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Characterization of Ubiquitin/Proteasome-Dependent Regulation of Hap2/3/4/5 Complex In *Saccharomyces cerevisiae*

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Characterization of Ubiquitin/Proteasome-Dependent
Regulation of Hap2/3/4/5 Complex
In *Saccharomyces cerevisiae*

An Honors Thesis

Presented to
the Department of Biological Sciences
at the University of New Orleans

In Partial Fulfillment
of the Requirements for the
Degree of Bachelor of Science,
with University Honors and Honors in Biological Sciences

by

Arielle Ruth Hunter

April 2012

Acknowledgments

I have often been quoted as saying, “my Honors thesis has changed my life.” This is a statement by which I resolutely stand. The expense in physical and mental strain that it has cost me are fully validated with the fulfillment and reward with which I have been repaid. From the increased level of comprehension in the biological sciences to the development of my skills in research, this undertaking has largely contributed to my maturity on both a personal and educational level. None of this would have been possible without the guidance, support, and inspiration from the persons listed here.

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Abstract

The Hap2/3/4/5 complex is a heme-activated, CCAATT binding, global transcriptional activator of genes involved in respiration and mitochondrial biogenesis in the yeast species *Saccharomyces cerevisiae*. Hap4 is the regulatory subunit of the complex and its levels determine the activity of the complex. Hap4 is known to play a signaling role in response to environmental conditions; however, little is known about the regulation of Hap4 levels or how it responds to a cell's functional state. The activity of the Hap2-5 complex is known to be reduced in respiratory-deficient cells. In Liu Lab, it has previously been found that a link between Hap4 stability, mediated through 26S proteasome-dependent degradation, and dependence on mitochondrial functional state plays a regulatory role on downstream targets of the Hap complex. However, the mechanism behind this regulation is still largely unknown. In normally functioning yeast cells, Hap4 is a highly unstable protein with a half-life of ~10 min. We have observed that loss of mitochondrial DNA in respiratory deficient ρ^0 cells has a role in the further destabilization of Hap4 to a half-life of ~4 min through the ubiquitin-proteasome pathway. Through the screening of a collection of mutants defective in E2 ubiquitin-conjugating enzymes, we show that Hap4 is greatly stabilized in *ubc1 Δ ubc4 Δ* double mutant cells. We also show that Hap4 stabilization in the *ubc1 Δ ubc4 Δ* mutant leads to increased activity of the Hap2-5 complex, indicating that mitochondrial biogenesis in yeast is regulated by the functional state of mitochondria through ubiquitin/proteasome-dependent degradation of Hap4. Furthermore, studies on Hap4 mutants involving two highly conserved cysteine residues led to a proposed mechanism behind the regulation of Ubc4 activity towards Hap4 in response to changes in the cellular redox state.

Key Terms: Hap2/3/4/5 complex, Hap4, mitochondrial biogenesis, glucose repression, rho^0 , rho^+
ubiquitin-proteasome pathway

Introduction

***Saccharomyces cerevisiae* as a model system**

As one of the best-studied eukaryotic organisms, yeast is a major research reagent in biological sciences. The simplicity of yeast, being one of the simplest eukaryotes, with genetic complexity marginally greater than that of most prokaryotes (Sherman, 2002), while simultaneously exhibiting high conservation of genes relative to mammalian cells, makes them an invaluable model organism (Botstein, Chervitz, & Cherry, 1997). The budding yeast species *Saccharomyces cerevisiae* is a well-established model organism in biology. As a non-pathogenic, fast growing, readily and affordably available yeast species, it is a highly convenient model to use in research (Sherman, 2002). Furthermore, protein structure homologies to mammalian proteins, ease of manipulation, and high experimental pliability make *S. cerevisiae* an archetypical model for research delving into the fundamental questions in biological sciences.

In 1996, the complete DNA sequence of *S. cerevisiae*'s ~6000-gene genome was released in electronic form, making it the first eukaryotic species to have its genome completely sequenced (Goffeau, et al., 1996). This pioneering event led to the use of *S. cerevisiae* as a “model organism” for interpreting and understanding mammalian DNA sequences (Botstein, Chervitz, & Cherry, 1997). The numerous structural and functional similarities in the genes and their protein products encoded in *S. cerevisiae*'s genome to those of higher eukaryotes make yeast a time and money efficient avenue for gaining insight into cellular mechanisms of higher organisms. Over the years, the worth of *S. cerevisiae* has been further established, with it acting as a valuable model for delving into underlying mechanisms of disease states in higher eukaryotes.

Yeast's high experimental pliability allows for ease and comfort in the discovery process. Its aptitude at up-taking and expressing genes through DNA transformation, which can either be integrated into the yeast genome or exist as a multi- or single-copy replicative molecule in the nucleus, has played a sizeable role in their usefulness as a system by providing means for in-depth studies of genes and the proteins they encode (Mumberg, Muller, & Funk, 1995) (Botstein, Chervitz, & Cherry, 1997). The well-defined genome of *S. cerevisiae* and protein homologies pairs wonderfully with this system, allowing for experiments on gene regulation, protein assay, genetic screening, random and targeted mutagenesis of specific genes, complete removal or "knock-out" of targeted genes. All of these approaches have the capability of further illuminating life on a molecular level.

The Metabolism of a Facultative Aerobe

Metabolism is the combination of anabolic and catabolic biochemical pathways for the building and breaking down of biomolecules in a cell. Energy generation and carbon metabolism are intimately connected in eukaryotic cells, with exothermic change from catabolic processes often driving endothermic anabolic processes (Walker, 1998). The pathways behind carbon metabolism are highly structured and complex, being mediated through a series of tightly regulated branching enzymatic reactions. In yeast, carbon metabolism is intrinsically linked to cell growth and proliferation as well as to basic cellular functions and vitality through energy and metabolite production.

S. cerevisiae is a facultative aerobe. In the presence of a preferred carbon source such as dextrose (glucose), it prefers fermentative metabolism through glycolysis and ethanol formation over respiratory metabolism and oxidative phosphorylation. Through evolutionary innovation, facultative aerobes have acquired the ability to utilize ethanol produced by fermentation as a

carbon source by switching to aerobic respiration when fermentable carbon sources are depleted (Botstein, Chervitz, & Cherry, 1997). The overview of these two pathways is shown in **Figure 1** and a brief outline will be given for understanding of the key factors regulating these two metabolic pathways.

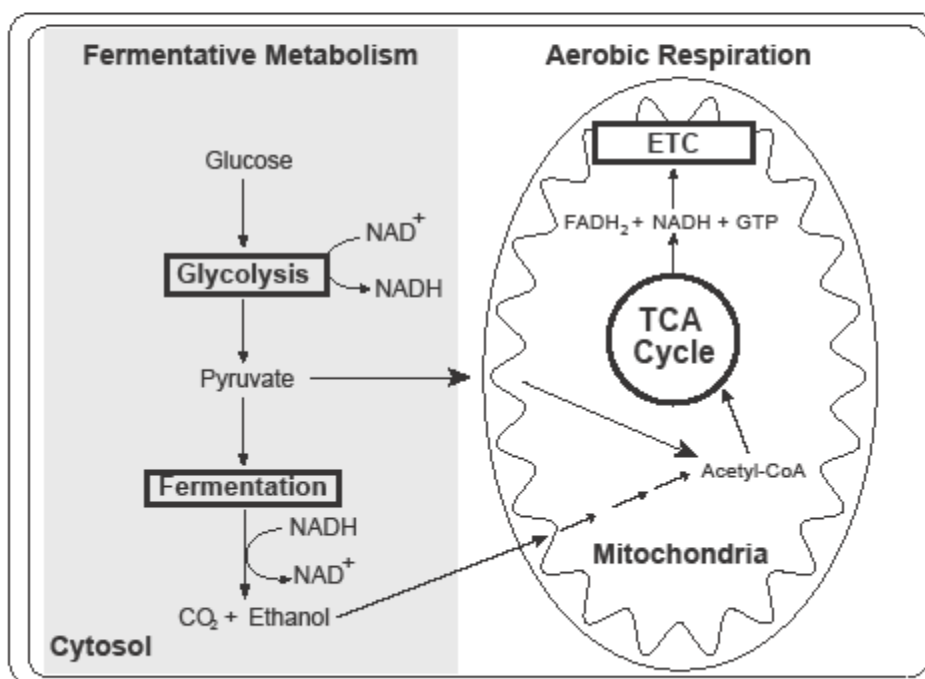


Figure 1: Fermentation and Aerobic Respiration

This model summarizes the two main metabolic pathways of facultative aerobes: Fermentative metabolism via glycolysis and fermentation and aerobic respiration via the Tricarboxylic Acid (TCA) cycle and the Electron Transport Chain (ETC). These two pathways are linked at two steps: with the conversion of pyruvate and/or ethanol to Acetyl-CoA, which is oxidized through the TCA cycle.

During fermentative metabolism, glucose is broken down through glycolysis, a ubiquitous catabolic pathway, with a title literally meaning, “splitting of sugar.” In this pathway, which occurs in the cytosol, the six-carbon sugar glucose is broken down into two three-carbon molecules of pyruvate through ten enzyme-catalyzed reactions. This process provides part of the total cellular energy, producing two adenosine triphosphates (ATP) per glucose molecule by way

of substrate level phosphorylation. In the sixth step of glycolysis, the oxidized form of nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH by the transfer of hydrogen from glyceraldehyde phosphate by the enzyme glyceraldehyde 3-phosphate dehydrogenase. The overall reaction of glycolysis thus produces two moles of ATP, two moles of pyruvate, and two moles of the reduced NADH from one mole of glucose.

For continuation of glycolysis, NAD^+ must be regenerated through oxidation of the NADH produced by glycolysis. *S. cerevisiae* utilize alcoholic fermentation, in which pyruvate is converted to ethanol and carbon dioxide for the regeneration of NAD^+ (Morton, 1980). This occurs in a two-step reaction whereby pyruvate is first decarboxylated by the enzyme pyruvate decarboxylase to form acetaldehyde, which is then reduced by NADH to form NAD^+ as catalyzed by a second enzyme, alcohol dehydrogenase (Walker, 1998). Glycerol is also generated in this pathway from dihydroxyacetone phosphate and later, as a non-fermentable carbon source, can be utilized in respiration (Morton, 1980).

Aerobic respiration occurs in the mitochondria. As the catabolic process with the highest efficiency for ATP production, it is the principal metabolic pathway for Gibbs free energy production in most eukaryotic cells (Raghevendran, Patil, Olsson, & Nielsen, 2006). The overall pathway can be broken down into two metabolic pathways: the citric acid cycle, also known as the Tricarboxylic acid (TCA) or Krebs cycle, and the mitochondrial electron transport chain (ETC). During respiration NADH produced in the TCA cycle is oxidized by oxygen through the electron transport chain with concomitant generation of a proton (H^+) gradient across the mitochondrial inner membrane, which drives ATP production through the F1F0-ATPase.

In 1937, Hans Krebs provided the early insights that led to the historical discovery of the circular pathway whereby the acetyl group of acetyl-CoA is oxidized into two molecules of

carbon dioxide through a series of eight highly conserved enzymatic reactions (Krebs, 1937). An overview of the TCA cycle with these eight enzymes and the 13 nuclear genes in *S. cerevisiae* that encode them are shown in **Figure 2**.

