protein Abf2p. *Nucleic Acids Res.* Jan 15; **24**(2): 386-393.

22. **Oswa, S., T. H. Jukes, K. Watanabe, A. Muto**. 1992. Recent evidence for evolution of the gentic code. *Microbiol Rev.* **56**(1):229-264

23. **Phadnis, N. Sia, R.A., and E.A. Sia.** 2005. Analysis of repeat-mediated deletions in the mitochondria genome of Saccharomyces cerevisiae. *Genetics*. 105.047092

24. **Prado, F. Cortes-Ledsma, P. Huertas, and A. Aguilera**. 2003. Mitotic recombination in *Saccharomyces cerevisiae*. *Curr. Genet.* **42**:185-198.

25. **Pringle, J.R., and L.H. Hartwell.** 1981. The Saccharomyces cerevisiae life cycle, p. 97-142. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast Saccharomyces: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor. NY.

26. **Sia, E. A., Butler, C., Dominska, M., Greenwell, P., Fox, T. D., Petes, T. 2000.** Analysis of microsatellite mutations in the mitochondrial DNA of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. Jan, 4; 97(1): 250-255.

27. **Sia, E. A., Dominska, M., Stefanovic, L., Petes, TD**. 2001. Isolation and Characterization of Point Mutations in Mismatch Repair Genes That Destabilize Microsatellite in Yeast. *Mol Cell Biol.* Dec, 21(**23**):8157-8167.

28. Sia, E. A., Jinks-Robertson, S., Petes, T. 1997. Genetic control of microsatelliete stability. *Mutation Research.* **383**:61-70

29. Sia, E. A., Kokoska, R. J., Dominska, M., Greenwell, P., and Petes, T. D. 1997. Microsatellite instability in yeast: dependence on repeat unit size and DNA mismatch repair genes. *Mol Cell Biol.* May; 17(**5**): 2851–2858.

30. Tautz, D. and C. Shlotterer. 1994. Simple Sequences. *Genetic Dev.*, **140**: 965-972.

31. Tzagoloff, A., Dieckmann CL. 1990. *PET* genes of *Saccharomyces cerevisiae. Microbiol Rev.* **54**:211–225

32. Usdin, K. 1998. NGG-triplet repeats form similar intrastrand structures: Implications for the triplet expansion diseases. *Nucl. Acids Res.* **26:** 4078-4085

33.Wilkinson, L.E., and J.R. Pringle. 1974. Transient G1 arrest of Saccharomyces cerevisiae of the mating types α-factor produced by cells of the mating type a. *Exp. Cell. Res.* 89:175-187.

34. Zassenhaus, H. P., T. J. Hofmann, R. Uthayashanker, R. D. Vincent, and M. Zona. 1988. Construction of a yeast mutant lacking the mitochondrial nuclease. *Nuc. Acids Res.* **16**:3283-32-96

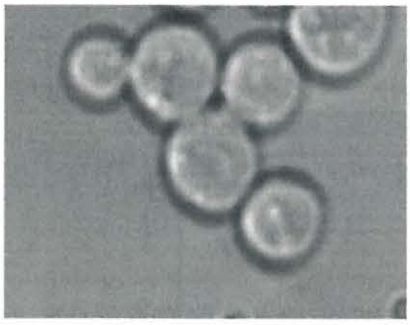
Figure: 1 **Yeast Strains And Assay Values**

	Strains:		
	Wild Type	*gyp7Δ::ura3*	Fold Difference
Assays:			
Respiration Loss			
	4.6x10-4	1.43x10-3	5.89↑
	3.2x10-4	3.18x10-3	
Average	**3.9x10-4**	**2.30x10-3**	
Directed Repeat			
	6.8x10-5	1.80x10-5	4.5↓
	9.5x10-5	1.80x10-5	
Average	**8.2x10-5**	**1.80x10-5**	
Microsatellite			
	1.49x10-6	1.10x10-6	2.14↓
	3.00x10-6	1.00x10-6	
Average	**2.3x10-6**	**1.05x10-6**	
Heteroplasmy			
	2.00x10-6	7.28x10-7	2.74↓

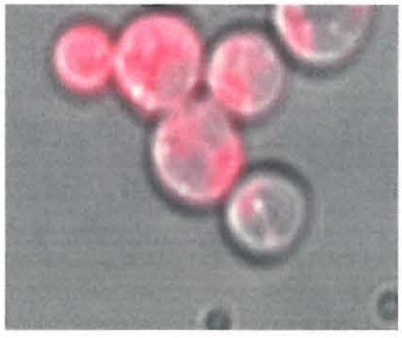
Units: Mutations / Cellular Division

Figure: 2

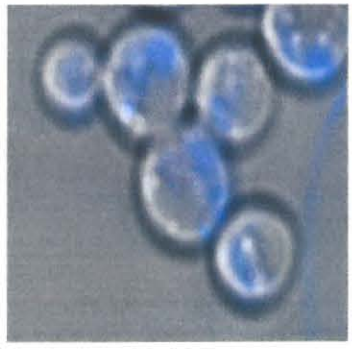
This figure shows the appearance of the mitochondria under 100x oil immersion stained with Mitotracker and DAPI staining compared to brightfield analysis when Gyp7p has been removed. You can see the mitochondria have been stained and have lost their wild-type structure while condensing to one side of the cell in image stained with mitotracker (red). The DAPI (blue) presents the location of the mitochondria DNA. The location of the mitochondrial DNA should be approximately in close proximity to the mitochondria. Also, you can see that the nuclear DNA has also become slightly stained by the DAPI and is visible in some of the cells.



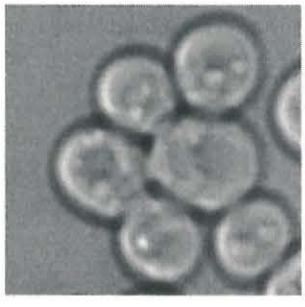
Wt - Brightfield



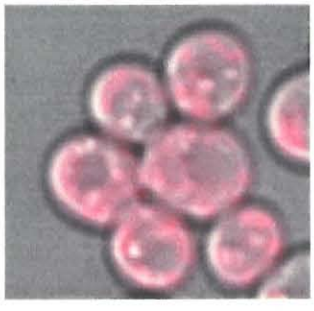
Wt - Mitotracker



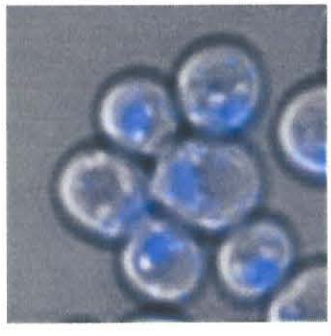
Wt - DAPI



Gyp7 (-) Brightfield



Gyp7 (-) Mitotracker



Gyp7 (-) DAPI

Figure: 3

Saccharomyces cerevisiae's GYP7 Protein-Protein Blast Results

A blast search using the 746 amino acid sequence of *GYP7* against other documented and published proteins within the National Center for Biotechnology Information's protein database (http://www.ncbi.nlm.nih.gov/BLAST/. This figure represents the general results in comparing the amino acid sequence of GYP7 to the relative conserved regions to proteins with similar or matching regions. Regions of high conservation between proteins can be indicated by color and length. The arrow pointing to the first line below the alignment score key can identify *GYP7*. The following figure presents a more detailed analysis of 8 different proteins from 8 other model organisms.

