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The Regulation of *HAP4* in *Saccharomyces cerevisiae*

A Thesis

Submitted by the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for degree of

Master of Science
in
Biological Sciences

By

Denise Capps
B.S. University of New Orleans, 2009

May, 2011

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Denise Capps

ACKNOWLEDGEMENTS

Graduate school has been challenging, to say the least. It was a trial of my intellectual abilities and stamina, and it tested my physical endurance and capabilities. There were many times when I couldn't imagine ever getting my mind wrapped around the particulars of my research, let alone write a thesis. However, my tenacity paired with an amazing support system helped me achieve a goal to feel comfortable in the lab and to produce original research which tells a story.

This journey would not have been possible, first and foremost, without the strong pathway led by my advisor, Dr. Zhengchang Liu. Every step of the way he encouraged, challenged, and guided me through the sometimes murky waters of research. Part researcher, part life-counselor, Dr. Liu has been a wonderful asset, both professionally and personally. I cannot thank him enough for his continued aid for without his direction, this research would not have been possible.

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my boyfriend, Michael Russo. He has been my daily support; he works for my goals just as much as I do.

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ABSTRACT

The CCAAT binding-factor (CBF) is a transcriptional activator conserved in eukaryotes. The CBF in *Saccharomyces cerevisiae* is a multimeric heteromer termed the Hap2/3/4/5 complex. Hap4, which contains the activation domain of the complex, is also the regulatory subunit and is known to be transcriptionally controlled by carbon sources. However, little is known about Hap4 regulation. In this report, I identify mechanisms by which Hap4 is regulated, including: (1) transcriptional regulation via two short upstream open reading frames (uORFs) in the 5' leader sequence of *HAP4* mRNA; (2) proteasome-dependent degradation of Hap4; and (3) identification of two negative regulators of *HAP4* expression, *CYC8* and *SIN4*. I also report differential patterns of Hap4 cellular localization which depends on (1) carbon sources, (2) abundance of Hap4 protein, and (3) presence or absence of mitochondrial DNA (mtDNA).

Keywords: Hap2, Hap3, Hap4, Hap5, Hap2-5, CCAAT-binding factor, CBF, cerevisiae, SSN6, CYC8, SIN4, upstream open reading frames, uORF, glucose repression, carbon catabolite repression

INTRODUCTION

CCAAT-Binding Factors

Transcription factors are proteins that bind DNA and regulate the expression of target genes by recruiting or hindering RNA polymerase II from transcribing DNA into mRNA. Since their first discovery, general patterns of their mechanism of action have begun to be understood. For example, to be considered a transcription factor, the protein or protein complex must have one or more DNA binding domains, which bind to a specific sequence of DNA adjacent to the genes that they regulate, and one or more transcriptional regulatory domains. Some common DNA binding domains of transcription factors include zinc finger, basic-helix-loop-helix, basic leucine zipper, helix-turn-helix, etc. These proteins can be transcriptional activators or repressors and can either (1) be involved in the formation of the preinitation complex to act generally, controlling the expression of a variety of genes (classes: TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH) (Orphanides, Lagrange et al. 1996); or (2) they can act more specifically, binding upstream of the initiation site to control a smaller range of genes (Charoensawan, Wilson et al.).

The CCAAT-binding factors, as the name implies, bind to a CCAAT sequence in the promoters of target genes. In *Saccharomyces cerevisiae,* the CCAAT-binding factor (CBF) is termed the Hap2/3/4/5 (Hap2-5) complex. This complex has been conserved from yeast, through millions of years to higher eukaryotes including humans (Chodosh, Olesen et al. 1988). CBFs in all species bind the consensus CCAAT sequence upstream of target genes. None of the subunits of the Hap2/3/4/5 complex, nor their homologs, have been shown to share sequence homology

with any of the known DNA-binding motifs. Despite the conservation of CBFs across species, the number and the type of genes that they regulate in different organisms have evolved. The CBF in humans controls a large number of genes of a variety of functions, whereas the yeast CBF controls a smaller set of genes (Mantovani 1999), encoding proteins in heme biogenesis and the electron transport chain, enzymes in the TCA cycle, and proteins involved in nitrogen assimilation (Olesen, Hahn et al. 1987; Pinkham, Olesen et al. 1987; Forsburg and Guarente 1989; Forsburg and Guarente 1989; Olesen and Guarente 1990; Zitomer and Lowry 1992; Xing, Fikes et al. 1993; McNabb, Xing et al. 1995; Dang, Bohn et al. 1996; DeRisi, Iyer et al. 1997; Riego, Avendano et al. 2002; Schuller 2003).

The first component of the Hap2-5 complex identified was Hap2, which was found to be required for expression of *CYC1*, encoding cytochrome c isoform 1, under derepressing conditions (Guarente, Lalonde et al. 1984). *CYC1* expression is up-regulated 5-10 fold in the presence of a non-fermentable carbon source (Zitomer, Montgomery et al. 1979). Detailed analysis of its upstream control region led to the identification of two upstream activating sequences UAS1 and UAS2. UAS1 is under control of the heme-dependent transcriptional activator Hap1, which is distinct from the Hap2-5 complex in that it does not bind CCAAT and is specifically involved in regulation of heme biosynthesis. UAS2 was found to be regulated by *HAP2* (Guarente, Lalonde et al. 1984). Characterization of *HAP2* led to the identification of *HAP3* (Pinkham and Guarente 1985). Hap2 and Hap3 were found to act as a dimer that binds to the CCAAT box in the promoter of target genes. Expression of Hap2/3-dependent genes were also responsive to changes in carbon sources (Pinkham and Guarente 1985). Further research led identification of Hap4 and Hap5 (Forsburg and Guarente 1989; McNabb, Xing et al. 1995).

Hap2 is 265 amino acids in length and contains a core domain of 65 amino acids that is evolutionarily conserved and sufficient for assembly and transcriptional activity of the CCAAT binding factor (Olesen and Guarente 1990). The core domain consists of two parts: a 21-amino acid DNA-binding domain and an 43-amino acid heterodimerization domain necessary for protein-protein interactions (Olesen and Guarente 1990). Although it was first assumed that Hap2 interacts with Hap3 directly, it was later found that Hap2 binds to Hap3 through Hap5 (McNabb, Xing et al. 1995; Schuller 2003).