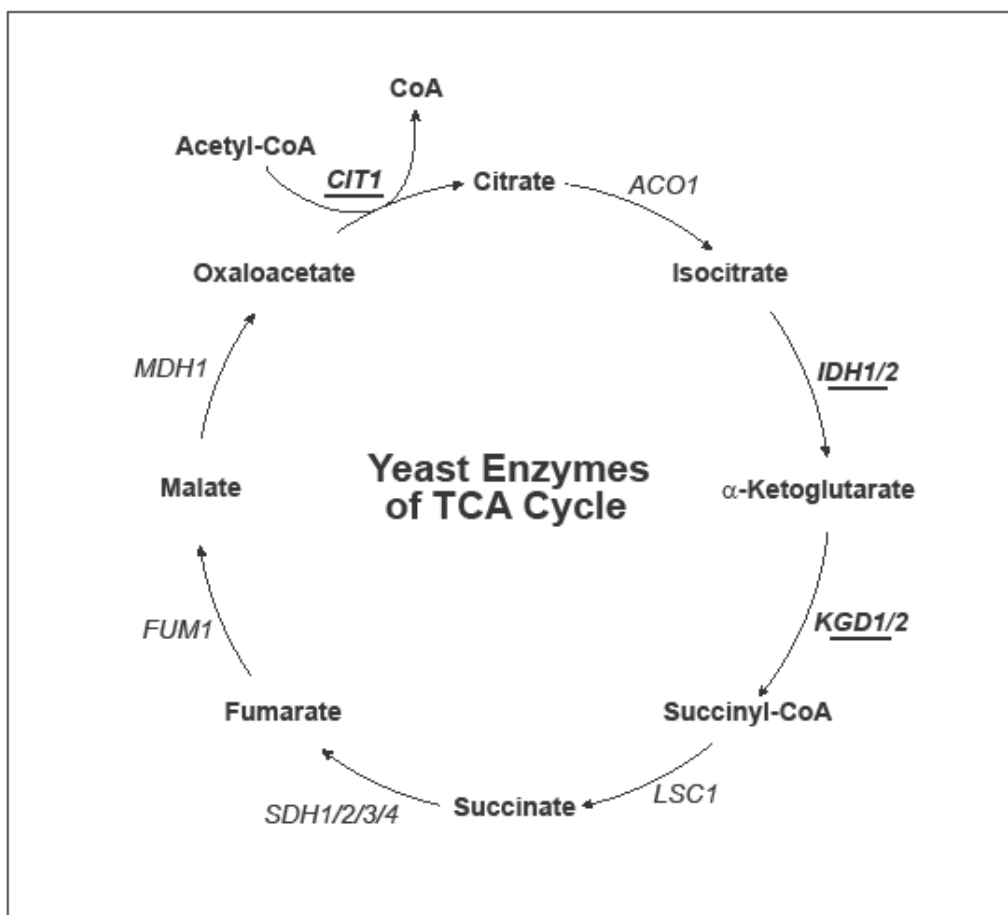


Figure 2: The TCA Cycle in *S. cerevisiae*

A diagram of the TCA cycle intermediates and the yeast genes encoding the enzymes that catalyze the reactions. Substrates are shown in bold, while genes encoding the TCA cycle enzymes are shown in uppercase italic font. Of these enzymes, those with a rate-controlling role of the cycle are denoted by a line beneath their gene name.

The first substrate of the cycle, acetyl-CoA, can be produced from pyruvate through oxidative decarboxylation by the multi-enzyme complex pyruvate dehydrogenase in the mitochondria matrix. This is the linking step between glycolysis and the TCA cycle. However, in

yeast, acetyl-CoA is largely synthesized from ethanol produced during alcoholic fermentation. This occurs by way of three enzyme-regulated steps whereby ethanol is oxidized to acetaldehyde, which is sub-sequentially converted to acetic acid. Acetic acid is, in turn, converted to acetyl-CoA through a reaction requiring ATP.

The FADH_2 and NADH produced in the TCA cycle act as electron carriers and are important in providing electrons for the next step of respiration, the electron transport chain (ETC), which harnesses free energy for the formation of ATP via oxidative phosphorylation. ATP synthesis occurs in the matrix of the mitochondria, where mobile and integral protein complexes act as electron carries, transporting electrons from NADH and FADH_2 to a final electron acceptor. In the case of aerobic respiration, this is O_2 . During this transfer, the electrons are shuttled from redox centers with more negative reduction potentials to those of more positive and relative greater values. These values correlate to greater affinity for electrons, thus this shuttling produces free energy as overall exergonic process (Voet, Voet, & Pratt, 2010).

In the ETC, there are four protein-lipid enzyme complexes located in the mitochondrial inner membrane containing flavins, quinoid compounds, and transition metal compounds, which act as electron carriers through redox reactions. In mammalian cells, these enzymes and their associated electron carriers are titled: complex I (NADH :ubiquinone oxidoreductase), complex II (succinate: ubiquinone oxidoreductase or succinate dehydrogenase), complex III (ubiquinol:ferrocytochrome oxidoreductase), and complex IV (cytochrome c oxidase). Electrons enter the electron transport chain through the oxidation of NADH to NAD^+ at Complex I and FADH_2 to FAD^+ at Complex II, the latter which is also the sixth enzyme in the TCA cycle. Complex I of mammalian cells is composed of 46 different subunits, 7 of which are encoded by mitochondrial DNA (Vilain, et al., 2012). Interestingly, yeast cells do not contain these multi-

subunit NADH-quinone oxidoreductases, rather they have a single protein, NADH dehydrogenase encoded by *NDH1*, which transfers electrons from NADH to ubiquinone in the respiratory chain without pumping protons (Vilain, et al., 2012).

The ETC is linked to ATP synthesis through a theory proposed by British biochemist, Peter Mitchell in 1961, titled the chemiosmotic theory (Mitchell, 1978). This theory, which won Mitchell the Nobel Prize in Chemistry in 1978 “for his contribution to the understanding of biological energy transfer” (Nobel Media), was first received with much skepticism, but came to be accepted as one of the fundamental processes in all living systems from plants to humans. It states, the proteins of electron transport and ATP-synthesizing systems are localized in a membrane with a well-defined orientation and are functionally linked to a directional transfer of protons (H^+), across the membrane (Mitchell, 1978). In eukaryotes, this occurs in mitochondria, via pumping of protons from the matrix into the intermembrane space at complexes I (except in yeast and other fungi), III, and IV, creating an electrochemical proton gradient across the inner mitochondrial membrane, the latter is then harnessed by the F1F0-ATP synthase for the production of ATP.

As shown in **Figure 1** fermentation and aerobic respiration each require their own enzymatic mechanisms and cellular machinery. Thus, the switch between fermentation and respiration requires a remodeling of gene expression, resulting in a characteristic growth pattern titled “diauxic shift” (Santangelo, 2006) (DeRisi, Lyer, & Brown, 1997). As the title implies, the diauxic shift refers to a two-sided curve that reflects the cells growth pattern as cells utilize a preferred carbon source first and then undergo remodeling of their metabolic pathways for the use of the second, and less preferred carbon source (Walker, 1998). In *S. cerevisiae*, this metabolic remodeling involves the use of non-fermentable carbon sources, such as lactate,

ethanol, glycerol, and pyruvate in respiration. This metabolic switch is regulated in response to external glucose levels, in a method that adjusts the energy metabolism in a process that is generally referred to as glucose repression (Rodrigues, Ludovico, & Leao, 2006).

By definition, glucose repression refers to the phenomenon whereby cells grown on glucose repress the expression of a large number of genes that are required for the metabolism of alternate carbon sources (Trumbly, 1992). Due to a preference for fermentative metabolism in *S. cerevisiae*, glucose repression affects the expression of enzymes required for metabolism of non-fermentable carbon sources as well as enzymes involved in respiration and mitochondrial biogenesis (Rolland, Winderickx, & Thevelein, 2002). This is a highly regulated process, involving gene and protein regulation at multiple levels in a number of signal transduction pathways. Energy metabolism is dependent on a few transcription factors, which regulate cellular processes by controlling the expression of multiple genes involved in a given cellular pathway. Transcription factors are key elements in regulating the expression of target genes (Fendt & Uwe, 2012). One of these transcription factors in *S. cerevisiae* is the Hap2-5 complex, which is an essential part of yeast's metabolic remodeling when entering respiratory growth and is responsible for the activation of roughly a hundred genes involved in respiration and other metabolic functions (Pinto & McNabb, 2005).

The Hap2/3/4/5 Complex

The majority of the genes encoding mitochondrial proteins are encoded in the nuclear genome. In yeast, the expression of these genes becomes de-repressed during the diauxic shift. This de-repression is principally regulated by a family of **heme-activated proteins**, known as *Hap* proteins (Liu & Butow, 1999). The *Hap* proteins are transcription factors that bind to DNA at distinct upstream activation sites (UAS) of genes involved in respiration and regulate their

transcription. These proteins are mainly responsible for the transcriptional regulation of respiration, and do so by controlling the expression of multiple genes encoding the proteins involved in respiration, such as components of the electron transport chain and the enzymes of the TCA cycle.

As their names imply, *Hap* proteins' activity depends on the cofactor heme. Heme is an iron-containing heterocyclic prosthetic group with electron carrying properties and a high affinity for oxygen. Its cellular concentration is linked to oxygen availability through its biosynthesis, which is oxygen-dependent (Kwast, Burke, & Poyton, 1998). Heme is known to regulate the activity of a diverse group of proteins, with heme-regulated signal transducers being found in organisms ranging from bacteria to humans (Zhang, et al., 2007). Heme is involved in oxygen sensing, acting as a positive modulator for the transcription of the aerobic genes and as a negative modulator for the transcription of the hypoxic genes (Kwast, Burke, & Poyton, 1998). Therefore, heme acts as an intermediary in the regulation of the expression of genes involved in respiration and thus oxidative phosphorylation. In *S. cerevisiae*, this regulation is accomplished through the *HAP* family. There are two components to the *HAP* system: Hap1 and the Hap2/3/4/5 complex. Together these two transcription factors represent an important mechanism for global control of the expression of key components in respiratory metabolism.

Hap1 was first identified as an activator of the hypoxic gene iso-cytochrome C gene (*CYC7*) in 1969 and termed *CYP1*. However, the gene was later re-designated based on the discovery in 1986 that it was the same protein reported as Hap1, an activator of *CYC1* expression (reviewed by Kwast, Burke, & Poyton, 1998). Analysis of the upstream regulation of *CYC1* expression led to the identification of two distinct upstream activation sequences, UAS1 and UAS2 (Pfeifer, Prezant, & Guarente, 1987) (Lalonde, Arcangioli, & Guarente, 1986). Upstream

activation sequences (UAS) are *cis*-acting elements upstream of TATA boxes, which act as a primary cellular loci for gene-specific regulation (Lalonde, Arcangioli, & Guarente, 1986). Hap1 is responsible for the regulation at UAS1 of the *CYC1* gene and it is also targeted to genes encoding the proteins involved in heme biosynthesis, electron transfer reactions and controlling oxidative damage (Gaisne, Becam, Verdiere, & Herbert, 1999) (Zhang & Hach, 1999). The other component of the *Hap* family, the Hap2-5 complex, regulates the transcription of genes involved in mitochondrial biogenesis and oxidative phosphorylation by acting on the CCAAT element .