Figure: 4

Saccharomyces cerevisiae's GYP7 Protein-Protein Blast Alignment

GYP7 protein sequence was obtained through (http://www.yeastgenome.org/). A Blast search using GYP7 protein sequence against other protein sequences published in the NCBI Protein-Protein database was then conducted. (http://www.ncbi.nlm.nih.gov/BLAST/). The results for the following specimens (See Key) were identified as having conserved regions in comparison to GYP7's protein sequence through the Blast search. Their protein sequence along with GYP7 were then aligned by ClustalW Multiple Sequence Analysis (http://www.ebi.ac.uk/clustalw/). Conserved regions can be identified by identical amino acid letter abbreviations matching in a vertical manner between the specimens.

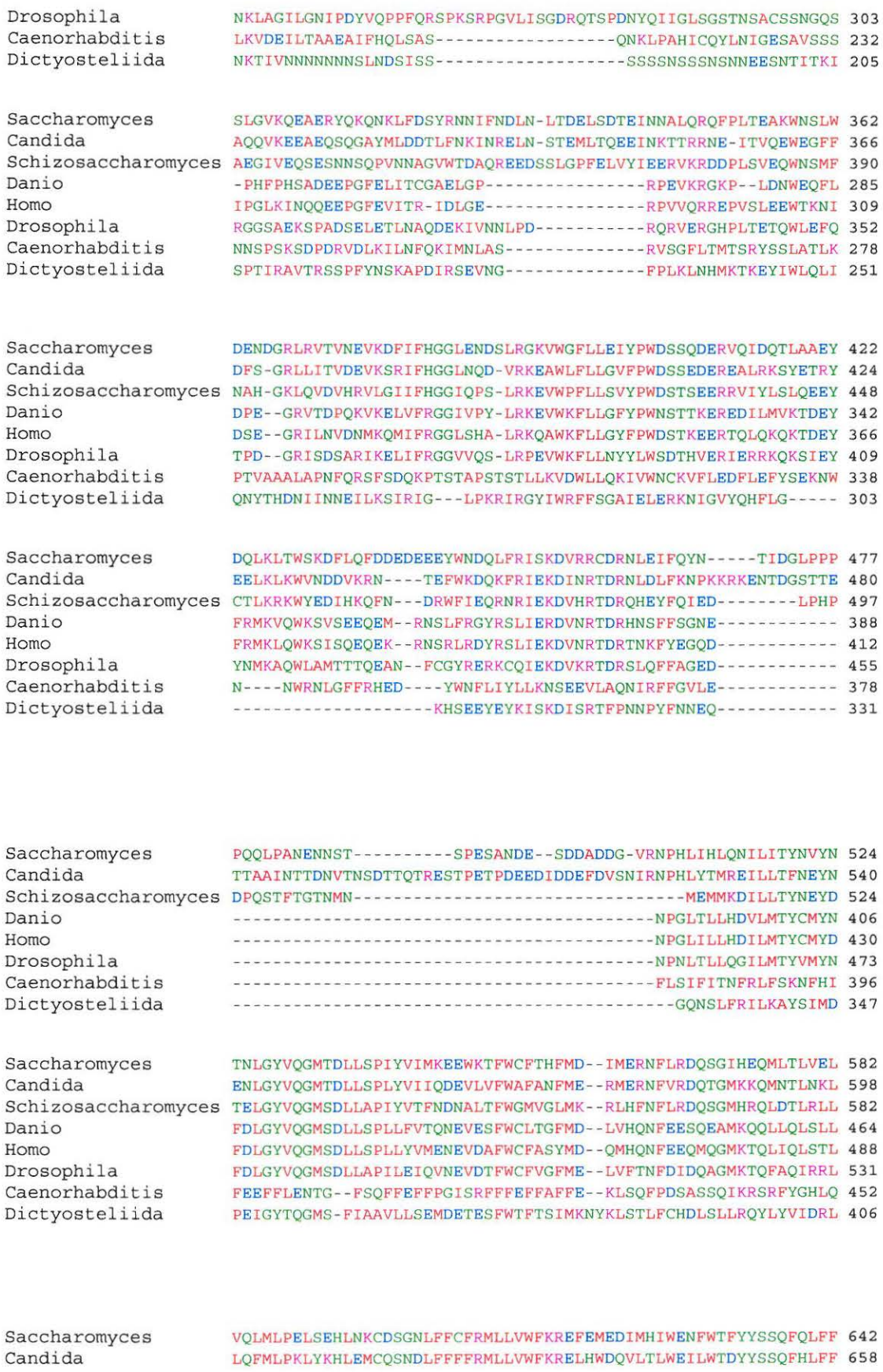
Key: Specimens	Accession #	Protein Definition:
Caenorhabditis elegans	T27026	Hypothetical protein Y48E1C.3
Candida albicans SC5314	AACQ01000166.1	Hypothetical protein CaO19.6706
Danio rerio (zebrafish)	BC066727.1	Zgc:110443 protein
Dictyosteliida dictyostelium	XP_63144.1	Hypothetical protein DDB0188123
Drosophila melanogaster (fruit fly)	NM_134659.2	CG11490-PA
Homo sapiens	AK022147.1	Unnamed protein product
Saccharomyces cerevisiae (baker's yeast)	NC_001136.6	GYP7 (GTPase-activating protein for yeast)
Schizosaccharomyces pombe (fission yeast)	AL109832.1	SPAC630.05

Amino Acid Aberviations	Color	Characteristics
AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RHK	MAGENTA	Basic
STYHCNGQ	GREEN	Hydroxyl + Amine + Basic - Q
Others	Gray	

(http://www.ebi.ac.uk/clustalw/color_frame.html)