Hap4 was cloned and initially characterized by Forsburg and Guarente (1989). The protein consists of 554 amino acids and has a C-terminal acidic region (between residues 330-554) functioning as the activation domain for the entire complex (Forsburg and Guarente 1989). Neither Hap2 nor Hap3 has such extensive regions of acidic residues as Hap4 (Pinkham, Olesen et al. 1987; Hahn, Pinkham et al. 1988). Olesen and Guarente showed that a LexA-Hap4 fusion is a potent activator at the *LexA* operator, even in the absence of *HAP2* and *HAP3* (Olesen and Guarente 1990). Further analysis revealed that Hap4 has two transcriptional activation domains: one at the C-terminus, between residues 359 and 476, and the other at the N-terminus, between residues 124-329. Although both domains are capable of stimulating transcription when fused to the *LexA* DNA-binding domain, the C-terminal activity is Gcn5-independent, while the N-terminal region is Gcn5-dependent (Gcn5 is a transcriptional co-activator and a histone acetyltransferase) (Stebbins and Triezenberg 2004). Despite that it contains the transcriptional activation domain of the Hap2-5 complex, Hap4 is not required for the binding of Hap2/3/5 to the CCAAT sequence. However, only after Hap4 binds is the complex able to activate target gene expression (Xing, Fikes et al. 1993).

Xing and others demonstrated that another protein was likely to be required for DNA binding activity of Hap2/3 (Xing, Zhang et al. 1994). McNabb and others (1995) later isolated Hap5 in a yeast two-hybrid screen to uncover Hap2-interacting proteins (McNabb, Xing et al. 1995). A *hap5*Δ mutant strain cannot grow on nonfermentable carbon sources, just like *hap2*Δ, *hap3*Δ, and *hap4*Δ mutant strains (Pinkham, Olesen et al. 1987; Hahn and Guarente 1988; Forsburg and Guarente 1989). Hap5 contains 216 amino acids and is a linker protein bringing together Hap2 and Hap3, although it is Hap2 and Hap3 that actually bind DNA (McNabb, Xing et al. 1995). Hap5 as well as Hap3 have core elements displaying amino acid sequence similarity to the histone fold motifs of histones H2A and H2B, respectively (Mantovani 1999).

Hap2, 3, and 5 have mammalian counterparts; rats have CBF-A (Hap3), CBF-B (Hap2), CBF-C (Hap5); mice and humans have NFY-A (Hap2), NFY-B (Hap3), and NFY-C (Hap5) (Schuller 2003). Hap4 homologs have yet to be identified in mammals but researchers have identified its homologs in *Kluyveromyces lactis* (Bourgarel, Nguyen et al. 1999) and the methylotrophic yeast *Hansenula polymorpha* (Sybirna, Guiard et al. 2005). The sequences necessary for Hap4 binding to Hap2/3/5-DNA are at its N-terminus (residues 23-46) and C-terminus (residues 165-180). The C-terminal region spanning amino acids 161-180 is serine rich and conserved across divergent species (McNabb and Pinto 2005).

Hap2, Hap3, and Hap5 are all required for DNA-binding activity, which made the Hap2/3/4/5 complex the first DNA-binding complex discovered to require three different subunits to bind DNA (McNabb, Xing et al. 1995). The three subunits in yeast assemble on DNA in a one-step fashion, which is in contrast to their mammalian counterparts that assemble in a

two-step manner (McNabb and Pinto 2005). Hap2/3/5 binding to CCAAT is a prerequisite for the binding of Hap4, which finally activates the complex to stimulate transcription (McNabb and Pinto 2005). In the Hap2-5 complex, each subunit is present as a single copy.

A genome-wide analysis of gene expression in cells that underwent a switch from the fermentative to respiratory growth led to the identification of genes whose expression requires the Hap2-5 complex (DeRisi, Iyer et al. 1997; Lascaris, Bussemaker et al. 2003). Many of such genes were necessary for metabolic assimilation of non-fermentable carbon sources. Expression of *HAP2, HAP3,* and *HAP5* is constitutive but the expression of *HAP4* is repressed in the presence of glucose (Forsburg and Guarente 1989). However, when yeast cells are switched from a medium containing glucose to a medium containing a nonfermentable carbon source, such as glycerol, *HAP4* expression is induced 3- to 4-fold from its basal level (Forsburg and Guarente 1989). McNabb and Pinto (2005) found similar results. They also found that *CYC1* transcript levels (a Hap2-5 target gene) increase by >10-fold (McNabb and Pinto 2005). It has been postulated that, in the glucose repression/derepression system, expression of target genes might be modulated by the level of Hap4 (Bourges, Mucchielli et al. 2009). When *HAP4* is overexpressed in cells grown in glucose medium, the metabolic state of the cell resembles that of cells grown under derepressing conditions: there is increased transcription of nuclear genes involved in respiratory functions as well as increased expression of mitochondrial genes. However, the metabolic state due to *HAP4* overexpression does not exactly mirror that of bona fide respiring cells and is considered to be a novel state (De Winde and Grivell 1995; Lascaris, Bussemaker et al. 2003).

HAP4 expression is regulated by carbon sources. Hxk2, the major form of hexose kinase, has also been found to be a negative regulator of *HAP4* expression (Lascaris, Piwowarski et al. 2004). It is clear that Hap4 plays an important role in allowing the cell to utilize nonfermentable carbon sources. However, molecular mechanisms by which Hap4 expression is regulated are still unclear.

Glucose Sensing

Saccharomyces cerevisiae is considered to be a "glucose-sensitive" yeast, which, along with *Schizosaccharomyces pombe,* prefers fermentation over respiration even under aerobic conditions (Rolland, Winderickx et al. 2002). This phenomenon has been is hypothesized to have evolved in the Cretaceous age, when trees first started bearing fleshy fruits (Thomson, Gaucher et al. 2005). Yeasts living on these fruits rapidly convert fermentable carbon sources such as glucose and fructose into ethanol, accumulation of which transforms a once desirable food source into an environment where other microorganisms are unable to thrive. Thus, the yeasts' unique metabolic strategy enables it to defend its food source. Importantly, once the fruits' sugars are exhausted, the yeast cells can turn to ethanol as a carbon source for oxidative catabolism (Rolland, Winderickx et al. 2002). This switch from fermentative growth to aerobic respiration upon depletion of glucose, referred to as the diauxic shift, is correlated with widespread changes in the expression of genes involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage (DeRisi, Iyer et al. 1997). To undergo such a major shift in the yeast's metabolic strategy, the cell must have mechanisms that integrate the nutritional and metabolic states of the cell.