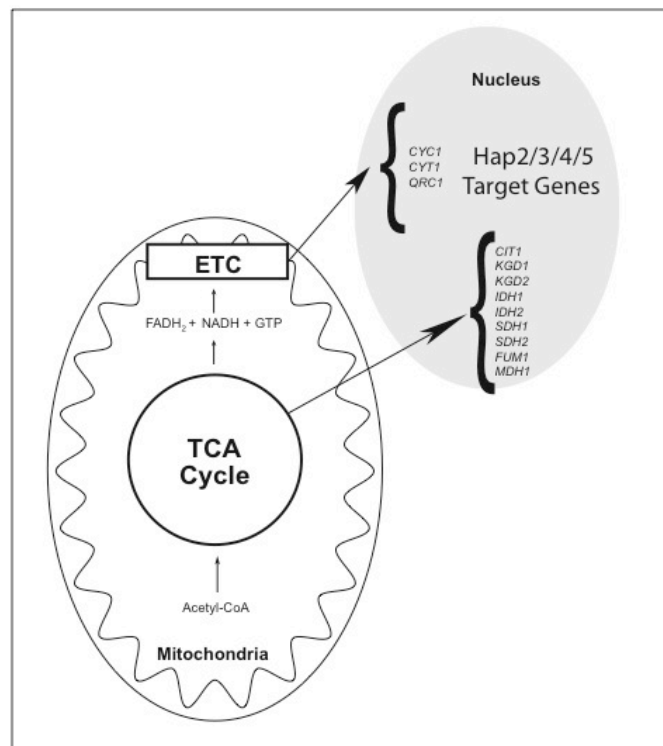


Figure 3: Hap2-5 Target Genes

A model for the role of the *Hap* Pathway in controlling the expression of genes involved in the TCA cycle and ETC. When the *Hap* pathway is activated the expression of key components of respiration increases and mitochondrial function and biogenesis is optimized as under the control of the *Hap* pathway.

As a global transcriptional activator, the Hap2-5 complex is responsible for the activation of about 100 genes responsible for respiration and other metabolic functions (Pinto & McNabb,

2005). Included in this group are genes encoding key enzymes in oxidative phosphorylation and mitochondrial biogenesis (**Figure 3**). These are required for respiration, with deletion or malfunction of any one of these subunits rendering cells unable to grow on non-fermentable carbon sources (Olesen, Hahn, & Guarente, 1987) (Bourgarel, Nguyen, & Bolotin, 1993). In de-repressed cells that are grown in low glucose media and that contain respiratory-competent mitochondria, the Hap2-5 complex is required for transcriptional activation of genes encoding key enzymes of the TCA cycle, including: *KGD1* (encoding the E1 component of α -ketoglutarate dehydrogenase), *IDH1* and *IDH2* (encoding the subunits of isocitrate dehydrogenase), *SDH1* (encoding the flavoprotein component of succinate dehydrogenase), *FUM1* (encoding the enzyme fumarate hydratase), and *MDH1* (encoding malate dehydrogenase) (Liu & Butow, 2006). Additionally, *CYC1* (encoding cytochrome *c*, isoform 1), *CYT1* (encoding cytochrome *c*1), *CIT1* (encoding the mitochondrial isoform of citrate synthase), *KGD2* (encoding the E2 component of mitochondrial α -ketoglutarate dehydrogenase complex), *QCR8* (encoding a subunit of the ubiquinone:cytochrome *c* oxidoreductase complex) and *SDH2* (encoding the iron-sulfur protein subunit of the succinate dehydrogenase complex) are also reported to be target genes of the Hap2-5 complex (Schüller, 2003).

The Hap2-5 complex binds to the 5'-CCAAT-3' consensus sequence in the promoter of target genes and is thus also known as the CCAAT-binding factor (CBF) complex in yeast. CBF's exist in many species and represents a conserved mechanism of heterologous gene regulation (Mantovani, 1999). The type and number of genes regulated by CBF's vary, but the 5'-CCAAT-3' consensus sequence that they bind to is conserved (Mantovani, 1999). Hap2, Hap3, and Hap5 each contain a core domain that is highly conserved during evolution and is required for DNA binding and subunit association. Among the four protein of the Hap2-5

complex, Hap2 was discovered first and its identification led to the discovery of Hap3 (Olesen, Hahn, & Guarente, 1987). Hap5 is required for interaction between Hap2 and Hap3, and thus the assembly and DNA-binding activity of the complex (McNabb, Xing, & Guarente, 1995) (Pinto & McNabb, 2005). The DNA binding domain of the Hap2-5 complex is located on the Hap2 and Hap3 subunits. These two subunits are functionally conserved in mammalian CBF (McNabb, Xing, & Guarente, 1995) (Xing, Fikes, & Guarente, 1993).

On the other hand, the Hap4 subunit of the complex is not required for DNA binding, but it is the regulatory subunit, with its levels determining the activity of the complex (Forsburg & Guarente, 1989)(Olesen & Guarente, 1990). In cells grown in low levels of glucose, *HAP4* expression is induced, leading to high levels of Hap4 and its migration into the nucleus, and the resultant activation of the Hap2/3/4/5 complex (as shown in **Figure 5**) (Liu Lab Unpublished Results). This activation is accomplished by two transcriptional activation domains one in the carboxyl- and one in the amino-termini (Stebbins & Triezenberg, 2004) (Forsburg & Guarente, 1989). The C-terminus contains a highly acidic region, a common characteristic of transcriptional activators (Forsburg & Guarente, 1989). *HAP4* expression is affected by the functional states of the mitochondria. In cells with robust mitochondrial respiratory activity, Hap4 expression is high, while in cells with dysfunctional mitochondria, for example due to loss of mitochondrial DNA as in ρ^0 petites, Hap4 expression is reduced (Ozcan, Dover, & Johnston, 1998).

As the regulatory subunit of the complex, Hap4 is an ideal point of focus for the study of regulation of the Hap pathway. Despite its central role in respiratory metabolism and mitochondrial biogenesis, little is known about the mechanism behind *HAP4* expression and how Hap4 levels are regulated. Further studies on the regulation of Hap4 levels could provide new

insights into the regulation of this important pathway. Regulation of Hap4 protein levels is the focus of this thesis.

Mitochondrial Biogenesis

Mitochondria are thought to have once existed as independent organisms. Their evolution into their current role as a cellular organelle is explained by the endosymbiotic theory, which postulates that mitochondria began as independent bacteria that were ingested through endosymbiosis. One important piece of evidence in support of this theory is that mitochondria still contain their own circular genome, known as mtDNA. Though most of the proteins utilized in the mitochondrial pathways are encoded in the nuclear genome, mtDNA contains 15,000-18,000 base pairs depending on the species. These base pairs are responsible for approximately thirteen protein-encoding genes required for respiration. However, the majority of genes involved in respiration are not located in mtDNA and are instead encoded in the nuclear genome. The remainder of the mitochondrial genome encodes the machinery necessary for translation of mtDNA. Mitochondrial number and function are altered in response to external stimuli; this is mediated through coordinated expression of genes encoded in the nuclear and mitochondrial genome. This coordinated expression is regulated by transcription/replication factors such as the Hap2-5 complex.

From the genetic standpoint, the respiratory chain is a unique cellular structure, being formed by means of the complementation of two separate genetic systems: the nuclear genome and the mitochondrial genome (Zeviani & Donato, 2004). Thus, one fundamental question in the biological sciences is how the coordinated expression between these two genomes occurs. For coordinated expression, cells must have a mechanism of sensing the state of the mitochondria and leading to changes in nuclear gene expression accordingly. The budding yeast is an ideal

organism for studying the signal transduction pathways involved in mitochondrial biogenesis: it is viable in ρ^0 (no mtDNA) and ρ^- (mutant mtDNA) states. Mitochondrial biogenesis is often studied through research on the regulation of key factors controlling the biosynthesis of the enzyme complexes of oxidative phosphorylation. This is often carried out by the same factors regulating the expression of genes implicated in glucose derepression, such as the Hap proteins.

The Ubiquitin-Protease System

Protein levels are controlled at transcriptional and post-translational levels. The ubiquitin-protease system (UPS) is the main degradation pathway in the cytosol in all eukaryotes and is key in post-translational regulation of protein levels (Seufert & Jentsch, 1990). Protein degradation is the breaking down of proteins via proteolysis, namely the breaking of peptide bonds. This is an important regulatory process that allows cells to dispose of mis-folded or damaged proteins, as well as to control the concentration of proteins within the cell by selective degradation. Selective degradation is key to the control of cellular signal transduction and regulation of cellular pathways by modulation of the levels of key regulatory factors (Hochstrasser, 1996). Through this method, the UPS controls multiple pathways by regulating the stability of target proteins that govern specific cellular function, including the cell cycle, class I antigen processing, transcriptional regulation, signal transduction pathways, receptor down regulation, and receptor-mediated endocytosis (Hochstrasser, 1996). Malfunction in this system leads to a number of disease states and pathological disorders including Alzheimer's, Huntington's, Parkinson's, and malignant transformation linked to cancer (Davies, Sarkar, & Rubinsztein, 2007) (Schwartz, 1999). Understanding of the factors regulating the UPS in targeted degradation thus has practical application in the cellular viability and disease mechanism.

In 2004, Aaron Ciechanover, Avram Hershko, and Irwin Rose won the Nobel Prize in Chemistry for their work on the discovery of the UPS. The UPS involves poly-ubiquitination of proteins through covalent binding of ubiquitin molecules to a target protein in an ATP-dependent manner at specific lysine residues for its subsequent degradation through the 26S proteasome (**Figure 4**) (Tongaronkar, Li, Lambertson, Ko, & Madura, 2000). Three types of enzymes catalyze the covalent attachment of ubiquitin to its target protein, which, with increasing specificity, ensure a system with the high degree of selectivity toward its many substrates (Ciechanover, Orian, & Schwartz, 2000). These are: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3).

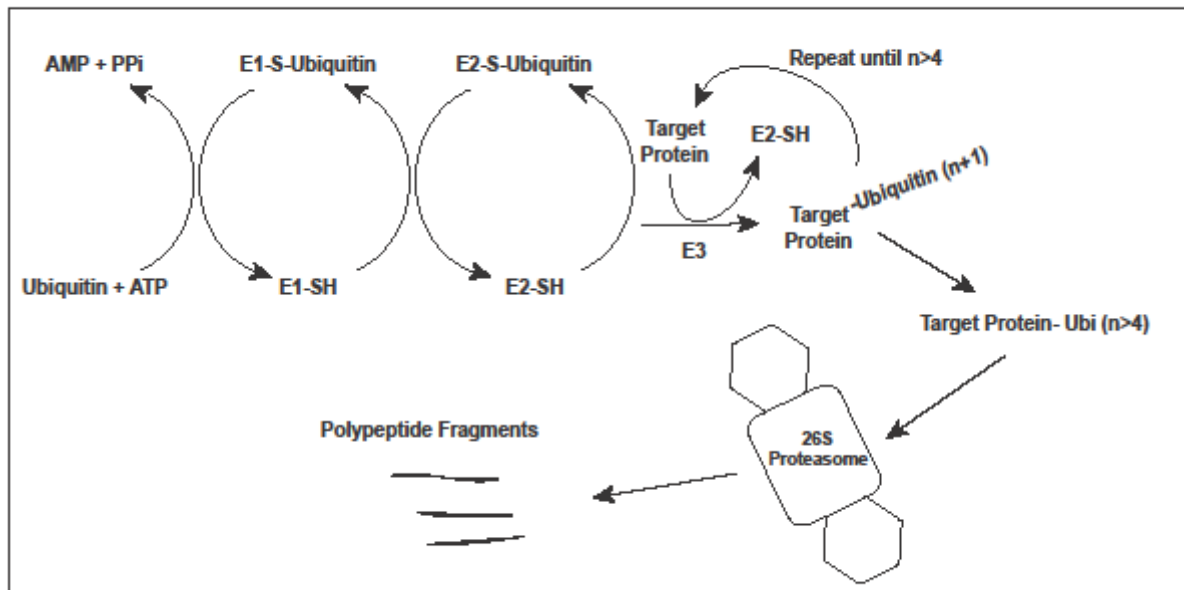


Figure 4: The Ubiquitin-Proteasome System

A simplified model of the Ubiquitin-Proteasome System. Whereby, a target protein is polyubiquitinated by repeated cycling through a three enzyme (E1, E2, and E3) cascade for its eventual targeting to the 26S-Proteasome for degradation.