Alignment of specimens:

```
Saccharomyces          ----------------------MS----KILFCKSKVFLHP--TSDARDNIAGFLLLTL 31
Candida                ----------------MPTKRTLSSNEVELLYVKSKVCLHP--SPSKKDNIAGFLTLSR 41
Schizosaccharomyces    MTTLESLDMPEMTEVEDDTVDIHIDNSKVALLFSKSKVFVHP--TSKMKDNISGYLSLSK 58
Danio                  ----------------------EAKPESHKLIFEKEGVYLHT--NAKRSN-QDTTIPGFI 35
Homo                   --------------------MAAAGVVSGKIIYEQEGVYIHS--SCGKTNDQDGLISGIL 38
Drosophila             ---------------------MDESVRYDRCHQVQLGVFNESQLSSVEEDVQNLPQQGTG 39
Caenorhabditis         ------------------------------QYLREKDVARTDRTVPFFQGDDNVNLVHLH 30
Dictyosteliida         ---------------------------------MDTKIDYQP------TIQEDKVVDIKY 21

Saccharomyces          EANKLSHQAILQYIPESGLSTLEISKLLKHEAKVGTCPTSTPFVIENSINFSNLVNTS-- 89
Candida                PPRATNLEILLSYVPESQLSTEELKIYQQVDVEDLDLNLGSVNNINHDHHKQNKASTSRI 101
Schizosaccharomyces    SKALGNSSVAGSDILLSWVPDSFLKNRPRDLSVFQNAETLSNGSIREWVEIPQHLDYS-- 116
Danio                  RIVERDGEPALEWSPVE--DDGRNAPAVFYTKKDG-----------EGGEEETKFDPG-- 80
Homo                   RVLEKDAEVIVDWGPL---DDALDSSSILYARKDSSSVVEWTQAPKERGHRGSEHLNS-- 93
Drosophila             NILFTHDGVLLKKASAEHIADLNTSGSLSLVEYSRSIAEMPRRRLLLEWQPNDSIMIAD- 98
Caenorhabditis         NVLMTYVMYNFDLGYVQGMSDFASPLLFVMKDEVDT------------------------ 66
Dictyosteliida         NISSSDDGNVINMSGGGGGGDVGNSCCISEDNHQKG------------------------ 57

Saccharomyces          ---------LGQAFEISLSQIYCIQFRPPSP---NGWYVGSLVIYPLTEQFTGFQPPVLF 137
Candida                VSKPSQSVLTGYAFNVQLSFIYSIQFRTPS----HGYWYGSIVLN----LQDGEKLPILF 153
Schizosaccharomyces    -------------FSVRLCSIYSIIFRPPR----YGWNYGSIVIN---LRDSGESLPPLF 156
Danio                  -------------YEPDWAVISTVKQDRR--------QEQPPVRETGHWSSFSLPLSELY 119
Homo                   -------------YEAEWDMVNTVSFKRKP----HTNGDAPSHRNGKSKWSFLFSLTDLK 136
Drosophila             ----------DSQDQGDWALVDRISGRTRTTSECRAFNTRPIEPSGGATTRSRVMRAQLE 148
Caenorhabditis         ----------------FWCFVGLMELTQKN------FETDQAFIKLQMNQLRDLVMIINP 104
Dictyosteliida         -------------------IIRNSSSGTIN-------------------NILGTDDNNNN 79

Saccharomyces          FHDQLCPSTTDK-LKRLRKSMNPFDDSDELYWGGVDLRNKINELMELKKSNLEPE----- 191
Candida                FHDNESPSSLKS-QKLQNQRFDPFGNDGELYWGGLDFLKVLQQLINVQRSTIEPS----- 207
Schizosaccharomyces    FHDDECISTIEYGKQITRDRFDPFDESGNMFWGGTHLLMQLKKYASLEQSSHESQ----- 211
Danio                  SLRRARFSLGRNFLVLTTRGGDPLPPLHFHRGGTRELLKAMQRYIRLAPSPMDGR----- 174
Homo                   SIKQNKEGMGWSYLVFCLKDDVVLPALHFHQGDSKLLIESLEKYVVLCESPQDKR----- 191
Drosophila             DLSSVEVRHRGQTIRFMRKGANGVHSEFFFQHGNADLFVRSMRDQHLIENAETSRSGGEV 208
Caenorhabditis         KLANYLESEKSDDMYFCFRWVLVWFKREFSFLDTCKLWEVLWSGQPCPR----------- 153
Dictyosteliida         NNNNNSNNKNSNNNDTTNSTITNSTSTNLPIKTYIESPKSISSDSSSVSSSLYEP----- 134

Saccharomyces          -FWLVNPSLNDLRNFVSKDLLESYNNSKKDTTELATAGVKLNEKFQEWK-------WNVM 243
Candida                -VYLVNPESNDLRNFAPFKEKQKVPEPSQEPFKLP----DVAKFFNTAK-------WKVL 255
Schizosaccharomyces    -LYLVNPSPEDTVAFQSVELQKVISNNRLNSSSTPPTPRSSSSIFNPFRRALHDLSFTVL 270
Danio                  -LFLAYPHDSGALSQS-FDELHLFDDTSAD----------------------------LV 204
Homo                   -TLLVNCQNK-SLSQS-FEN--LLDEPAYG----------------------------LI 218
Drosophila             YAILTTENQKLKKTFAELDIGQIKASQLPRES-------------------------WLP 243
Caenorhabditis         FLLLICVAILDSQTNIIIDNQFGLTEILKHINDLS----------------------MH 190
Dictyosteliida         ----IVKEINEFINPPSITSSQTIINNQEEKD-------------------------IE 164

Saccharomyces          SKIADVTTKSTNFIDSWLTNNSPIQKSQIDNEYLQKLLNNEKVKQIEQDYDSARVYLANW 303
Candida                STVATLSAKTKNQVLDIIEDNAPKP-------IKDLVLQQPEVIKIGDEFDSARIYLAKW 308
Schizosaccharomyces    ERFSRVTNYGKSEVDRLMEHKVTKSILPHLPRELQVLLESKRVQKLTEEYDPARMFLARW 330
Danio                  SRFIQDPYATTFGGFSKVTN------------FFRGALRNPESPLNNRSPQD-------- 244
Homo                   QKIKKDPYTATMIGFSKVTN------------YIFDSLRGSDPSTHQRPPSEMADFLSDA 266
```