Decades of research has been devoted to the subject of glucose sensing and signaling in yeast; several reviews have been published recently (Rolland, Winderickx et al. 2002; Santangelo 2006; Gancedo 2008). Although glucose is both a signaling molecule and a catabolite, researchers have found it possible to distinguish clearly between glucose's regulatory and nutrient functions. Most glucose-induced signaling pathways have not been shown to require any further metabolism other than simple phosphorylation (Gancedo 2008). The availability of glucose is transduced to the nucleus by three main signaling pathways, the Mig1-Hxk2 glucose repression pathway, the Snf3-Rgt2 glucose sensing pathway, and the Gpr1-cAMP pathway (Ahuatzi, Riera et al. 2007). The Mig1-Hxk2 signaling pathway is responsible for the effects of carbon catabolite repression. Briefly, it is a pathway that senses glucose and then down-regulates specific sets of genes including those involved in gluconeogenesis and catabolic metabolism of alternative carbon sources (Rolland, Winderickx et al. 2002). The Snf3-Rgt2 glucose sensing pathway specifically senses extracellular glucose and then activates the transcription of genes required for rapid import of the sugar. Finally, the Gpr1-cAMP pathway activates enzymes involved in the metabolism of alternative carbon sources. Its physiological role is unique in that it is confined to the short transition from the derepressed state to the repressed state.

Hexose transporters, encoded by genes such as *HXT1*, *HXT2*, *HXT3*, *HXT4*, and *HXT7*, are only produced after the cell senses glucose in the extracellular environment (Rolland, Winderickx et al. 2002). A schematic diagram of the regulation of *HXT* genes by Sin3.Rtg2 is seen in Illustration 1. This pathway begins with the glucose sensors Snf3 and Rgt2 that bind extracellular glucose. Glucose binding activates the membrane-bound casein kinase Yck1/2 which then phosphorylates Mth1/Std1 (Moriya and Johnston 2004). Phosphorylated Mth1/Std1

is a target for ubiquitination and subsequent degradation via the SCF^{Grr1} complex. Subsequent decrease in the levels of Mth1/Std1 allows phosphorylation of Rgt1. Rgt1 binds to the promoter of *HXT* genes along with Cyc8 and Tup1, acting to repress *HXT* gene transcription under noninducing conditions. Glucose-induced phosphorylation of Rgt1 leads to its cytoplasmic translocation and the expression of *HXT* genes is derepressed (Gancedo 2008). Thus, the glucose signal, through the action of several downstream proteins and regulatory events, allows the derepression of genes encoding proteins which uptake glucose into the cell. *HXT* genes can be subdivided by their substrate affinities (low, intermediate, and high). Under conditions of low extracellular glucose, the cell expresses high-affinity *HXT* genes; under high extracellular glucose conditions, the cell expresses low-affinity *HXT* genes (Rolland, Winderickx et al. 2002)

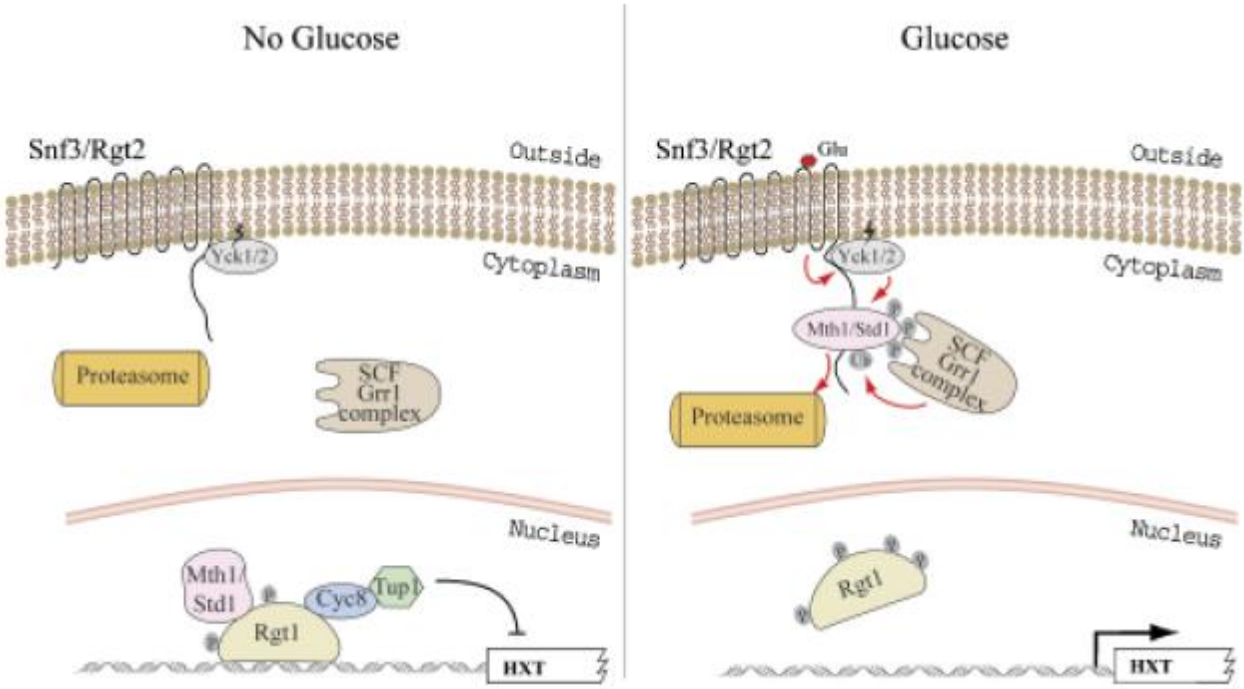

Illustration 1: The Snf3/Rgt2-signaling pathway
In the absence of glucose, a repressing complex of Rgt1, Mth1/STD1, Cyc8 and Tup1 binds to the promoters of the *HXT* genes, blocking their transcription. When glucose is present, it binds to the Snf3/Rgt1 sensors and activates kinase Yck1/2 which then phosphorylates Mth/Std1, bound to the C-terminal tails of Snf3 and Rgt2. Phosphorylated Mth/Std1 are recognized by the SCF^{Grr1} complex which ubiquitinationates them, thus tagging them for degradation by the 26S proteasome. Absence of Mth/Std1 allows Rgt1 to be phosphorylated and to dissociate from the promoter of *HXT* genes, thus derepressing the *HXT* genes. Illustration 1 was taken from Gancedo 2008.

When glucose (or fructose) is unavailable to the cell, the yeast must undergo a major reprogramming of its metabolism. As such, there are a variety of genes under the control of the Mig1-Hxk2 carbon catabolite repression pathway (Illustration 2). In this pathway, when the cell senses extracellular glucose, genes encoding proteins involved in the breakdown and assimilation of nonfermentable carbon sources are repressed by a complex of the zinc-finger containing Mig1 protein and the general transcriptional co-repressors Cyc8 and Tup1. In the nucleus, Mig1 also interacts with Hxk2, which, in turn, binds to Med8, a subunit of the Srb/mediator complex (Palomino, Herrero et al. 2005). This is hypothesized to interfere with the interaction of RNA polymerase II in the promoter of target genes. Mig1 acts as a transcriptional repressor only in its unphosphorylated form (Ahuatzi, Riera et al. 2007). Under glucose repressing conditions, the protein phosphatase activity of the Glc7-Reg1 complex, whose targets include Mig1, is enhanced. This, coupled with a low activity of the Snf1 kinase towards Mig1, allows both Mig1 and Hxk2 to translocate from the cytoplasm to the nucleus where they repress target gene expression (Ahuatzi, Riera et al. 2007). However, when glucose is depleted, the Snf1 kinase complex is activated and Glc7-Reg1 is inhibited, resulting in hyperphosphorylation of Mig1. Subsequently, both Mig1 and Hxk2 leave the nucleus, their repressing activity is abolished, and Med8 is able to recruit RNA polymerase II to transcribe the target genes (Gancedo 2008).