The UPS starts with ubiquitin's activation by E1 in an ATP-dependent reaction, which resulting in the linking of ubiquitin to E1 by means a high-energy thioester bond at a highly

conserved cysteine residue. Ubiquitin is then transferred to E2, where it forms a thioester bond at a conserved cysteine residue on the surface of E2. Then, ubiquitin is transferred to a targeted protein substrate as facilitated by E2 and E3, which together catalyze the isopeptide bond formation between ubiquitin and a lysine residue(s) on the substrate (Hochstrasser, 1996). This enzyme cascade is repeated to form a chain of isopeptide-linked ubiquitin on the substrate, a structure that targets the protein to the 26S proteasome complex, a large, multi-protein complex that catalyzes proteolysis of the ubiquitinated protein.

Polyubiquitination of target proteins is a highly regulated process and varies greatly among substrate proteins. While much is known about the overall mechanism of the UPS, the exact signals that target specific proteins for degradation remain to be discovered in many signal transduction pathways. A few of the known factors that affect the regulation of protein turnover include: phosphorylation as a signal for ubiquitination (as in the case of Mos, oocyte protein kinase and beta-catenin and NF- κ B regulatory protein I κ B α), glucose (as with fructose biphosphate in yeast), glycosylation (for the elimination of denatured glycoproteins), amino acid starvation (as for Gcn4 a transcription factor in yeast), and even red light (seen in Phytochrome, a plant light-responsive receptor) (Hochstrasser, 1996) (Hunter, 2007) (Orford, Crockett, Jensen, Weissman, & Byers, 1997).

The enzymes of the UPS are highly conserved in all eukaryotic cells, though the number and variety is more complex in mammalian cells as compared to lower eukaryotic organisms. Though there are many homologues between yeast and mammalian E2's, the terminology is completely different and does not reflect the functional or structural homologies (Glickman & Ciechanover, 2002). In *S. cerevisiae* there exists a single E1, designated (UBA1), thirteen E2's (UBC), and an unknown number of E3's. Of the thirteen identified E2's in the yeast genome,

two, Ubc9 and Ubc12, do not conjugate to ubiquitin, but are nonetheless considered part of the UBC family.

As stated previously, Hap4 levels are dramatically decreased in cells with dysfunctional mitochondria as compared to those with robust mitochondrial respiratory activity (**Figure 5**). This change in protein level is not fully explained by *HAP4* transcription, indicating that Hap4 might be subject to posttranslational regulation (Capps, 2011) (Ozcan, Dover, & Johnston, 1998).

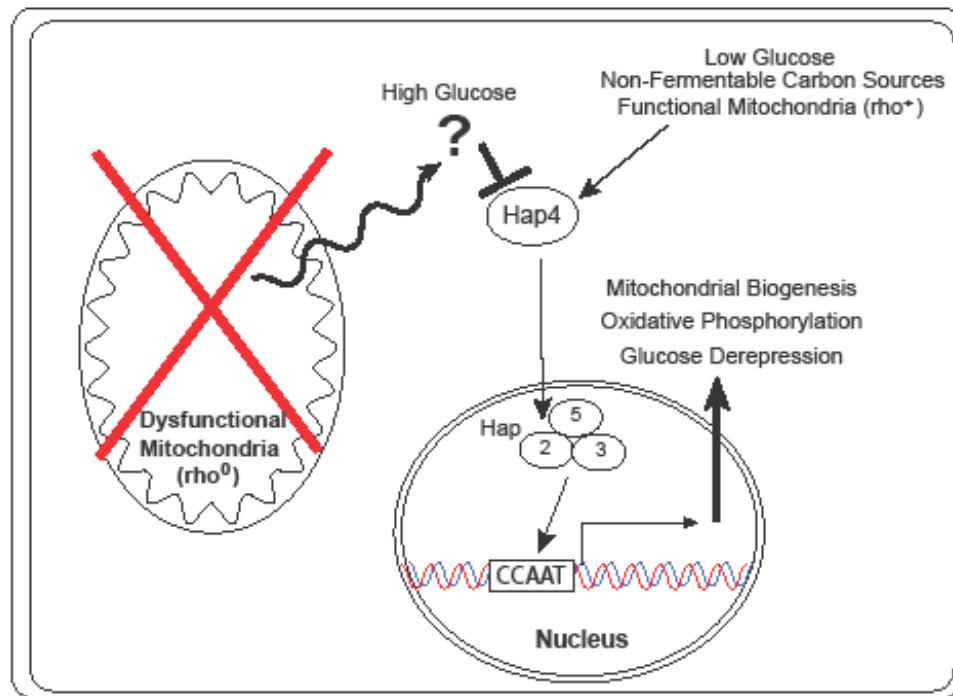


Figure 5: Hap4 Activity in Response to Dysfunctional Mitochondria

A model for the regulation of the Hap2-5 complex. To the right are de-repressing conditions under which Hap4 levels are high, leading to its migration into the nucleus for interaction with the Hap2/3/4/5 complex for its activation and, consequently, the transcription of genes involved in respiratory growth. To the left are repressing conditions under which Hap4 is inhibited by high glucose and some unknown mechanism in cells with dysfunctional mitochondria, leading in turn to a downregulation of the *HAP* pathway and respiratory growth.

Materials and Methods

Media and Growth Conditions

Yeast strains were grown in media at 30°C. The media used were: YPD medium (1% Bacto-yeast extract, 2% Bacto-Peptide, 2% dextrose), YNB medium (0.67% yeast nitrogen base) supplemented with 1% Casamino Acids, 5% or 2% dextrose (YNBcasD) or 2% raffinose (YNBcasR) (Amberg, Burke, & Strathern, 2005). For plates, agar was used as well (2% Bacto-Agar). When required, tryptophan (30 mg/liter) was added to YNBcasD or YNBcasR media to allow *trp1* mutant cells to grow.

Yeast Strains

All yeast strains used in this project were of one of two parent strain backgrounds: Y000 (*MATa his3-Δ200 leu2-3,2-112 lys2-801 trp1-1(am) ura3-52*) or BY4741 (*MATa ura3Δ leu2Δ his3Δ1 met15Δ*). Specific strains that were used in ρ^0 mutant generation and plasmids for transformation were all acquired from Liu Lab stock.

Yeast Expression Plasmids for Constructing Epitope Tagged Proteins

In this project, two yeast expression vectors were used as plasmid templates: pRS414 (*TRP1*) and pRS416 (*URA3*) (Mumberg, Muller, & Funk, 1995). The gene encoding the protein under investigation was fused at its 3'-end, to a DNA sequence encoding a 10-alanine linker and a epitope tag (-myc3 or -HA3).

Generation of strains lacking mtDNA (ρ^0)

Respiratory capable, ρ^+ yeast strains were treated with ethidium bromide (EB), a mutagenic known to preferentially intercalate into mtDNA. To generate ρ^0 cells, ρ^+ cells were grown in YPD medium supplemented with 15 ug/ml EB at 30°C to saturation. Cultures

were streaked out on YPD plate to obtain single colonies. Single colonies were then streaked on to YPGlycerol plates and YPD plates. Cells lacking mtDNA were selected for by growth phenotype, as ρ^0 cells unable to form colonies on YPGlycerol plates (due to inability to undergo respiration). Loss of mtDNA was further verified by 4,6-diamidino-2-phenylindole (DAPI) staining of individual colonies and fluorescence microscopy using MetaMorph computer program.

Yeast High-efficiency Transformation

Fresh cells were inoculated in rich YPD media (or appropriate selective media) using a sterile loop to OD ~0.2. Cells were incubated 30°C for 3-5 hours with agitation. Cells were harvested by centrifuge and re-suspended in 0.1M LiOAc. Cells were incubated with plasmid DNA and transformation mix (single strand DNA, LiOAc, PEG) at 42°C for 60 minutes. Cells were then plated on selective media to select for transformants.

Protein Collection

Yeast strains were transformed with plasmids containing the gene encoding the protein of interest with an epitope tag. Cells were lysed using extraction buffer (18.5% 10N NaOH, 7.4% beta-mercaptoethanol) on ice for 10-30 min. Total cellular proteins were collected through precipitation using 8% trichloroacetic acid. Pellets were neutralized using 1M unbuffered Tris and boiled in SDS-PAGE loading buffer for 4 min. For samples where cysteine bond formation was examined, samples were collected using extraction buffer lacking β -Mercaptoethanol.

Immunoblotting (Western Blotting)

Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein loading was balanced based on the OD₆₀₀ readings of cultures before collection. For samples in which cysteine bond formation was to be examined, urea (8M) was

used for denaturing and DTT was omitted to prevent reduction of disulfide bonds. Proteins were transferred to nitrocellulose membranes by electroblotting. For immunoblotting, nitrocellulose membranes were probed with primary antibody, either rat monoclonal high affinity anti- HA IgG or mouse monoclonal anti-Myc anti-body in 1X TBS-Tween solution with 5% milk powder. Membranes were then probed with HRP-conjugated goat anti-rat, goat anti-mouse, or goat anti-rabbit antibody. Chemiluminescence was produced using the ECL reagent and images were captured using the Bio-Rad Chemi-Doc system. For loading controls, membranes were stripped in deprobing buffer (67.5 mM Tris-HCl, pH 6.7, 2% SDS, 0.08% β -mercaptoethanol) at 60°C for 30 min and reprobed with rabbit polyclonal anti-Pgk1 antibody (ubiquitous and stable protein phosphoglycerate kinase) .

PCR mutagenesis of Cysteine to Serine mutant of Ubc4

Polymerase Chain Reaction (PCR) SOEing was utilized to carry out site-directed mutagenesis. To this end, primers were designed with nucleotide mismatches for the introduction mutation of cysteine residue 22 to serine (C22S) or cysteine residue 108 to serine (C108S) by point mutation of guanine to cytosine (**Figure 6**). Mutants were confirmed by sequencing. Plasmids containing confirmed mutations were transformed into respective yeast strains and selected for as outlined in Yeast High Efficiency Transformation.