Drosophila NKLAGILGNIPDYVQPPFQRSPKSRPGVLISGDRQTSPDNYQIIGLSGSTNSACSSNGQS 303
Caenorhabditis LKVDEILTAAEAIFHQLSAS----------QNKLPAHICQYLNIGESAVSSS 232
Dictyosteliida NKTIVNNNNNNNSLNDSISS----------SSSSNSSSNSNNEESNTITKI 205
Saccharomyces SLGVKQEAERYQKQNKLFDSYRNNIFNDLN-LTDELSDTEINNALQRQFPLTEAKWNSLW 362
Candida AQQVKEEAEQSQGAYMLDDTLFNKINRELN-STEMLTQEEINKTTRRNE-ITVQEWEGFF 366
Schizosaccharomyces AEGIVEQSESNNSQPVNNAGVWTDAQREEDSSLGPFELVYIEERVKRDDPLSVEQWNSMF 390
Danio -PHFPHSADEEPGFELITCGAELGP----------RPEVKRGKP--LDNWEQFL 285
Homo IPGLKINQQEEPGFEVITR-IDLGE----------RPVVQRREPVSLEEWTKNI 309
Drosophila RGGSAEKSPADSELETLNAQDEKIVNNLPD----------RQRVERGHPLTETQWLEFQ 352
Caenorhabditis NNSPSKSDPDRVDLKILNFQKIMNLAS----------RVSGFLTMTSRYSSLATLK 278
Dictyosteliida SPTIRAVTRSSPFYNSKAPDIRSEVNG----------FPLKLNHMKTKEYIWLQLI 251
Saccharomyces DENDGRLRVTVNEVKDFIFHGGLENDSLRGKVWGFLLEIYPWDSSQDERVQIDQTLAAEY 422
Candida DFS-GRLLITVDEVKSRIFHGGLNQD-VRKEAWLFLLGVFPWDSSEDEREALRKSYETRY 424
Schizosaccharomyces NAH-GKLQVDVHRVLGIIFHGGIQPS-LRKEVWPFLLSVYPWDSTSEERRVIYLSLQEEY 448
Danio DPE--GRVTDPQKVKELVFRGGIVPY-LRKEVWKFLLGFYPWNSTTKEREDILMVKTDEY 342
Homo DSE--GRILNVDNMKQMIFRGGLSHA-LRKQAWKFLLGYFPWDSTKEERTQLQKQKTDEY 366
Drosophila TPD--GRISDSARIKELIFRGGVVQS-LRPEVWKFLLNYYLWSDTHVERIERRKQKSIEY 409
Caenorhabditis PTVAAALAPNFQRSFSDQKPTSTAPSTSTLLKVDWLLQKIVWNCKVFLEDFLEFYSEKNW 338
Dictyosteliida QNYTHDNIINNEILKSIRIG---LPKRIRGYIWRFFSGAIELERKNIGVYQHFLG----- 303
Saccharomyces DQLKLTWSKDFLQFDDEDEEEYWNDQLFRISKDVRRCDRNLEIFQYN-----TIDGLPPP 477
Candida EELKLKWVNDDVKRN----TEFWKDQKFRIEKDINRTDRNLDLFKNPKKRKENTDGSTTE 480
Schizosaccharomyces CTLKRKWYEDIHKQFN---DRWFIEQRNRIEKDVHRTDRQHEYFQIED--------LPHP 497
Danio FRMKVQWKSVSEEQEM--RNSLFRGYRSLIERDVNRTDRHNSFFSGNE------------ 388
Homo FRMKLQWKSISQEQEK--RNSRLRDYRSLIEKDVNRTDRTNKFYEGQD------------ 412
Drosophila YNMKAQWLAMTTTQEAN--FCGYRERKCQIEKDVKRTDRSLQFFAGED------------ 455
Caenorhabditis N----NWRNLGFFRHED----YWNFLIYLLKNSEEVLAQNIRFFGVLE------------ 378
Dictyosteliida --------------------KHSEEYEYKISKDISRTFPNNPYFNNEQ------------ 331
Saccharomyces PQQLPANENNST----------SPESANDE--SDDADDG-VRNPHLIHLQNILITYNVYN 524
Candida TTAAINTTDNVTNSDTTQTRESTPETPDEEDIDDEFDVSNIRNPHLYTMREILLTFNEYN 540
Schizosaccharomyces DPQSTFTGTNMN----------MEMMKDILLTYNEYD 524
Danio ----------NPGLTLLHDVLMTYCMYN 406
Homo ----------NPGLILLHDILMTYCMYD 430
Drosophila ----------NPNLTLLQGILMTYVMYN 473
Caenorhabditis ----------FLSIFITNFRLFSKNFHI 396
Dictyosteliida ----------GQNSLFRILKAYSIMD 347
Saccharomyces TNLGYVQGMTDLLSPIYVIMKEEWKTFWCFTHFMD--IMERNFLRDQSGIHEQMLTLVEL 582
Candida ENLGYVQGMTDLLSPLYVIIQDEVLVFWAFANFME--RMERNFVRDQTGMKKQMNTLNKL 598
Schizosaccharomyces TELGYVQGMSDLLAPIYVTFNDNALTFWGMVGLMK--RLHFNFLRDQSGMHRQLDTLRLL 582
Danio FDLGYVQGMSDLLSPLLFVTQNEVESFWCLTGFMD--LVHQNFEESQEAMKQQLLQLSLL 464
Homo FDLGYVQGMSDLLSPLLYVMENEVDAFWCFASYMD--QMHQNFEEQMQGMKTQLIQLSTL 488
Drosophila FDLGYVQGMSDLLAPILEIQVNEVDTFWCFVGFME--LVFTNFDIDQAGMKTQFAQIRRL 531
Caenorhabditis FEEFFLENTG--FSQFFEFFPGISRFFFEFFAFFE--KLSQFPDSASSQIKRSRFYGHLQ 452
Dictyosteliida PEIGYTQGMS-FIAAVLLSEMDETESFWTFTSIMKNYKLSTLFCHDLSLLRQYLYVIDRL 406
Saccharomyces VQLMLPELSEHLNKCDSGNLFFCFRMLLVWFKREFEMEDIMHIWENFWTFYYSSQFQLFF 642
Candida LQFMLPKLYKHLEMCQSNDLFFFFRMLLVWFKRELHWDQVLTLWEILWTDYYSSQFHLFF 658

```
Schizosaccharomyces IEFMDPELFAHLEKTDSSNLFCFFRMLLIYFKREFDWEVLLKLWDVLFTNYLSYDYHIFV 642
Danio               LRALDPELCDYLDSQDSGSLCFCFRWLLIWFKREFSLEDILSLWEVLWTRLPCENFHLLM 524
Homo                LRLLDSGFCSYLESQDSGYLYFCFRWLLIRFKREFSFLDILRLWEVMWTELPCTNFHLLL 548
Drosophila          IEFANAPLFNYMRSHDSDNMYFCFRWLLVWYKRELNSEDVLKLWECLWTRLPCPNFHLLF 591
Caenorhabditis      GRRSIRRSHFFEKVGWPDTADVTYRESSHWIG--SCVYENEDVIVVMWRLTTLDSSFMSY 510
Dictyosteliida      IETLLPKLFSHFKEIGVTPVLFASEWISTLFTYNFDLPISKRLLDVFFIEG--RFYLHRM 464

Saccharomyces       MLAILQKNSQAILQHLNQFDQILKFFNELNGKLDWNDLMVRAELLFKKFEKMMHVMERDL 702
Candida             ALSILSDNERIIIQNLKQFDEVLKYMNDLSMKLHLNPLLIRSELLFLKFKRMLDIIDRDT 718
Schizosaccharomyces AYAIAERHREVLLNQTSAFDEVLKYFNELSGKLALEPTLICAEQCFYQFKNKLALIDRKQ 702
Danio               ACSILESQKEELIGSNHDFNSILKHINELTMKLDLQSVLCGAEAIYLQLTHCKELSPKVQ 584
Homo                CCAILESEKQQIMEKHYGFNEILKHINELSMKIDVEDILCKAEAISLQMVKCKELPQAVC 608
Drosophila          SVAILDQETRVIIDSQYEFTEILKHVNELSGNIDVQKTLQVAEGIYLQLKGSETLPNDIR 651
Caenorhabditis      FPSFITKKENKMIVKEGALDVQIDREGHVLRIVNRPITASDREGAKSLAKMKEEQHQEYV 570
Dictyosteliida      SLAILKIYEKQLIE--FEFEDAVEFLKKLGTQIDPDLLLKTSDSLPLTIPMIES------ 516

Saccharomyces       ------------QNVSSSS----------SSSSTGVLPCQSERLTLLLSKKPIIRHEG 738
Candida             SLNALRHDDPYRNGNATGTG----------SSENSNNGEIIGDELRELLRKDLVIQKEV 767
Schizosaccharomyces ------------MEETNSDE----------DGSKETDLPTIAPYLRNLLKTSQPQYTPS 739
Danio               EVLGLR---IPSGSSEQSPD---------SEACEMDTL--LSHSQARGATSCQPFNGHTP 630
Homo                EILGLQGGEVTTPDSDVGEDENVVMTPCPTSAFQSNALPTLSASGARNDSPTQIPVSSDV 668
Drosophila          SIIGEPLLPAAAGEEIDGGMVD------EEPTYSDDGFDELVKELTPEEKVRQQALLEEA 705
Caenorhabditis      Q----------AEEKEMRK----------EFDRQYHTIFSQPSDCPFFWAKKRFFLKM 608
Dictyosteliida      ----------------------------------------FEKEFHPDTHPSDYL----- 531

Saccharomyces       QRSKNSVK-- 746
Candida             ERPEGVGGG- 776
Schizosaccharomyces GKQE------ 743
Danio               QRPPPTYP-- 638
Homo                CRLTPA---- 674
Drosophila          CERSLFLQFH 715
Caenorhabditis      FETTGS---- 614
Dictyosteliida      ----------
```