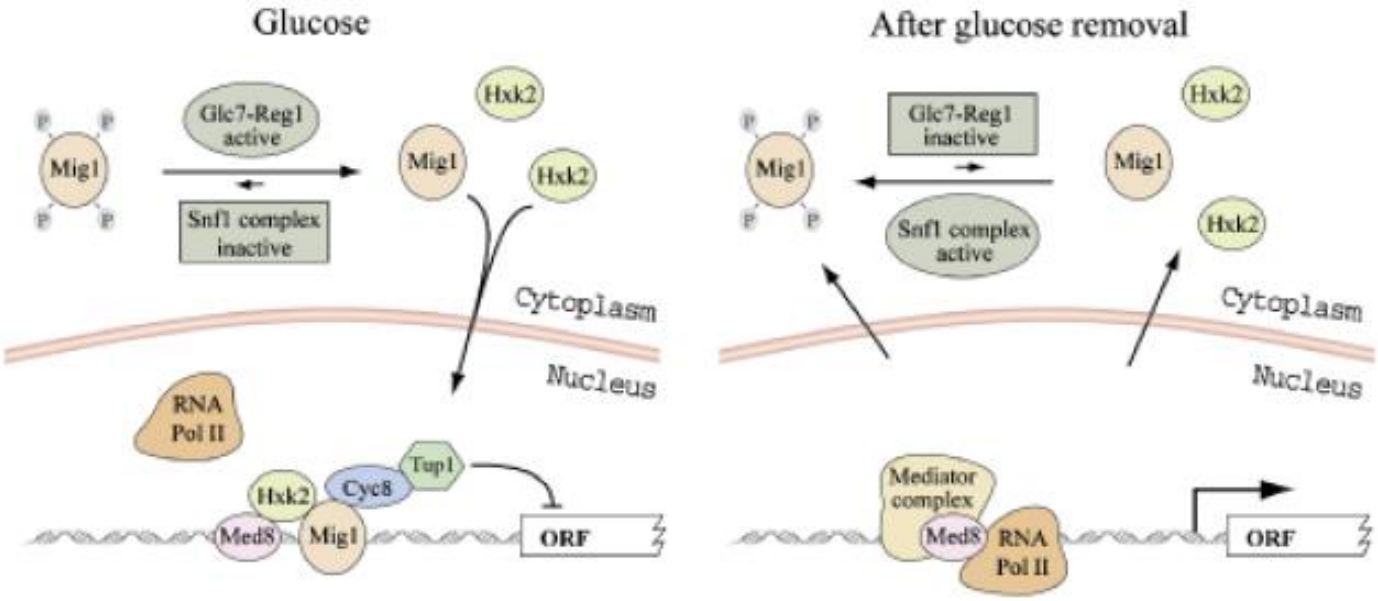

Illustration 2: Model for the role of Hxk2 in the nucleus in glucose signaling
During growth on glucose, the repressing protein Mig1 is mainly in its unphosphorylated form, due to the protein phosphatase activity of the Glc7-Reg1 complex, coupled with a low activity of the Snf1 complex. In these conditions Mig has a nuclear localization and Hxk2 is partially retained within the nucleus through its binding to Mig1: this binding hinders the contact between Mig1 and any active molecule of Snf1 in the nucleus and would thus reinforce the repressing capacity of the Mig1-Cyc8-Tup1 complex. Nuclear Hxk2 is also able to bind Med8, a subunit of the Srb/Mediator complex, and this binding may interfere with the capacity of RNA polymerase II (RNA Pol II) to interact with the promoter of the regulated gene. When glucose is removed, the Snf1 complex is activated, Mig1 is phosphorylated and both Mig1 and Hxk2 leave the nucleus. As Hxk2 dissociates from Med8, the mediator complex would be able to recruit RNA Pol II and transcription may proceed. Illustration 2 was taken from Gancedo 2008.

Sensing of Mitochondrial Functional State

Yeast cells can sense not only extracellular nutrient availability, but also their internal environments, such as the functional state of the mitochondria. *S. cerevisiae* can exist as having normal mtDNA (*rho*$^{+}$; ρ^{+}), mutated mtDNA (*rho*$^{-}$; ρ^{-}) or complete lack of mtDNA (*rho*^{0}*;* ρ^{0}; petites); phenotypic characteristics of petite cells includes smaller colony sizes. Since the mitochondrial genome encodes a limited number of proteins (the majority of mitochondrial proteins are encoded in nuclear DNA), most of which are essential components of oxidative phosphorylation (Foury, Roganti et al. 1998), *rho*^{0} cells are viable as long as they are not grown on a medium containing only nonfermentable carbon sources, such as lactate or glycerol. The retrograde response transduces information from the mitochondria to the nucleus and affects changes in nuclear genetic expression (Liu and Butow 2006). For example, in *rho*^{0} cells (or cells with reduced respiratory function), the expression of the early TCA cycle genes switches from Hap2-5 control to control by the Rtg1/3 complex (Liu and Butow 1999). Genes with respiratory functions can still be expressed in cells with mitochondrial dysfunction (e.g., via retrograde control), however, these genes are often repressed (Liu and Butow 1999; Bourges, Mucchielli et al. 2009).

Upstream Open Reading Frames

The scanning mechanism of eukaryotic translation entails that translational initiation occurs at the first AUG to be encountered, nearest to the 5' end of the mRNA. When *HAP4* was first described by Forsburg and Guarente in 1989, they identified two upstream ATGs which initiate ORFs of nine and three residues, respectively. More recently, upstream open reading

frames (uORFs) are becoming more well known as post-transcriptional regulatory elements that can be present in the 5' leader regions of mRNA transcripts (Liu, Dilworth et al. 1999). The presence of an active (i.e., it is recognized and translated by a scanning ribosome) uORF often negatively affects expression of the downstream main open reading frame (Hinnebusch 2005). uORF incidence is predicted in ~10-45% of all eukaryotic mRNAs (Calvo, Pagliarini et al. 2009) and genetic mutations which add or delete a uORF are implicated in human diseases such as thrombocythaemia (Ghilardi and Skoda 1999) and melanoma (Liu, Dilworth et al. 1999). It has also been postulated that uORFs may play roles in diseases such as familial hypercholesterolemia and hereditary pancreatitis, among others (Calvo, Pagliarini et al. 2009).