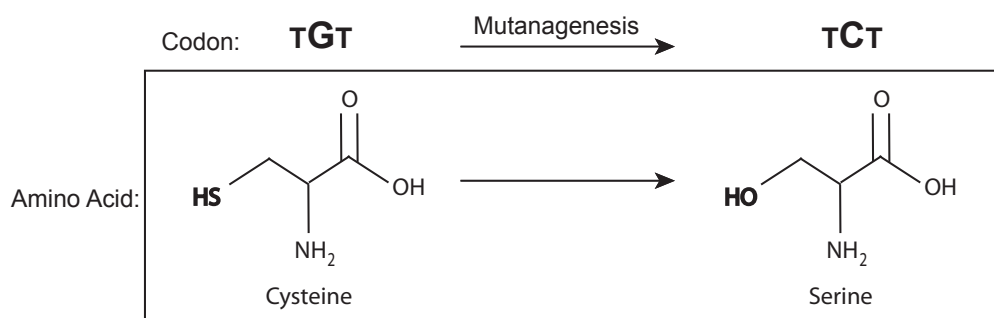


Figure 6: Point Mutagenesis of Cysteine Residue to Serine

Cycloheximide Chase Analysis

Cells were grown overnight in liquid YNBcas5%D or YNBcasRaff medium to log phase (OD_{600} 0.6~0.8). Before cycloheximide treatment, 1.0 ml culture was collected as zero time point. Cycloheximide was used to inhibit protein synthesis by acting on the ribosome to prevent translation. At fixed time intervals (five or ten min.), 1.0 ml culture was collected for generating total cellular extracts. Proteins of interest were visualized by Immunoblotting. Two to three independent experiments were carried out and scrutinized for reproducibility. Quantification was carried out using Bio-Rad QuantityOne software and protein half-life ($T_{1/2}$) was calculated using Microsoft Excel.

β -Galactosidase Assay

Plasmids expressing a *lacZ* gene fused to the promoter of the gene under analysis were transformed into yeast strains. Cells were grown to undergo a minimum of six generations and collected at OD_{600} 0.6-0.8. β -galactosidase assays were carried out as described (Amberg, Burke, & Strathern, 2005). Briefly, cells were collected by centrifugation and resuspended in breaking buffer (100 mM Tris-HCl, pH 8, 1 mM dithiotreitol, 20% glycerol). Samples were stored at -20 °C until assay was conducted. For assays, cells were thawed on ice and treated with 1 mM phenylmethylsulfonyl fluoride [PMSF] to prevent protein degradation. Total cellular extracts were obtained by vortexing in the presence of glass beads (0.45-0.50 mm). β -galactosidase activities of a *lacZ* reporter gene were reported in nanomoles of colorimetric product o-nitrophenyl- β -d-galactopyranoside [ONPG] per milligram of protein per minute. Specific activity was calculated in relation to the total protein amount in the initial extract, determined using the

Bradford assay. For each plasmid-strain combination, two or three independent cultures were grown and β -galactosidase activities were determined.

Results

Preliminary Findings from Liu Lab

As reported by Denise Capps in her thesis for the requirements for a degree of Master of Science in Biological Sciences at the University of New Orleans (Capps, 2011).

Mitochondrial Functional State Regulates Hap2-5 Pathway Activity

In previous experiments from Liu Lab, it has been reported that expression of *KGDI*, a Hap2-5 complex reporter gene, is reduced in ρ^0 cells. The *KGDI* activity is similar to that seen in a *hap4* deletion mutant, where the pathway is inactivated, suggesting the Hap2-5 pathway is down regulated in response to compromised mitochondrial function. The promoter activity of Hap4 was also found to be down regulated in ρ^0 cells, however only a 2.5-fold decrease from ρ^+ to ρ^0 was observed, leaving the question of where the discrepancy in pathway activity and *HAP4* expression is originating. Hap4 protein levels were examined using Western blotting and found to show a six-fold decrease from ρ^+ to ρ^0 cells. The discrepancy in *HAP4* promoter activity and Hap4 protein levels suggest that Hap4 is subject to post-transcriptional regulation. It was then found that Hap4 is highly unstable in ρ^+ cells. In ρ^0 cells, Hap4 instability is further increased. Hap4 instability was found to be mediated through proteasome-dependent degradation.

Goals of Research

The overall objective of my research is to understand the cellular mechanisms regulating the coordinated expression of genes involved in mitochondrial biogenesis and oxidative

phosphorylation between mitochondria and the nuclear genome. A fundamental question is how cells sense functional states of mitochondria and adjust nuclear gene expression accordingly, thus coordinating the expression of genes between the two genomes. Hap4, the regulatory subunit of the Hap2-5 complex in this pathway, is the focus of my study. More specifically, my central focus is on how Hap4 degradation is regulated through the Ubiquitin-Proteasome system and the mechanism underlying the increased degradation of Hap4 in ρ^0 cells.

Research Results

Hap4 has increased instability in ρ^0 cells, as compared to ρ^+ cells

Hap4 is a highly unstable protein (Capps, 2011). To confirm that Hap4 is further destabilized in cells lacking functional mitochondria, ρ^+ and ρ^0 cells containing a plasmid encoding 3xHA-tagged Hap4 were assayed for Hap4 stability using cycloheximide chase and immunoblotting. The results of this experiment are shown in **Figure 7**.

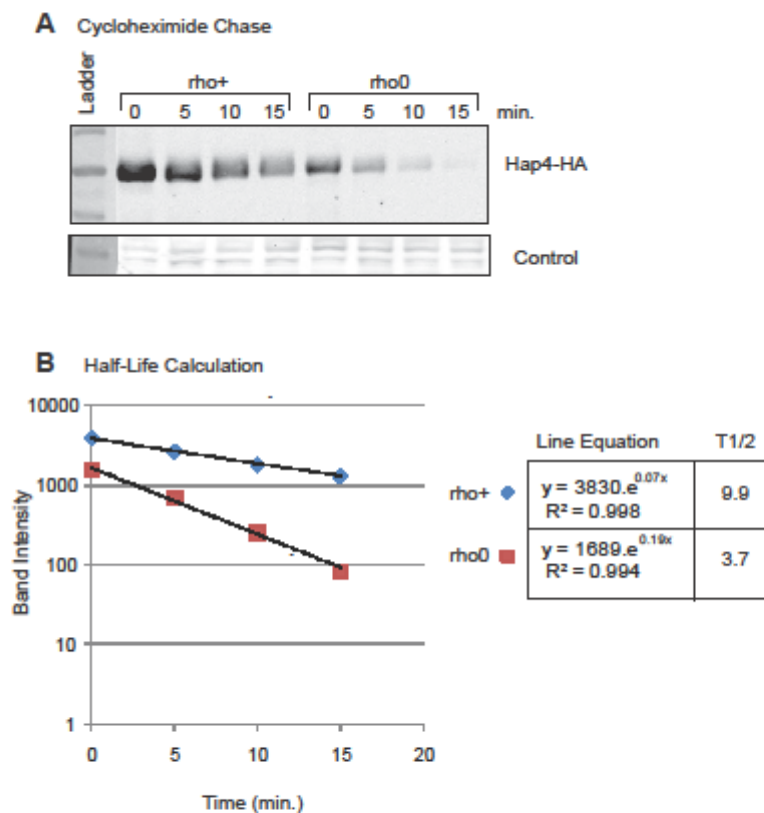


Figure 7: Analysis of Hap4 Stability by Cycloheximide Chase

(A) Strains containing a *HAP4*-HA plasmid were assayed for Hap4 stability using cycloheximide chase and immunoblotting. (B) Hap4 levels in panel A were determined using Bio-Rad QuantityOne software and the half-life ($T_{1/2}$) of Hap4-HA was calculated using Microsoft Excel.

Consistent with Denise Capps's result, Hap4 is an unstable protein in cells with normal functioning mitochondria (rho^+ cells), with an estimated half-life of ~10 minutes. In cells with dysfunctional mitochondria (rho^0 cells), Hap4 is greatly destabilized, with an estimated half-life of ~4 min. Based on these results, it is clear that there exists some cellular mechanism by which Hap4 stability is regulated by the functional states of mitochondria.

Hap4 is stabilized in a *ubc1* Δ *ubc4* Δ double mutant

To identify the ubiquitin-conjugating (Ubc) enzyme(s) responsible Hap4 degradation in rho^0 cells, I analyzed Hap4 stability in a collection of fifteen strains lacking designated Ubc enzymes as described in Figure 8. To this end, all mutant strains were acquired from Liu Lab Yeast Stock and rho^0 petite derivatives of the mutants were generated as described in Materials and Methods. These rho^0 cells were transformed with plasmids encoding 3xHA-tagged *HAP4* and transformants were analyzed for Hap4 stability using cycloheximide chase. Figure 8 shows that only a *ubc1 ubc4* double mutation greatly stabilizes Hap4, indicating that Ubc1 and Ubc4 play a redundant role in mediating ubiquitin-proteasome dependent degradation of Hap4.

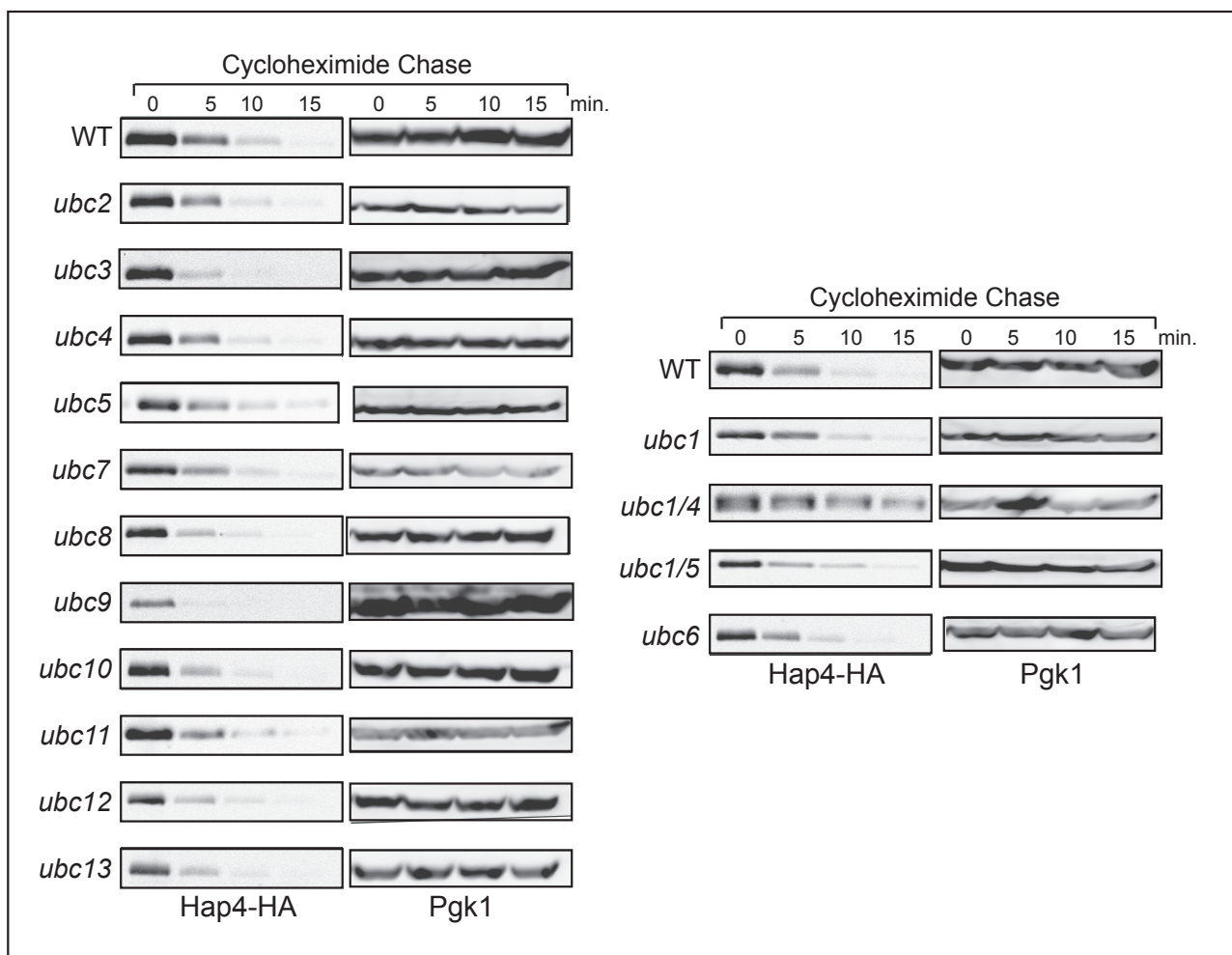


Figure 8: Analysis of Hap4 Stability in *ubc* Mutants by Cycloheximide chase
 Rho⁰ wild-type and isogenic *ubc* mutant cells were grown in YNBcasR media to mid-log phase and Hap4 stability was analyzed by cycloheximide chase and immunoblotting. Pgk1 protein levels were determined using anti-Pgk1 antibody and included as loading controls.