Figure: 5

Gyp7p homology sequence alignment. "Saccharomyces" denotes the protein sequence of gyp7 gene in *Saccharomyces cerevisiae* and "Homo" denotes the protein sequence of Gyp7p in *Homo sapiens*. Sequence alignment was produced by using Clustalw software database (http://ebi.ac.uk/clustalw).

CLUSTAL W (1.82) multiple sequence alignment

```
Saccharomyces       -------MSKILFCKSKVFLHPTSDARDNIAGFLLLTLEANKLSHQAILQYIPESGLSTL 53
Homo                MAAAGVVSGKIIYEQEGVYIHSSCGKTNDQDGLISGILRVLEKDAEVIVDWGPLD--DAL 58
                           .**:: :. *::*.:..  ::  *::   *.. : . :.*::: * .  .:*

Saccharomyces       EISKLLKHEAKVGTCPTSTPFVIENSINFSNLVNTSLGQAFEISLSQIYCIQFR-PPSPN 112
Homo                DSSSILYARKDSSSVVEWTQAPKERGHRGSEHLNS-----YEAEWDMVNTVSFKRKPHTN 113
                    : *.:*  . . .:    *   *.. . *: :*:     :* . . :  :.*:  * .*

Saccharomyces       GWYVGSLVIYPLTEQFTGFQPPVLFFHDQLCPSTTDKLKRLRKSMNPFDDSDELYWGGVD 172
Homo                G---------DAPSHRNGKSKWSFLFSLTDLKSIKQNKEGMGWSYLVFCLKDDVVLPALH 164
                    *           ..: .* .   ::*      * .:: : :  *   *  .*::   .:.

Saccharomyces       LRNKINELMELKKSNLEPEFWLVNPSLNDLRNFVSKDLLESYNNSKKDTTELATAGVKLN 232
Homo                FHQGDSKLLIES----LEKYVVLCESPQDKRTLLVNCQNKSLSQSFENLLDEPAYGLIQK 220
                    :::  .:*:  .      :: ::  * :* *.:: :   :* .:* ::  : .: *:  :

Saccharomyces       EKFQEWKWNVMSKIADVTTKSTNFIDSWLTNNSPIQKSQIDNEYLQKLLNNEKVKQIEQD 292
Homo                IKKDPYTATMIG-----FSKVTNYIFDSLRGSDPSTHQRPPSEMADFLSDAIPGLKINQQ 275
                     * : :. .::.      :* **:* . * ...*  :.:  .*  : * :     :*:*:

Saccharomyces       YDSARVYLANWSLGVKQEAERYQKQNKLFDSYRNNIFNDLNLTDELSDTEINNALQRQFP 352
Homo                EEPGFEVITRIDLGERPVVQRRE-----------------------------------P 299
                     :.   ::. .** :  .:* :                                      *

Saccharomyces       LTEAKWNSLWDENDGRLRVTVNEVKDFIFHGGLENDSLRGKVWGFLLEIYPWDSSQDERV 412
Homo                VSLEEWTKNID-SEGRI-LNVDNMKQMIFRGGLS-HALRKQAWKFLLGYFPWDSTKEERT 356
                    ::  :*..  * .:**: :.*:::*::**:***. .:** :.* ***  :****:::**.

Saccharomyces       QIDQTLAAEYDQLKLTWSKDFLQFDDEDEEEYWNDQLFRISKDVRRCDRNLEIFQYNTID 472
Homo                QLQKQKTDEYFRMKLQWKS--ISQEQEKRNSRLRDYRSLIEKDVNRTDR----------- 403
                    *:::  : ** ::** *..  :. ::*..:.  .*    *.***.* **

Saccharomyces       GLPPPPQQLPANENNSTSPESANDESDDADDGVRNPHLIHLQNILITYNVYNTNLGYVQG 532
Homo                ------------------------TNKFYEGQDNPGLILLHDILMTYCMYDFDLGYVQG 438
                                           ::.  :*  ** ** *::**:** :*: :******

Saccharomyces       MTDLLSPIYVIMKEEWKTFWCFTHFMDIMERNFLRDQSGIHEQMLTLVELVQLMLPELSE 592
Homo                MSDLLSPLLYVMENEVDAFWCFASYMDQMHQNFEEQMQGMKTQLIQLSTLLRLLDSGFCS 498
                    *:*****:  :*::* .:****: :** *.:** .: .*:: *:: *  *::*: . :..

Saccharomyces       HLNKCDSGNLFFCFRMLLVWFKREFEMEDIMHIWENFWTFYYSSQFQLFFMLAILQKNSQ 652
Homo                YLESQDSGYLYFCFRWLLIRFKREFSFLDILRLWEVMWTELPCTNFHLLLCCAILESEKQ 558
                    :*:. *** *:**** **: *****.: **:::** :**   .::*:*::  ***:.:.*

Saccharomyces       AILQHLNQFDQILKFFNELNGKLDWNDLMVRAELLFKKFEKMMHVMER-----DLQNVSS 707
Homo                QIMEKHYGFNEILKHINELSMKIDVEDILCKAEAISLQMVKCKELPQAVCEILGLQGGEV 618
                     *:::   *::***.:***. *:* :*:: :** :  :: *  .: :      .**. .

Saccharomyces       SSSSSSTG----VLPCQSERLTLLLSKKPIIRHEGQRSKNSVK------------- 746
Homo                TTPDSDVGEDENVVMTPCPTSAFQSNALPTLSASGARNDSPTQIPVSSDVCRLTPA 674
                    ::..*..*     *:   .   ::  .  * :  .* *.....:
```

"*" denotes amino acids are identical in sequence alignment

":" denotes conserved substitutions used in sequence alignment

"." Denotes semi-conserved substitutions used in sequence alignments

Figure: 6

DNA Sequence of *GYP7*

GYP7/YDL234C Chr 4 Reverse Complement

The sequence below oriented with respect to the Crick (bottom) strand. The sequence shown here is the 5'-3' direction of the Crick strand and is the reverse complement of the Watson (top) strand.