The best mechanistic studies on uORF regulation have been through experiments with yeast *GCN4* that has four short open reading frames in the 5' leader sequence of its mRNA (Hinnebusch 1984). The uORFs mediate translational control of *GCN4*; under normal conditions, *GCN4* uORF3 and uORF4 repress translation while uORF1 and uORF2 are positive regulators. The cell can modulate the negative regulatory effects of *GCN4* uORFs: under amino acid starvation conditions, the cell overcomes uORF4 inhibition of *GCN4* expression via the protein kinase Gcn2 (Hinnebusch 1997).

An important regulatory step of translational control in response to environmental stresses is the phosphorylation of the alpha subunit of translation initiation factor 2 (eIF-2α). Unlike higher eukaryotes, there is only one eIF-2α kinase, Gcn2, in yeast (Ramirez, Wek et al. 1992). Gcn2 stimulates the expression of *GCN4* in response to starvation of amino acids, purine and glucose limitation, and impaired tRNA synthetase activity (Hinnebusch 1997; Yang, Wek et

al. 2000; Hinnebusch 2005). Upon nutrient starvation, Gcn2 phosphorylates serine 51 of eIF-2α. This impairs the conversion of eIF-2-GDP to eIF-2-GTP, a reaction carried out by the guanine nucleotide exchange factor eIF-2B. Only the GTP-bound form of eIF-2 is able to form a ternary complex with the initiator $tRNA^{Met}$ and catalyze a new round of translational initiation. Under normal conditions with ample eIF-2-GTP-Met-$tRNA_i^{Met}$ ternary complexes, scanning ribosomes can translate all four *GCN4* uORFs. This eventually leads to an inability to translate the main ORF. However, under amino acid-limiting conditions, there is a lower concentration of the eIF-2-GTP-Met-$tRNA_i^{Met}$ ternary complex and as a consequence, many of the ribosomes which translate uORF1 and resume scanning cannot acquire the ternary complex needed for reinitiation. Only after the ribosome successfully scans past uORF2, uORF3, and uORF4 is the ribosome able to bind another ternary complex and initiate translation at the main ORF.

Although this particular mechanism of uORF action has been well studied, uORFs may affect gene expression through other mechanisms, as well. For example, uORFs may alter mRNA stability by triggering the nonsense-mediated decay pathway (Morris and Geballe 2000). A complete analysis of uORF effects on gene expression is important in understanding the full spectrum of ways that the cell can regulate protein synthesis. It was hypothesized that the uORFs of *HAP4* could possibly provide a mechanism of translational regulation of *HAP4*, as is seen in *GCN4* (Forsburg and Guarente 1989). Since their study, there have been several reports identifying *HAP4* uORFs as being potentially active (Zhang and Dietrich 2005; Cvijovic, Dalevi et al. 2007; Selpi, Bryant et al. 2009). However, there have not been any detailed reports on the activity of individual *HAP4* uORFs on *HAP4* expression and their potential roles in the regulation of Hap2-5 target genes.

Research Aims

Very little is known about the regulation of Hap4, the regulatory component of the *S. cerevisiae* CCAAT-binding factor, the Hap2/3/4/5 complex. Since the Hap2-5 complex controls the expression of genes involved in mitochondrial respiratory functions, Hap4 regulation is likely to be important in mitochondrial biogenesis. Further elucidation of this pivotal transcription factor can provide additional insights into the regulation of glucose sensing pathways. Understanding the regulation of Hap4 is not just important for yeast biology. There exists a large body of literature on the regulation and function of the mouse, rat, and human CCAAT binding factors, since they are conserved among eukaryotes. A better understanding of the regulation of Hap2-5 in yeast can help us better understand the regulation of CCAAT binding factors in higher eukaryotes.

One particularly interesting feature of *HAP4* is its upstream open reading frames, which are post-transcriptional regulatory elements that are increasingly known as important modulators of protein synthesis. uORFs can have a drastic effect on translational efficiency of the downstream main open reading frame and have been recently implicated in some human diseases. *HAP4* uORFs have been identified and marked as potentially active, although to date no specific studies have been published on *HAP4* uORFs apart from genome-wide studies of uORF effects on expression of main ORFs.

Although it is well known that *HAP4* is regulated transcriptionally by carbon sources, the factors that regulate *HAP4* expression have been elusive. Thus, the purpose of my project is to dissect the regulatory mechanisms by which Hap4 is controlled. The research presented here

focuses on (1) the regulatory function of *HAP4* uORFs; (2) regulation of *HAP4* by the mitochondrial functional state; (3) subcellular localization of Hap4; (4) and the identification of novel regulators of *HAP4* expression.

MATERIALS AND METHODS

Primers, Plasmids and Strains

Primers, plasmids, and yeast strains used in this study are listed in Table 2, Table 3, and Table 4, respectively.

Growth Media and Conditions

Yeast cells were grown at 30°C in minimal SD medium (0.67% yeast nitrogen base and 2% D-glucose), S5%D medium (0.67% yeast nitrogen base and 5% D-glucose), SR (0.67% yeast nitrogen base and 2% raffinose), YNBcasD (0.67% yeast nitrogen base, 1% casamino acids, and 2% D-glucose), YNBcas5%D (0.67% yeast nitrogen base, 1% casamino acids, and 5% D-glucose), YNBcasRaff (0.67% yeast nitrogen base, 1% casamino acids, and 2% raffinose), and YPD (2% peptone, 1% bacto yeast extract, and 2% D-glucose). When necessary, the amino acids L-leucine, L-lysine, L-histidine or L-methoionine, and/or uracil were added were added to growth media to a final concentration of 30 mg/L to meet auxotrophic requirements (Amberg et al 2005). The solid media contains 2% agar in addition to the components described above. When required, MG132 (50 µM) was added to the growth media to inhibit proteasome activity (Lee and Goldberg 1996).

Site-directed Mutagenesis for uORF Mutations

HAP4 uORF mutants were created using site directed mutagenesis via PCR SOEing (splicing by overlap extension) as described (Horton 1995). Four primers were selected; two

were designed to amplify the entire *HAP4* promoter; two were designed to anneal to the specific region to be mutated. The region to be mutated was the ATG start codon of either uORF1 or uORF2; it was mutated to ATA. To create the mutant, two separate rounds of PCR took place. The first round had two reactions, one with the 5' outer flanking primer and the 3' inner mismatch primer (to make the left [L] segment), and the other with the 3' outer flanking primer and the 5' inner mismatch primer (to make the right [R] segment). Both reactions used pWCJ-HAP4 plasmid DNA as the template (Table 3). After 25 cycles of amplification, the PCR products were ethanol precipitated, gel purified, and then DNA was extracted via GENEclean protocol. The resulting L and R fragments were then mixed, amplified via PCR without primers and after 8 cycles, the original 5' and 3' outer flanking primers were added and allowed to amplify for a total of 25 rounds of PCR. All reactions were carried out with the Pfu DNA polymerase.