Hap4 stabilization in *ubc1Δubc4Δ* mutant cells under various conditions

We next examined the effect of a *ubc1 ubc4* double mutation on Hap4 stability in rho⁺ cells grown in YNBcasR media and in both rho⁺ cells and rho⁰ cells grown in YNBcasD media. I found that Hap4 is stabilized in *ubc1 ubc4* double mutant cells under all four conditions (rho⁺, rho⁰, YNBcasR, YNBcasD) (**Figure 9**). These data suggest that Ubc1 and Ubc4 play a house-keeping role in turning over Hap4. However, they do not exclude the possibility that the activity

of Ubc1 and Ubc4 towards Hap4 is increased in response to a loss of mitochondrial respiratory function.

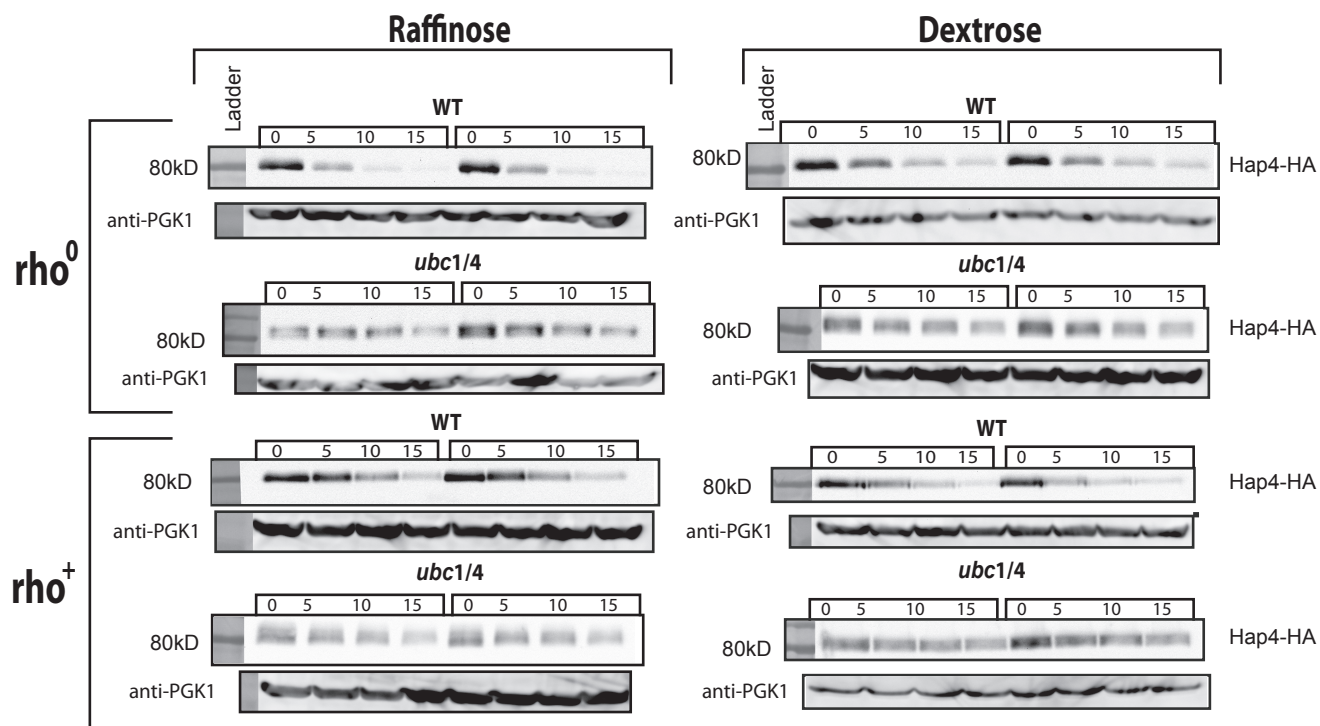


Figure 9: Hap4 Stability in $ubc1\Delta ubc4\Delta$ mutant

ρ^+ and ρ^0 wild-type and isogenic $ubc1 ubc4$ mutant cells were grown in YNBcasD or YNBcasR media and Hap4 stability was analyzed by cycloheximide chase and immunoblotting. Pgk1 was used as loading controls.

Hap4 stabilization due to a $ubc1\Delta ubc4\Delta$ double mutation correlates with increased expression of Hap2-5 target genes

Hap4 is stabilized in a $ubc1 ubc4$ double mutant, however the question remains of does Ubc1/4-dependent degradation of Hap4 affect pathway activity. To this end, the expression of two Hap2-5 target genes, *KGD1* and *SDH1*, was analyzed in ρ^+ wild-type and isogenic $ubc1\Delta ubc4\Delta$ double mutant cells grown in YNBcasD media. Figure 10 shows that expression of a *lacZ* reporter gene under the control of *KGD1* or *SDH1* promoter is increased ~6-fold in $ubc1 ubc4$ double mutant cells compared to wild-type cells. To determine whether an increase in the

expression of *KGD1*- and *SDH1*-lacZ reporter genes in the *ubc1 ubc4* mutant is Hap4-dependent, their expression was analyzed in a *hap4* single mutant and a *ubc1 ubc4 hap4* triple mutant. Figure 10 shows that a *hap4* mutation largely abolishes increased expression of *KGD1*- and *SDH1*-lacZ reporter genes in the *ubc1 ubc4* mutant. Together, these data indicate that Ubc1/4-dependent degradation of Hap4 negatively regulates the expression of Hap2-5 target genes.

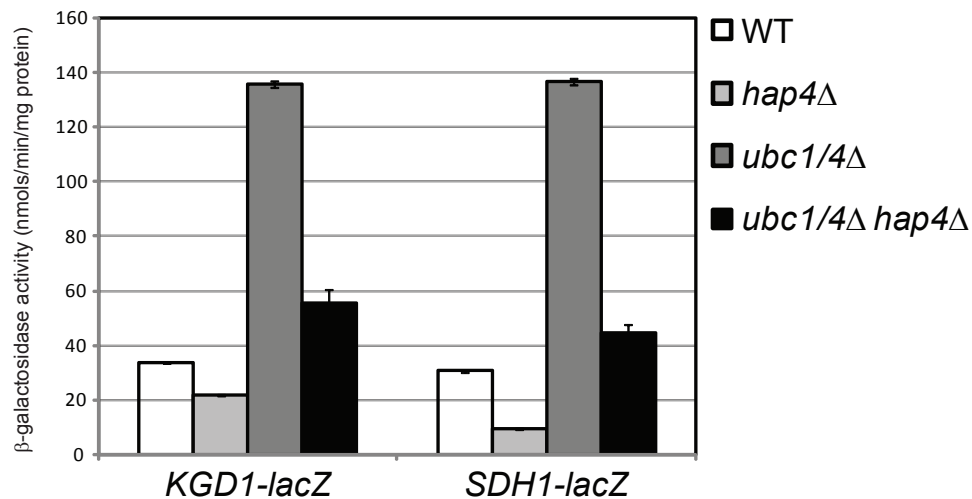


Figure 10: Hap2-5 Pathway Activity is Up-Regulated by Hap4 Stabilization

Rho⁺ wild-type and isogenic *hap4* single, *ubc1/4* double and *ubc1/4 hap4* triple mutant cells carrying either a *KGD1-lacZ* or a *SDH1-lacZ* fusion gene were grown in YNBcasD media and β-galactosidase activities were determined as described in Methods and Materials.

Two cysteine to serine mutants of Ubc4 are functional based on their ability to restore robust cell growth to a *ubc4 ubc5* double mutant

Ubc4 and Ubc5 share three highly conserved cysteine residues. Of the three, one is the catalytic residue and the other two serve no known function. Cysteine residues are among the least common amino acid residues and are known to be involved in sensing the redox environment of the cell. A loss in the mitochondrial respiratory function might affect the redox balance in the cell, which in turn might affect the activity of Ubc4 towards Hap4. To test this

possibility, the conserved non-catalytic cysteine residues at the 22nd and 108th positions of Ubc4 were mutated to a serine residue and the resulting mutants will be referred to as C1S and C2S for C22S and C108S mutations, respectively. We determined whether Ubc4(C1S) and Ubc4(C2S) mutants are functional based on their ability to restore fast cell growth to a *ubc4 ubc5* double mutant (Figure 11). A *ubc5Δ* single deletion mutant that expresses wild-type Ubc4 shows robust growth. In contrast, in cells lacking both Ubc4 and Ubc5, the growth is strongly reduced. In *ubc4 ubc5* double mutant cells transformed with a plasmid encoding myc-tagged *UBC4*, robust cell growth is largely restored. This growth is matched by the *ubc4*(C1S) and *ubc4*(C2S) mutants, indicating that these two mutants are functional.

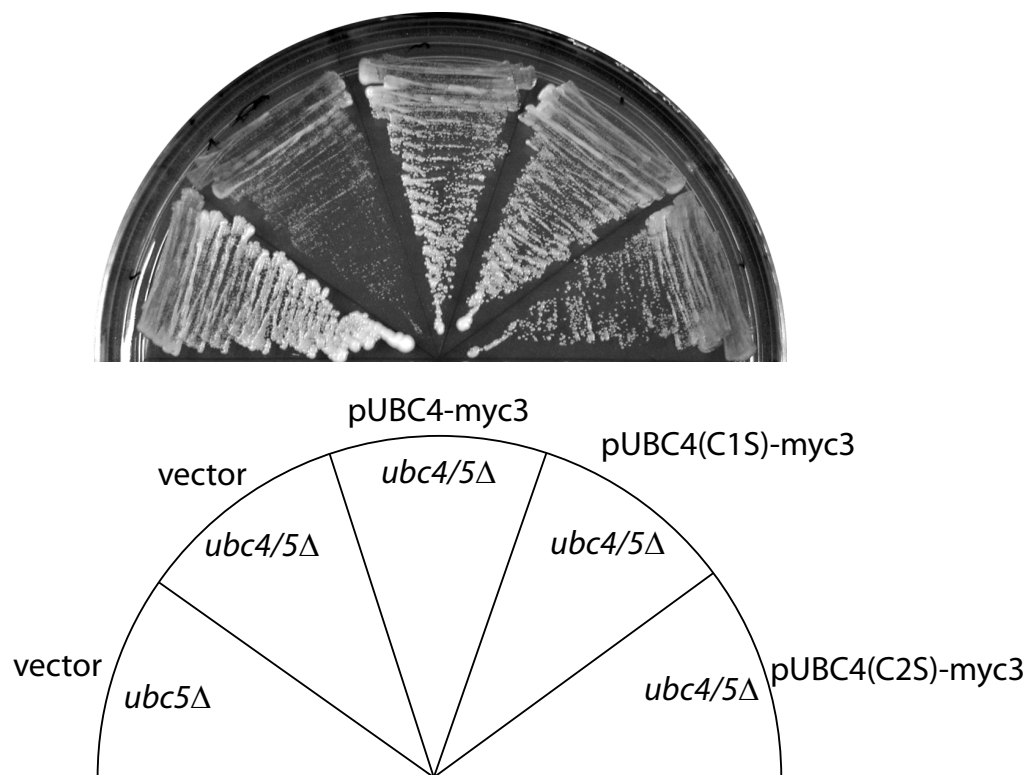


Figure 11: *ubc4* Cysteine to Serine Mutant Functionality

Plasmids encoding myc3-tagged *UBC4* wild type, *C1S* or *C2S* mutant were transformed into a *ubc4 ubc5* double mutant and transformants were grown on YNBcasD media. As controls, a *ubc5* single and a *ubc4 ubc5* double mutant each carrying an empty vector were also tested for growth. The picture was taken after 48 h. The slow growth phenotype seen in *ubc4Δ ubc5Δ*

double mutant cells carrying an empty vector is not shared with the strains transformed with a plasmid encoding any of the *UBC4-myc3* (WT or mutant).