ATGAGTAAGATACTATTCTGCAAATCTAAAGTGTTCTTACACCCAACAAGCGATGCTAGA
GATAATATTGCTGGTTTCCTGCTACTCACATTGGAAGCGAATAAGCTATCCCACCAAGCA
ATTCTCCAATATATCCCAGAATCTGGCTTATCCACATTAGAGATATCAAAACTACTAAAA
CATGAAGCTAAAGTGGGCACATGTCCAACTTCTACACCATTTGTTATTGAAAATTCAATA
AACTTTAGTAACCTGGTCAATACTTCTTTAGGTCAAGCTTTCGAAATTTCTCTCTCACAA
ATATACTGCATCCAATTTAGGCCTCCTAGTCCAAATGGTTGGTATGTCGGATCTCTGGTA
ATATATCCCTTGACAGAACAGTTTACGGGCTTTCAACCTCCTGTCTTGTTCTTTCATGAC
CAACTCTGCCCATCAACTACGGACAAGTTAAAAAGACTACGCAAATCCATGAACCCATTT
GATGACTCGGACGAATTGTATTGGGGTGGCGTAGATTTGAGAAACAAAATCAACGAACTG
ATGGAACTCAAGAAATCAAACTTAGAGCCAGAGTTTTGGTTAGTAAATCCCTCTTTGAAT
GATTTGAGAAATTTCGTTTCCAAGGATCTATTGGAAAGTTATAACAATTCGAAGAAAGAT
ACAACTGAACTCGCTACCGCCGGTGTGAAACTAAACGAGAAATTCCAAGAATGGAAATGG
AACGTAATGAGCAAGATAGCAGACGTGACTACCAAGTCTACCAATTTTATCGATAGTTGG
CTGACTAACAACTCACCCATACAAAAATCACAAATCGACAATGAATACTTGCAAAAGCTT
CTGAACAATGAGAAGGTTAAACAAATTGAACAGGATTATGACTCTGCAAGAGTGTATTTG
GCAAACTGGTCATTAGGAGTAAAACAGGAAGCTGAGAGGTATCAAAAGCAGAACAAACTC
TTTGATTCGTATAGGAATAACATTTTCAACGACCTGAATCTAACTGATGAATTAAGTGAT
ACTGAAATAAATAATGCTCTGCAAAGACAATTTCCTTTAACGGAAGCGAAATGGAATTCG
CTATGGGATGAGAACGATGGGAGGCTGAGGGTCACGGTAAACGAAGTTAAGGATTTCATA
TTTCATGGTGGGTTAGAAAATGACAGTTTGCGAGGGAAAGTTTGGGGTTTTCTCTTAGAA
ATATATCCGTGGGATTCTTCTCAAGACGAGAGGGTACAAATCGATCAAACTTTAGCCGCG
GAATACGATCAATTGAAACTAACCTGGTCAAAAGATTTTTTACAATTTGACGATGAAGAT
GAAGAGGAATATTGGAACGATCAATTATTCAGAATATCTAAAGATGTGAGACGCTGTGAT
AGAAACTTGGAGATATTTCAATACAATACCATCGATGGATTACCACCACCGCCACAACAA
CTCCCAGCAAATGAAAATAACAGTACCAGCCCCGAATCCGCTAATGACGAGAGCGATGAT
GCAGACGATGGAGTCAGGAACCCACATTTGATACATTTACAAAACATTCTTATCACTTAT
AATGTCTACAACACAAACTTAGGCTACGTGCAGGGGATGACCGATCTTTTATCGCCTATT
TATGTCATCATGAAGGAAGAATGGAAAACGTTTTGGTGTTTTACGCACTTCATGGACATT
ATGGAAAGAAATTTTCTAAGAGACCAGAGTGGCATCCATGAACAAATGTTAACTCTCGTG
GAATTGGTACAATTAATGCTACCCGAATTGAGCGAACATTTAAACAAGTGTGATTCAGGA
AACTTGTTCTTTTGCTTTCGAATGCTTCTAGTATGGTTCAAGAGAGAATTTGAAATGGAA
GATATTATGCACATTTGGGAGAATTTCTGGACTTTCTACTACAGTTCACAATTCCAATTA
TTCTTCATGTTGGCCATTCTACAGAAAAACTCACAAGCTATTTTACAACATCTAAATCAG
TTCGACCAAATATTAAAATTTTTTAATGAACTTAACGGGAAGTTGGATTGGAATGACCTA
ATGGTTAGAGCAGAGCTTTTGTTCAAGAAGTTTGAGAAAATGATGCACGTCATGGAAAGA
GATTTACAAAACGTATCCTCCTCTTCATCCTCATCCTCTACCGGCGTATTGCCTTGTCAG
AGCGAGAGGTTGACCCTGCTTCTTTCTAAAAAACCTATCATAAGACATGAAGGGCAAAGG
AGCAAAAATTCCGTTAAATAG

Figure: 7

Protein Sequence of *GYP7*

YDL234C |GYP7||GTPase-activating protein for Ypt7p

MSKILFCKSK VFLHPTSDAR DNIAGFLLLT LEANKLSHQA ILQYIPESGL STLEISKLLK
HEAKVGTCPT STPFVIENSI NFSNLVNTSL GQAFEISLSQ IYCIQFRPPS PNGWYVGSLV
IYPLTEQFTG FQPPVLFFHD QLCPSTTDKL KRLRKSMNPF DDSDELYWGGDLRNKINEL
MELKKSNLEP EFWLVNPSLN DLRNFVSKDLLESYNNSKKDTTELATAGVKLNEKFQEWKW
NVMSKIADVT TKSTNFIDSW LTNNSPIQKS QIDNEYLQKL LNNEKVKQIE QDYDSARVYL
ANWSLGVKQE AERYQKQNKL FDSYRNNIFN DLNLTDELSD TEINNALQRQ FPLTEAKWNS
LWDENDGRLR VTVNEVKDFI FHGGLENDSL RGKVWGFLLE IYPWDSSQDE RVQIDQTLAA
EYDQLKLTWS KDFLQFDDED EEEYWNDQLF RISKDVRRCD RNLEIFQYNT IDGLPPPPQQ
LPANENNSTS PESANDESDD ADDGVRNPHL IHLQNILITY NVYNTNLGYV QGMTDLLSPI
YVIMKEEWKT FWCFTHFMDI MERNFLRDQS GIHEQMLTLV ELVQLMLPEL SEHLNKCDSG
NLFFCFRMLL VWFKREFEME DIMHIWENFW TFYYSSQFQL FFMLAILQKN SQAILQHLNQ
FDQILKFFNE LNGKLDWNDL MVRAELLFKK FEKMMHVMER DLQNVSSSSS SSSTGVLPCQ
SERLTLLLSK KPIIRHEGQR SKNSVK

DNA and Protein sequences were obtained through (http://www.yeastgenome.org/).

Figure 8:

Calculating Mutation rates

Calculating the mutational rates using Lea and Coulson's method of the median.

1. Count colonies on selected plates.

2. Rank the values collected from the plates and give values from lowest to highest and select the median value. The median value is designated r_0. From the following tables (Pages 62-65) generated by Lea and Coulson paper (J. Genet. **49**:264-285) determine the r_0 and r_0/m values. Determine where your experimental r_0 value falls within. Once this has been determined this will allow you to determine *m,* the estimated average number of mutational events in the culture.