Yeast Transformations and β-galactosidase Activity Assays

Various yeast strains were transformed as described by Chen et. al (1992). β-galactosidase assays, using the glass bead lysis method, were performed according to the method of Amberg et al (2005). For each plasmid-strain combination, the reported *lacZ* activities (in nanomoles of hydrolyzed *o*-nitrophenyl-β-d-galactopyranoside [ONPG] per milligram of protein per minute) are averages of results from triplicate or duplicate assays of usually two independent samples. Specific activity was calculated in relation to the total protein amount in the crude extract, using bovine serum albumin as the standard. Independent experiments were carried out at least two or three times.

Cellular Extract Preparation and Immunoblotting

Total cellular protein extracts were prepared by lysing live yeast cells in extraction buffer (1.85 N NaOH – 7.5% β-mercaptoethanol) followed by precipitation with trichloroacetic acid (TCA) as described (Yaffe and Schatz 1984). TCA pellets were neutralized with 1 M unbuffered Tris and resuspended in 1X SDS-PAGE loading buffer. Immunoblotting was carried out by incubating blotted nitrocellulose membranes with primary antibody, either rat monoclonal anti-HA (3F10, Roche) or mouse monoclonal anti-GFP (FL, Santa Cruz Biotechnology, Inc), followed by either goat anti-rat or goat anti-rabbit polyclonal HRP-conjugated secondary antibodies. Chemiluminescence was induced by an ECL reagent and images of Western blots were captured using the Bio-Rad Chemi-Doc photo documentation system (Bio-Rad Laboratories, inc., Hercules, CA). For loading controls, blots were first stripped by incubation in stripping buffer (2% SDS – 0.08% β-mercaptoethanol) for 45 min at 60°C with agitation, and then blots were reprobed with anti-Ilv5 (Acetohydroxyacid reductoisomerase) rabbit monoclonal antibodies. For the determination of the half-life of a protein, band intensities on Western blots were quantified using the Bio-Rad QuantityOne software.

Cycloheximide Chase Assay

Cells expressing indicated HA-tagged proteins were grown in liquid YNBcas5%D or YNBcasRaff medium to OD_{600} 0.6 – 0.8. Protein synthesis was inhibited by addition of 50 µg/mL cycloheximide (Tumusiime, Zhang et al.). Every five or ten minutes, a 1 mL sample of the cell culture was withdrawn and immediately subject to TCA precipitation. Total cellular extracts were then prepared as described above. HA-tagged protein levels were determined by

probing Western blots with anti-HA antibody (3F10, Roche). For loading controls, blots were stripped and reprobed with either anti-Ilv5 (Acetohydroxyacid reductoisomerase) or anti-Pgk1 (3-phosphoglycerate kinase) rabbit monoclonal antibodies.

EMS Mutagenesis

To induce DNA mutations, the mutagen ethyl methanesulfonate (EMS), which induces a high frequency of C-G base pair substitutions, was used to treat two wild-type yeast strains PSY142 and BY4741, each carrying a pWCJ-HAP4p-lacZ (*URA3 CEN*) plasmid. A 10 mL culture of the strain to be mutated was grown in YNBcasD overnight at 30°C. 1 mL of the saturated culture was pelleted and subjected to EMS mutagenesis as described in Methods Enzymology (Lawrence 1991). After mutagenesis, cells were plated on YNBcasD + X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) medium for blue/white screening. Constitutive activating mutants were selected by dark blue colony phenotype on X-gal plates and subject to confirmation by β-galactosidase assays as described above.

Library Complementation of *HAP4* Activating Mutants

Three highly activating mutants were transformed with YCp50, a *URA3 CEN* library plasmid previously constructed (Liu, Spirek et al. 2005). Transformants were plated on YNBcasD plates. After 2-3 days, cells were transferred onto S5%D + X-gal plates via velvet replication. Activating mutants appeared dark blue on X-gal plates, and colonies which appear light blue or white were candidates that may contain a complementing library plasmid. When a transformant was confirmed to contain a complementary plasmid, the plasmid was recovered as described (Hoffman and Winston 1987) and sent for sequencing using the dideoxynucleotide

substitution technique at the W.M. Keck Conservation and Molecular Genetics Laboratory in the Department of Biological Sciences at the University of New Orleans. The primers used were designed to anneal 30 base pairs upstream and downstream of the BamHI site in YCp50 and are listed in Table 2.

Mating-type Switch

Haploid yeast cells can have a mating type of either *MAT*α or *MAT***a,** encoded by the *MAT* locus. They also have an *HML* (Hidden MAT Left) or *HMR* (Hidden MAT Right) which typically carry a silenced copy of the *MATα* or *MAT**a*** allele, respectively (Herskowitz and Jensen 1991) . Wild-type haploid yeast strains are able to switch mating-type from α to **a** and back via the Homothalism (HO) DNA endonuclease. Laboratory yeast strains typically have the *HO* gene deleted. However, mating-type switching can be induced in a controlled manner by the introduction of the *HO* gene encoded on a glactose-inducible plasmid (Jensen and Herskowitz 1984). Mating-type switching occurs after HO creates double strand breaks in the DNA, which only occurs at the *MAT* locus. After restriction, exonucleases degrade DNA including the portion encoded by the *MAT* allele. The resulting gap in the DNA is repaired by copying in the genetic information present at either *HML* or *HMR*, filling in a new allele of either the *MAT**a*** or *MATα* gene. Thus, the silenced alleles of *MAT**a*** and *MATα* present at *HML* and *HMR* serve as a source of genetic information to repair the HO-induced DNA damage at the active *MAT* locus (Herskowitz and Jensen 1991).

To induce mating-type switching, yeast cells were transformed with a plasmid encoding HO under control of a galactose-inducible promoter (Jensen and Herskowitz 1984).

Transformants were grown overnight in 10 mL YNBcasRaff medium supplemented with 2% galactose and then plated on solid YNBasD medium to isolate single colonies. Colonies were assayed for mating type by mixing with a tester strain of either *MAT*α or *MAT***a.** For example, since only strains of opposite mating type can form a diploid, if the original *MAT*α cells could form diploids with the *MAT*α tester, then the original *MATα* cells must have undergone a mating-type switch to be *MAT**a***.