The steady-state level of Hap4 is increased in *ubc4(C1S)* mutant

To determine whether the *C1S* and *C2S* mutations in *UBC4* affect Hap4 stability, we first examined the effect of these two mutations on the steady-state level of Hap4. To this end, ρ^0 *ubc1 ubc4* double mutant cells co-expressing Hap4-HA and Ubc4-myc, *ubc4(c1S)*-myc or *ubc4(C2)*-myc were grown in YNBcasD or YNBcasR media and analyzed for Hap4 expression by SDS-PAGE and immunoblotting. Figure 12 shows that the *C1S* mutation, not the *C2S* mutation, significantly increases the steady-state level of Hap4, suggesting that the cysteine residue 22 might be required for Hap4 degradation.

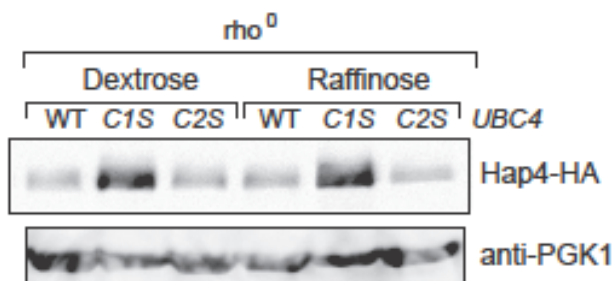


Figure 12: Hap4 Steady State Levels in *ubc4* (Cys to Ser) Mutants

ρ^0 *ubc1Δ ubc4Δ* strain containing plasmids encoding *HAP4-HA* and *UBC4* alleles as indicated were grown in dextrose or raffinose media and total cellular proteins was prepared using TCA precipitation. The steady-state level of Hap4 was analyzed using immunoblotting. Pgk1 protein levels were determined to show equal sample loading.

To determine whether the effect of the *UBC4(C1S)* mutant on Hap4 expression observed in Figure 12 is post-translational or transcriptional, I determined the activity of a *HAP4-lacZ* reporter gene, which provides information on *HAP4* transcription, in *ubc1 ubc4* double mutant cells expressing myc-tagged wild-type *UBC4*, a *C1S* or a *C2S* mutant. Figure 13 shows that the promoter activity of *HAP4* is similar in cells expressing wild-type *UBC4*, the *C1S* or the *C2S*

mutant. The data in Figure 12 and 13 provide evidence for a post-transcriptional mechanism behind the increase in the steady-state level of Hap4 observed in the *ubc4(C1S)* mutant.

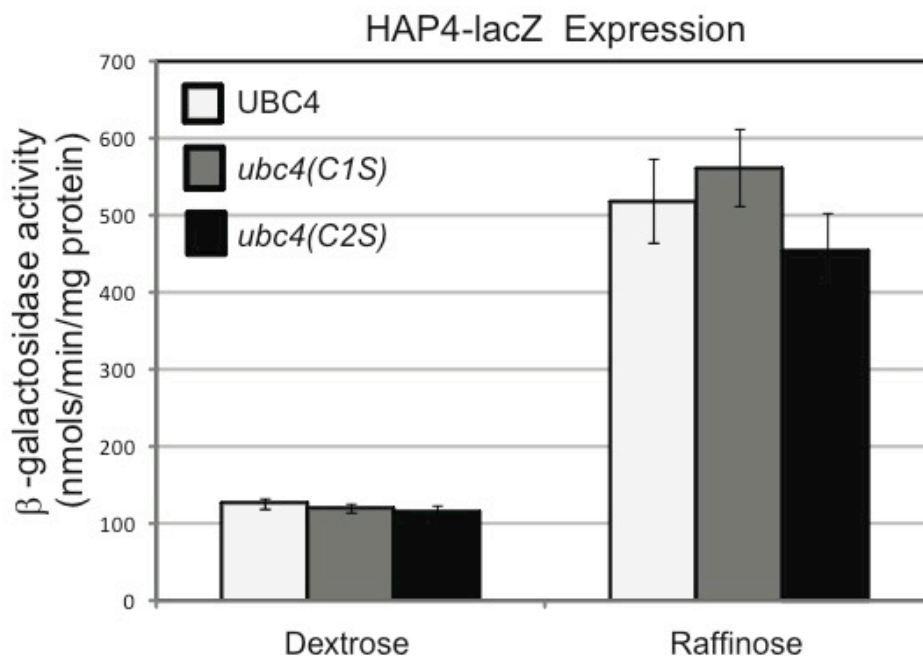


Figure 13: *HAP4-lacZ* promoter activity in *ubc4* (Cys to Ser) mutation

Rho⁰ *ubc1Δ ubc4Δ* mutant cells containing plasmids encoding *HAP4-lacZ* and *UBC4* alleles as indicated were grown in dextrose or raffinose media and media and β-galactosidase activities were determined as described in Methods and Materials.

Hap4 is stabilized due to a *ubc4(C1S)* mutation

To confirm that Hap4 is stabilized in a *ubc4(C1S)* mutant, I determined Hap4 stability in *ubc1 ubc4* double mutant cells expressing myc-tagged Ubc4 or a C1S mutant using cycloheximide chase. Hap4 is destabilized in *ubc1 ubc4* double mutant cells transformed with a plasmid encoding *UBC4-myc3* as compared to a control vector. In *ubc1 ubc4* double mutant cells transformed with a plasmid encoding the *C1S* mutant allele, Hap4 is dramatically stabilized. This stabilization is comparable to that in the *ubc1Δubc4Δ* double mutant with a control vector. Together, these data suggest that the cysteine residue 22 of Ubc4 is required for its activity

towards Hap4.

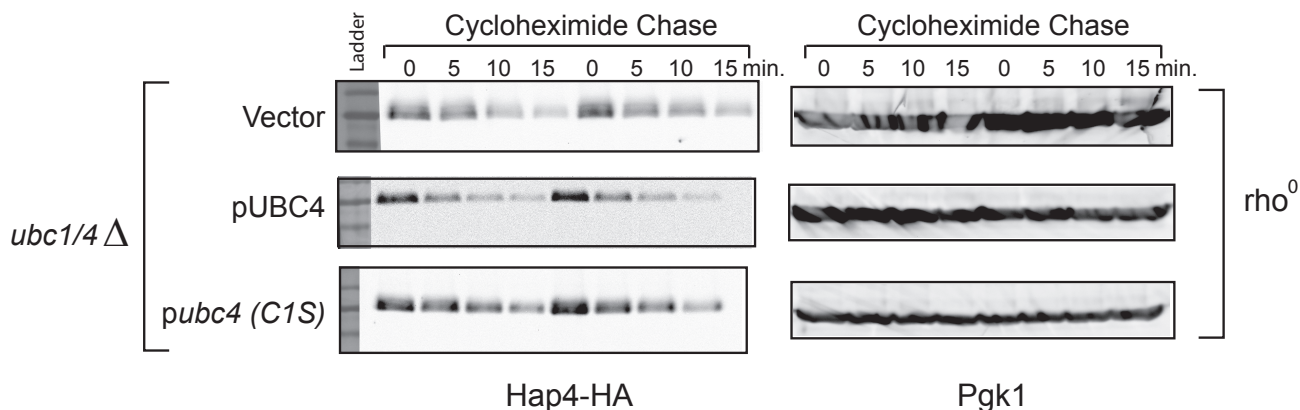


Figure 14: *ubc4*(C1S) mutation leads to Hap4 stabilization

Rho^0 *ubc1Δ ubc4Δ* strain containing plasmids encoding *HAP4-HA* and *UBC4* alleles as indicated were grown in raffinose media and Hap4 stability was analyzed by cycloheximide chase. Pgk1 protein levels were determined to show equal sample loading.

The *ubc4*(C1S) mutation affects Ubc4 modification

As a first step towards understanding the effect of a *C1S* mutation on Ubc4 activity, I determined whether the *C1S* mutation affect post-translational modification of Ubc4. It has been shown previously that Yap1 contains a redox active cysteine residue that causes mobility shift on polyacrylamide gel. To observe whether similar modification exists to Ubc4, I prepared cellular extracts without the use of reducing reagents. In cells expressing myc-tagged wild-type Ubc4 (WT) or a *ubc4*(C2S) mutant, Ubc4 exists as two distinct bands on Western blots. In contrast, the *ubc4*(C1S) mutant exists as a single band, corresponding to the slower-mobility form of the wild-type Ubc4 or the C1S mutant. In samples prepared in the presence of the reducing reagents DTT and β -mercaptoethanol, the fast-mobility form of Ubc4 disappears, suggesting that cysteine residue 22 of Ubc4 might be involved in redox sensing.

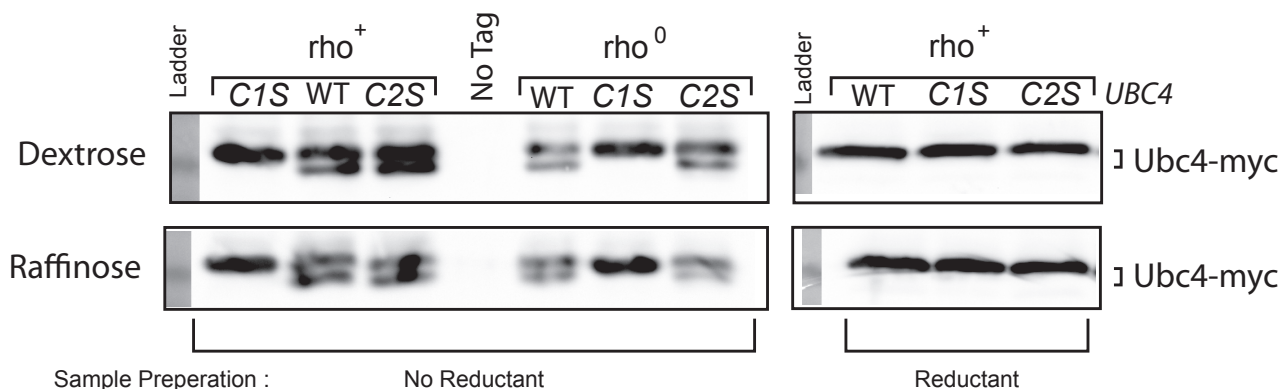


Figure 15: Post-translational modification of Ubc4

Rho⁺ and Rho⁰ *ubc1Δ ubc4Δ* strain containing a plasmid encoding myc-tagged wild-type *UBC4*, a *C1S* or a *C2S* mutant were grown in YNBcasD or YNBcasR media and Ubc4 was analyzed for post-translational modification by immunoblotting. Protein preparation and SDS-PAGE were done using two different procedures: reducing and non-reducing. Reducing SDS-PAGE was carried out using the reducing reagents dithiothreitol (DTT) and β-Mercaptoethanol (BME) during sample preparation. For samples that received non-reducing treatment, no reducing reagent was used.