3. Example calculation: Experimental $r_0 = 544$ then interpolate from tables:

r_0	r_0/m			
493	5.7	544- 493 = 51		
			(51 / 61)(0.1) = 0.083	0.015+5.7 = 5.784= r_0/m
554	5.8	554- 493 = 61		

Solve for *m:* r_0 / m : $544 / m = 2.515$ $m = 94.05$

Take (m) and then divide it by the selected median value of the plates times the dilution factor of that plate and quantity of cells plated onto the plates.

Example:

Dilution factor = (10,000) $521.2 \times 10{,}000 = 5.212 \times 10^6$
Median = (521.2)

Next divide *m* by the estimated number of colonies:

$94.05 / 5.212 \times 10^6 = 1.8 \times 10^{-5}$ (Actual Directed-Repeat Rate)

R_o and R_o / m Charts

	r_o	r_o / m			r_o	r_o / m
	1.4	1.3				
0.2				5956		
	1.6	1.4			57099	9..9
0.3				6641		
	1.9	1.5			63740	10.0
0.4				7909		
	2.3	1.6			71149	10.1
0.4				8262		
	2.7	1.7			79411	10.2
0.5				9212		
	3.2	1.8			88623	10.3
0.5				10271		
	3.7	1.9			98894	10.4
0.6				11452		
	4.3	2.0			110346	10.5
0.7				12767		
	5.0	2.1			123113	10.6
0.7				14231		
	5.7	2.2			137344	10.7
0.9				15863		
	6.6	2.3			153207	10.8
1.1				17681		
	7.7	2.4			170888	10.9
1.1				19705		
	8.8	2.5			190593	11.0
1.3				21940		
	10.1	2.6			212553	11.1
1.5				24470		
	11.6	2.7			237023	11.2
01.7				27267		
	13.3	2.8			264290	11.3
2.0				30381		
	15.3	2.9			294671	11.4
2.1				33847		
	17.4	3.0			328518	11.5
2.5				37707		
	19.9	3.1			366225	11.6
2.8				42006		
	22.7	3.2			408231	11.7
3.2				46790		
	25.9	3.3			455021	11.8

3.6				52116		
	29.5	3.4			507137	11.9
4.0				58047		
	33.5	3.5			565184	12.0
4.6				64646		
	38.1	3.6			629830	12.1
5.2				71993		
	43.3	3.7			701823	12.2
5.9				80169		
	49.2	3.8			781992	12.3
6.6				89269		
	55.8	3.9			871261	12.4
7.4				99396		
	63.2	4.0			970657	12.5
8.4				110667		
	71.6	4.1			1081324	12.6
9.5				123207		
	81.1	4.2			1204531	12.7
10.6				137207		
	91.7	4.3			1341696	12.8
12.3				137165		
	104	4.4			1494387	12.9
13				152691		
	117	4.5			1664351	13.0
15				169964		
	132	4.6				
18						
	150	4.7				
19						
	169	4.8				
21						
	190	4.9				
25						
	215	5.0				
27						
	242	5.1				
31						
	273	5.2				
34						
	307	5.3				
39						
	346	5.4				
43						
	389	5.5				
49						

	438	5.6				
55						
	493	5.7				
61						
	554	5.8				
69						
	623	5.9				
77						
	700	6.0				
87						
	787	6.1				
97						
	884	6.2				
109						
	993	6.3				
122						
	1115	6.4				
136						
	1251	6.5				
153						
	1404	6.6				
171						
	1575	6.7				
192						
	1767	6.8				
214						
	1981	6.9				
240						
	2221	7.0				
269						
	2490	7.1				
301						
	2791	7.2				
336						
	3127	7.3				
376						
	3503	7.4				
421						
	3924	7.5				
471						
	4395	7.6				
525						
	4920	7.7				
587						
	5507	7.8				

655						
	6162	7.9				
742						
	6904	8.0				
815						
	7719	8.1				
921						
	8640	8.2				
1021						
	9661	8.3				
1149						
	10810	8.4				
1279						
	12089	8.5				
1429						
	13518	8.6				
1595						
	15113	8.7				
1782						
	16895	8.8				
1989						
	18884	8.9				
2220						
	21104	9.0				
2479						
	23583	9.1				
2767						
	26349	9.2				
3087						
	29437	9.3				
3446						
	32883	9.4				
3845						
	36728	9.5				
4290						
	41018	9.6				
4786						
	45804	9.7				
5339						
	51143	9.8				

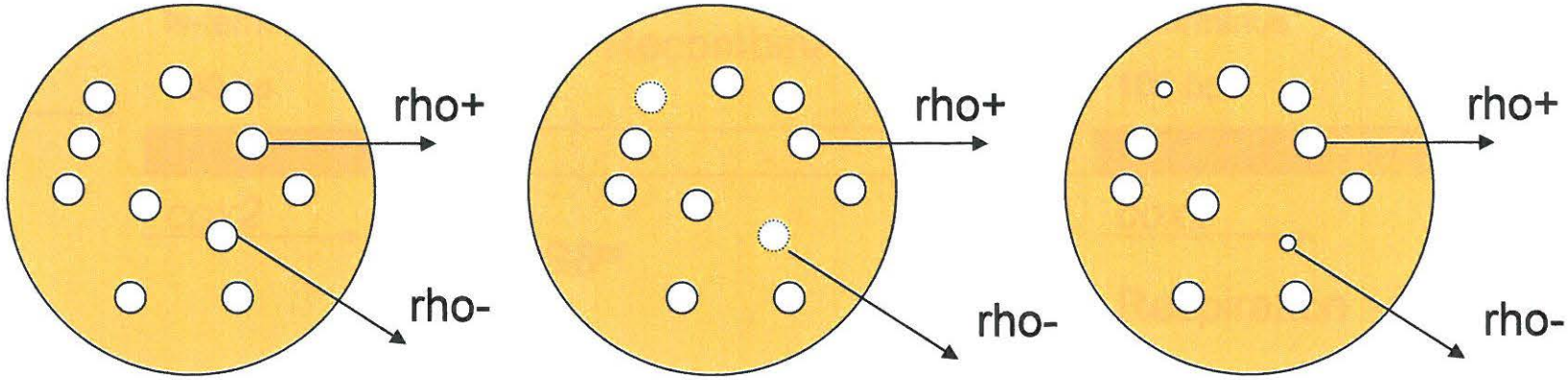
Formula for the above calculation: ro/m-logm =1.24 (From Lea and Coulson, J. Genetics. 49:264-285

Figure: 9

Alterations In Mitochondria Amino-Acid Codons Compared To Other Organisms

Codon:	**Standard Code: Nuclear-Encoded Proteins**	**Mirochondria**				
		Mammals	Drosophila	Neurospora	**Yeasts**	Plants
UGA	Stop	Trp	Trp	Trp	Trp	Stop
AGA, AGG	Arg	Stop	Ser	Arg	Arg	Arg
AUA	Ile	Met	Met	Ile	Met	Ile
AUU	Ile	Met	Met	Met	Met	Ile
CUU, CUC	Leu	Leu	Leu	Leu	Thr	Leu