Fluorescence Microscopic Analysis of Intracellular Localization of GFP-tagged Hap4

Cells expressing GFP-tagged Hap4 were grown in the indicated medium to mid-log phase (Table 2 and Table3). Cells were concentrated by pelleting at 3000 x G for 2 minutes. Live cells were observed immediately by fluorescence microscopy on a Nikon Eclipse E800 microscope equipped with an HBO 100 W/2 mercury arc lamp, a Nikon Plan Fluor 100X objective lens, and epifluorescence with Nikon B-2E/C media band excitation bandpass filter set (excitation light 465-495 nm, emission light 515-555 nm). Digital images were acquired with Photomectrics Coolsnap fx CCD camera and Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA) and processed using ImageJ (National Institutes of Health) and Adobe Photoshop (Mountain View, CA).

Determination of Hap4 Half-life

MG132 (carbobenzoxyl-leucinylleucinyl-Leucinal [Cbz-LLLal]) is a specific, potent, reversible proteasome inhibitor. It inhibits the degradation of ubiquitin-conjugaded proteins by inhibiting the 26S proteaseome (Lee and Goldberg 1996). MG132 is not normally permeable to

the *S. cerevisiae* cell membrane; however, deletion of ergosterol biosysnthesis gene *ERG6* confers cell membrane permeability to MG132 (Lee and Goldberg 1998). *erg6*Δ mutants expressing indicated HA-tagged proteins were grown in liquid YNBcas5%D or YNBcasRaff medium. When cultures reached OD_{600} 0.4, cells were treated with either MG132 or DMSO, which was the solvent for MG132. After the cells reached an OD_{600} of at least 0.6, a cycloheximide chase assay was performed as described above. Treatment of *erg6*Δ mutants with MG132 prior to a cycloheximide chase assay enables determination of whether protein degradation is proteasome-dependent.

DAPI Staining

4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to DNA and is used in fluorescence microscopy for visualization of both nuclear and mtDNA. To stain nuclear DNA, locating the nucleus in live cells, cultures were grown in 10 mL of S5%D or SR medium to OD_{600} 0.8, diluted to OD 0.1, and were inoculated with 5 uL DAPI to a concentration of 1 µg/mL at OD_{600} 0.3. Three hours later, after cells reached OD ~0.6, cells were pelleted and viewed under a fluorescence microscope.

Creation of *rho*^{0} Petites

To induce loss of mtDNA, normal *rho*$^+$ cells were grown in YPD liquid medium to saturation with 15 µg/mL ethidium bromide (EB). A sterile loop was dipped in the saturated culture and then streaked over a YPD plate so as to isolate single colonies. Single colonies were then picked individually and grown overnight to saturation and then fixed and stained with DAPI. Loss of mitochondrial DNA was confirmed via fluorescence microscopy and by inability

to grow on YPGlycerol plates. Individual colonies with confirmed loss of mtDNA were used for further analyses.

RESULTS

Upstream Open Reading Frames of *HAP4* Are Active

My first aim was to determine whether *HAP4* upstream open reading frames are active (i.e., whether they are recognized by scanning ribosomes and translated) and whether these two uORFs regulate the translation of the main open reading frame (ORF). To characterize translation of *HAP4* from the main ORF, I constructed a *HAP4p-lacZ* reporter gene which included a 1.8 Kb *HAP4* promoter sequence plus the start codon of *HAP4* fused in-frame to the *E. coli* gene *lacZ* (Table 2). To test the activity of *HAP4* uORFs, I created truncations of the wild-type promoter-*lacZ* fusion: for characterization of uORF1 activity, *lacZ* was fused in-frame to uORF1; for activity of uORF2, *lacZ* was fused in-frame with uORF2 (Figure 1 and Table 2). Although the construct designed to test uORF2 activity also includes uORF1, this design is consistent with published research on uORF activity (Hinnebusch, Asano et al. 2004).

Translation initiation of *HAP4* by RNA polymerase II is 367 nucleotides upstream of the main open reading frame. All constructs began 1800 base pairs upstream of the ATG start codon of the main ORF, so each contains the transcription initiation site.

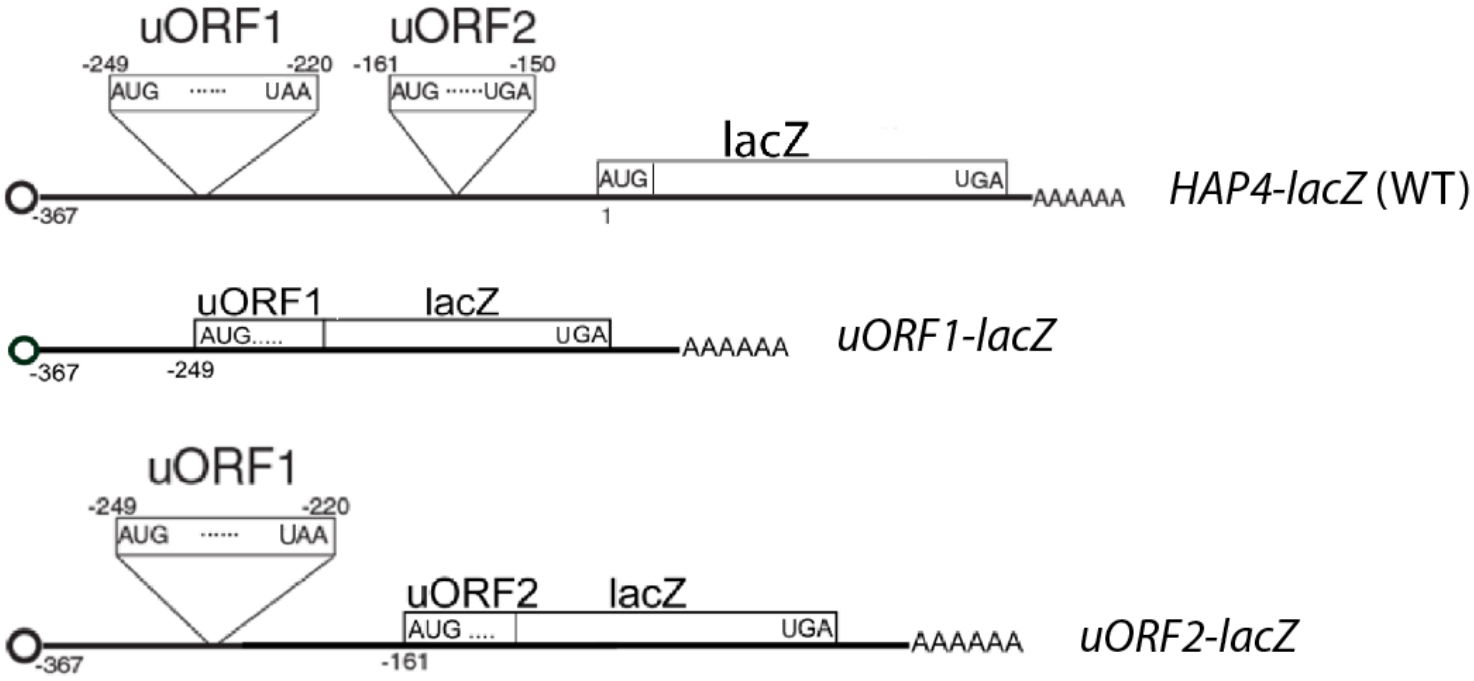

Figure 1: Three constructs used for the determination of *HAP4* uORF activity
From top: full-length *HAP4* promoter *lacZ* fusion used to assay the activity of the *HAP4* main ORF; uORF1 activity is measured via *lacZ* fusion in frame with uORF1; uORF2 activity is measured via *lacZ* fusion in frame with uORF2.