Discussion

In rho⁰ cells, where respiratory growth is not possible due to loss of components of oxidative phosphorylation, it is energetically favorable for cells to repress the expression of nuclear genes involved in respiratory metabolism for efficient use of cellular resources. Hap4, as the regulatory subunit of a major transcriptional activator of genes involved in oxidative phosphorylation and mitochondrial biogenesis, is an ideal target for this repression. Selective degradation of Hap4 as mediated through the UPS is largely implicated as the mechanism behind the decreased Hap2-5 pathway activity seen in rho⁰ cells. The discrepancy seen in the half-life of Hap4 in rho⁰ versus rho⁺ cells, which is not explained by Hap4 promoter activity, indicates a post-translational mechanism mediating Hap4 stability. This implicates the UPS in controlling the cellular response to dysfunctional mitochondria through increased degradation of Hap4.

Of the 13 ubiquitin-conjugating E2 enzymes encoded in the yeast genome, Ubc4 and Ubc5 are the main factors that mediate bulk protein degradation (Seufert & Jentsch, 1990). They are also involved in targeting a number of short-lived, abnormal, misfolded proteins (Glickman & Ciechanover, 2002). The redundant function of Ubc4 and Ubc5 is shared with Ubc1. However, Ubc1 has been implicated in acting mostly in the early stages of cell growth and therefore may have a more limited role in bulk protein degradation (Seufert, McGrath, & Jentsch, 1990). In this study, I found that Ubc1 and Ubc4 play a redundant role in mediating Hap4 degradation. Successful identification of the E2 enzymes responsible for Hap4 degradation through the UPS opens the gateway to research on the factors and cellular mechanisms regulating the selective targeting of Hap4 in response to mitochondria functional state.

Mitochondria are a major source of reactive oxygen species (ROS), which are produced as byproducts of aerobic respiration. These byproducts include hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot), and the superoxide radical (O_2^\cdot), all of which are highly reactive and capable of damaging cellular components. There is a large debate in the scientific community over the redox environment of rho^0 cells. One school of thought suggests that due to decreased respiratory activity, oxidative stress is reduced in cells lacking mtDNA (Chandel & Schumacker, 1999). However, it is also argued that the cellular environment is subject to increased oxidative stress due to high concentrations of ROS produced due to blocks in the respiratory chain. Our data shown in Figure 15 suggests that Ubc4 is oxidized via cysteine residue 22 based on its similar modification to another well-studied protein Yap1.

A number of short-lived proteins have been shown to be subject to increased targeting by Ubc4 and Ubc5 in response to oxidative stress (Medicherla & Goldberg, 2008). These enzymes are also the only two E2s in the yeast genome that share the highly conserved cysteine 22 and

108 cysteine residue (the numbering is based on Ubc4) (**Figure 16**). In combination with the enzymes' known overlapping functions, these observations suggest these two cysteine residues are potential sites of regulation in response to changes in the cellular redox environment. As previously stated, cysteine residues are rare in proteins and are often involved in cellular redox sensing. This is accomplished at cysteine's sulfhydryl group (SH), which responds to the oxidative state of the cell through the formation of redox sensitive bonds either to another cysteine residue (disulfide bonds) or is oxidized to sulfenic acid, sulfinic acid, or sulfonic acid.

Two proteins commonly involved in redox sensing are thioredoxin and glutaredoxin, both of which exist in *S. cerevisiae*. These two proteins work in a similar manner to sense and respond to oxidative stress for maintaining homeostasis of the cellular environment. Yeast have two types of glutaredoxin proteins. The first of these consists of two oxioreductases, Grx1 and Grx2, both of which contain two highly conserved redox-active cysteine residues. These proteins catalyze the formation of protein mixed disulfides with glutathione, a prevalent cysteine modification (Mieyal, Gallogly, Qanungo, Sabens, & Shelton). The other is a fairly novel group of three proteins (Grx3, Grx4, and Grx5), which contain only a single active cysteine residue (Collinson, Wheeler, Garrido, Avery, Avery, & Grant, 2002). As a mechanism catalyzed by a number of highly conserved enzymes, further insight on the factors controlling the regulation of this system has large implications in research on disease states such as diabetes, cancer, cardiovascular and lung disease, and neurodegenerative disease (Mieyal, Gallogly, Qanungo, Sabens, & Shelton, 2008). This is particularly relevant due to the fact that oxidative stress is a ubiquitous occurrence in all aerobic growing organisms, leading to conservation in the cellular responses to oxidative damage (Jamieson, 1998).

The fast migrating band seen in wild-type Ubc4 and the *ubc4(C2S)* mutant in Figure 15 is similar to what is observed for Yap1, a transcription factor that is activated in response to oxidative stress (Delaunay, Isnard, & Toledano, 2000). In Dr. Delaunay's paper on Yap1's role in H₂O₂ sensing, evidence was provided for two conserved cysteine residues as playing a central role in Yap1's *in vivo* oxidation. In their research, an unknown cause results in the redox-dependent migration shift of Yap1 when run on non-reducing PAGE after treatment with H₂O₂, but not with other oxidants (Delaunay, Isnard and Toledano, 2000). A similar model can be proposed for Ubc4, whereby an unknown mechanism leads to oxidation of Ubc4 at cysteine residue 22.

The research presented here has deep implications for the redox-sensing properties of Ubc4, possibly through the redox-sensitive cysteine residue 22, as contributing to the regulation of Hap2-5 pathway activity. Based on these findings, a novel model for Hap4 regulation can be proposed, whereby the steady-state level of Hap4 is regulated through the redox regulation of Ubc4.

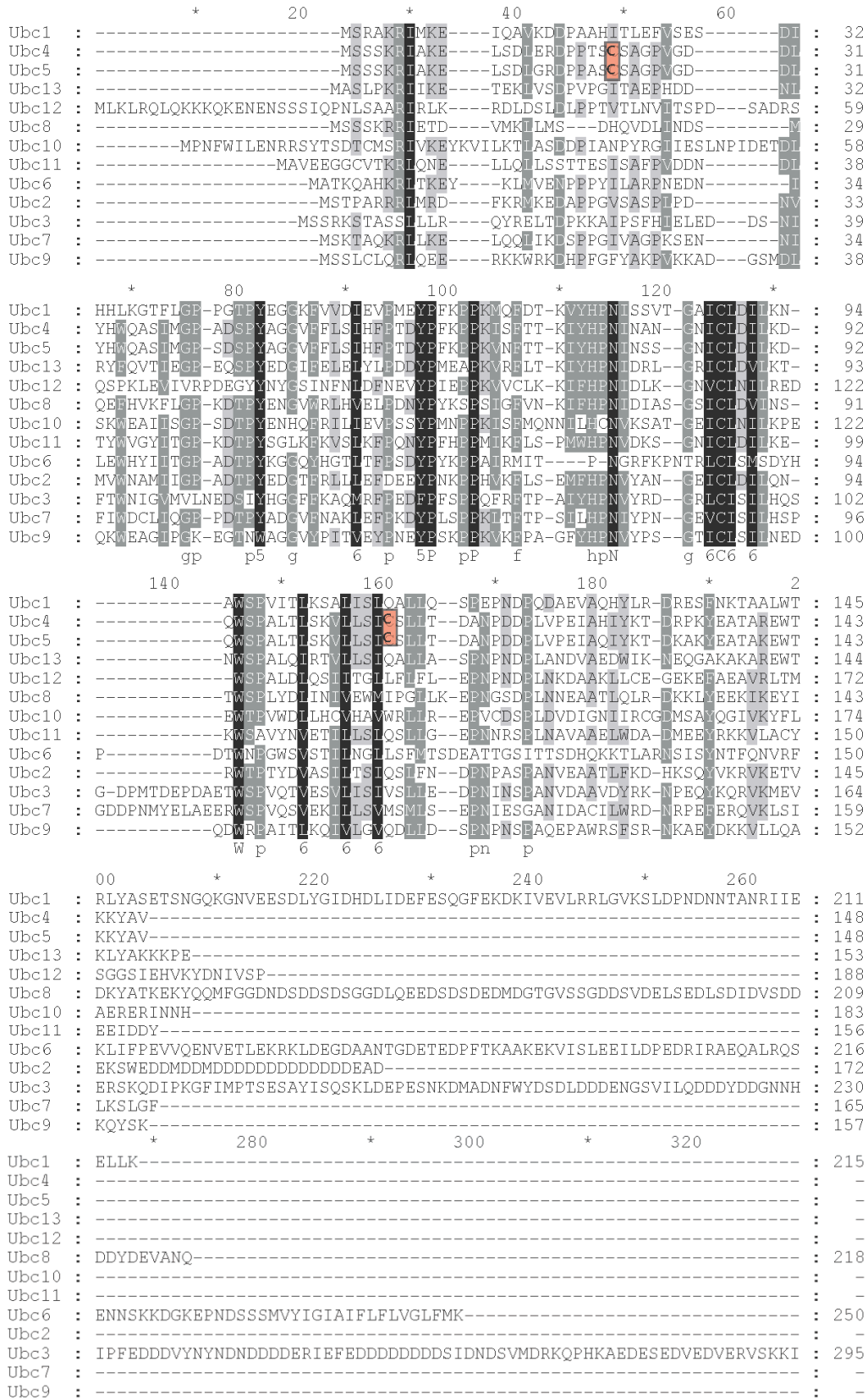


Figure 16: Sequence Alignment of Yeast's Thirteen Ubc Enzymes

Conclusion

The results of my research implicate post-translational regulation of Hap4 through the UPS as the underlying mechanism regulating Hap4 levels in response to changes in mitochondrial functional state. In this work, the isolation of the ubiquitin-conjugating enzymes responsible for Hap4 degradation provides a novel mechanism for the regulation of mitochondrial biogenesis in response to changes in the functional state of mitochondria. Through mutant screening, the ubiquitin conjugating-enzymes, Ubc1 and Ubc4, were found to be the primary E2s responsible for increased Hap4 instability in rho⁰ cells and subsequent down-regulation of Hap2-5 target genes. Mutant analyses of two highly conserved cysteine residues of Ubc4 led us to propose that regulation of Hap4 stability in response to changes in the cellular respiratory function is mediated through redox-regulation of Ubc4.

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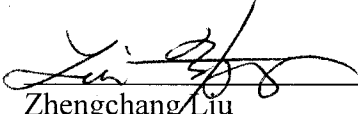
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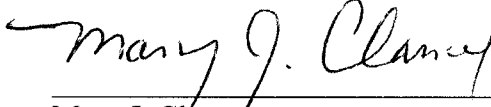
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APPROVAL SHEET

This is to certify that Arielle Ruth Hunter has successfully completed
her Senior Honors Thesis, entitled:

*Characterization of Ubiquitin/Proteasome-Dependent Regulation of
Hap2/3/4/5 Complex in Saccharomyces cerevisiae*


Zhengchang Liu Director of Thesis


Mary J. Clancy for the Department


Carl D. Malmgren for the University
Honors Program

May 1, 2012
Date