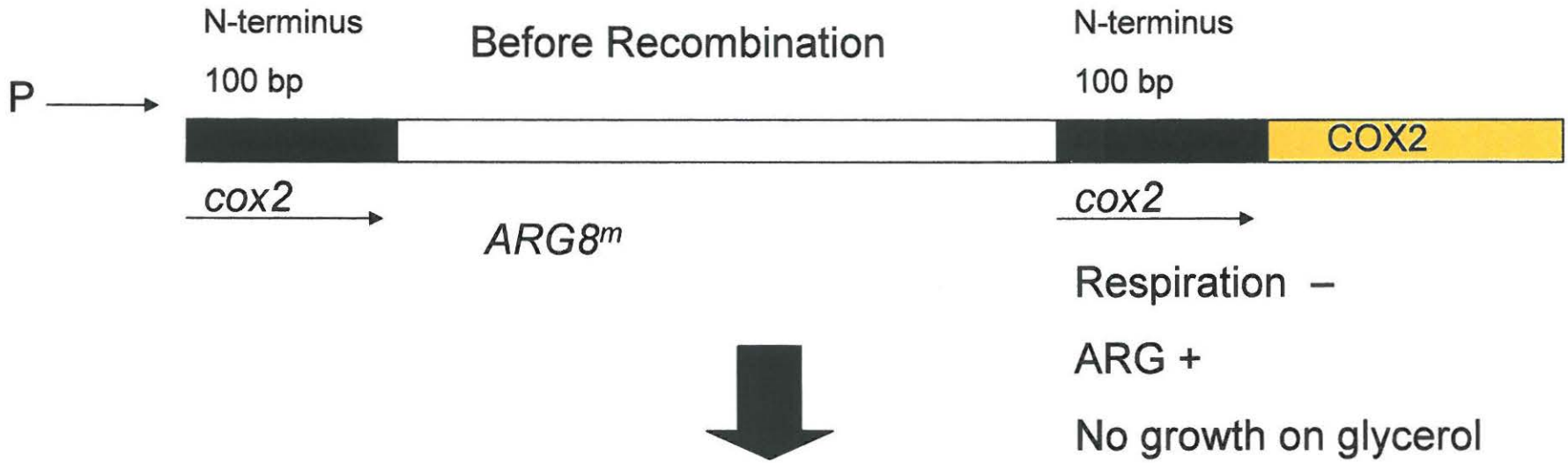
Respiration Loss Assay



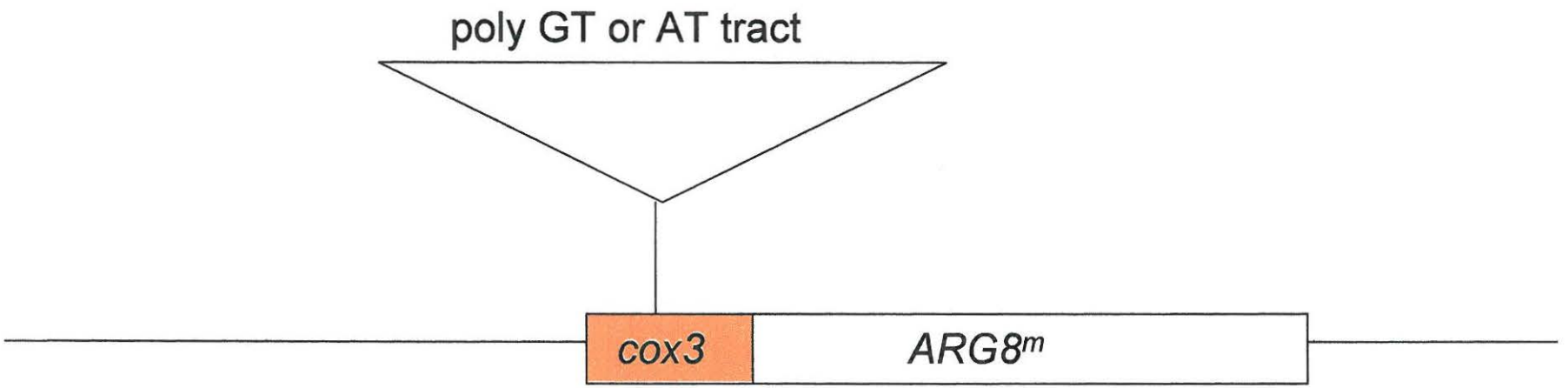
YPD		YPG		YPG + 0.1% Dextrose	
rho+	→ grow	rho+	→ grow	rho+	→ grow
rho-	→ grow	rho-	→ no growth	rho-	→ grow then stop growing

Selection on non-fermentable carbon source can distinguish cells with functional (rho+) or non-functional (rho-) mitochondria

Recombination Reporter



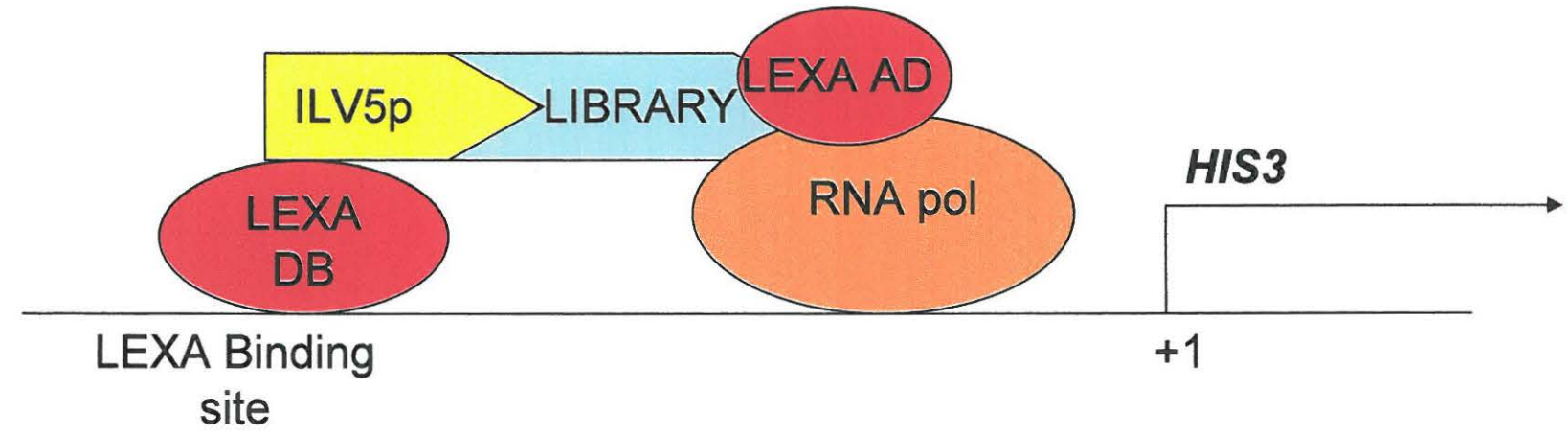
Microsatellite Reporter



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+

Yeast Two-Hybrid Assay



Assay uses the LEXA protein and its ability to be divided into the DNA binding and activation domains

When a gene of interest interacts with the ILV5 gene the two domains are able to activate the HIS3 reporter gene