Each construct was created in the *URA3* centromeric plasmid pWCJ (Liu, Thornton et al. 2008) and transformed in two yeast strains PSY142 and BY4741. Two strains were used to determine whether *HAP4p-lacZ* expression is strain-independent. Transformants were selected by growth on YNBcasD medium. Individual transformants were assayed for β-galactosidase activity as described in Materials and Methods. Since *HAP4* expression is carbon-source dependent, cells were grown in liquid medium that either represses *HAP4* expression (YNBcas5%D) or derepresses *HAP4* expression (YNBcasRaff). Figure 2 shows the β-galactosidase activity of *HAP4p-lacZ* in both PSY142 and BY4741 background stains grown YNBcas5%D or YNBcasRaff medium. In YNBcas5%D grown cells, *HAP4p-lacZ* activity is low; in YNBcasRaff grown cells, there is a ~10-fold increase of *HAP4p-lacZ* expression, consistent with previously published results (Forsburg and Guarente 1989). Interestingly, activity of both *uORF1-lacZ* and *uORF2-lacZ* was similar to that of *HAP4p-lacZ* (WT), indicating that both uORFs in the 5' leader sequence of *HAP4* are translated. Figure 2 also shows that, in both strains, expression of *HAP4-uORF1-lacZ* and *HAP4-uORF2-lacZ* in cells grown in YNBcasRaff medium increased ~10-fold compared to that of cells grown in YNBcas5%D. These data clearly indicate that both uORFs in the *HAP4* transcript are active. Data in Figure 2 also shows that β-galactosidase activity in uORF1 is consistently higher than from the main ORF (WT) and uORF2. Since uORF1 exists in both *HAP4p-lacZ* and *uORF2-lacZ* constructs, it is likely that uORF1 may have a negative regulatory effect on the activity of both uORF2 and the main ORF of *HAP4*.

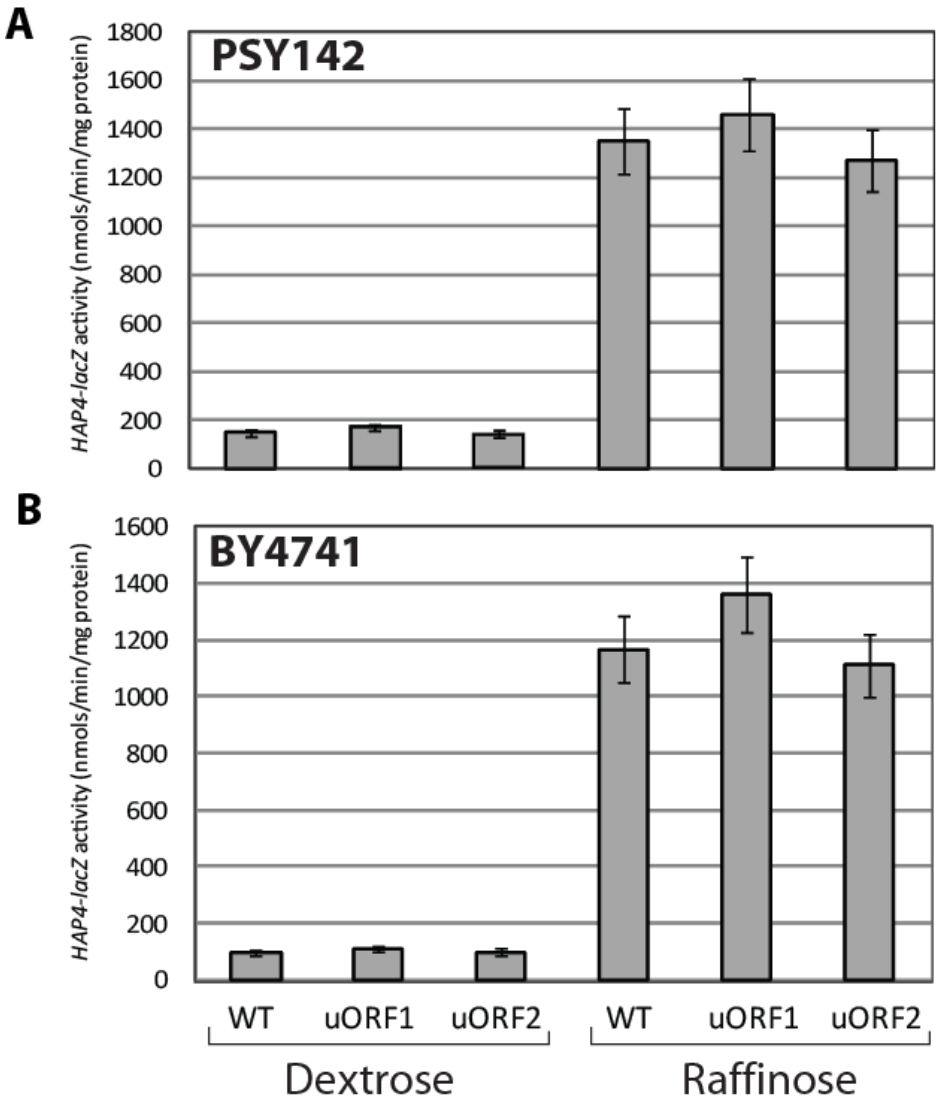

Figure 2: Upstream open reading frames of *HAP4* are translated
HAP4p-lacZ (WT), *uORF1-lacZ* (uORF1), and *uORF2-lacZ* (uORF2) in (A) PSY142 and (B) BY4741 background strains. WT represents the whole *HAP4* promoter fused to *lacZ*, uORF1 represents only the first upstream open reading frame fused to *lacZ*, and uORF2 represents the second upstream open reading frame fused to *lacZ*. Activity was measured in cells grown in either repressing (Dextrose) or derepressing (Raffinose) medium. Both strains show similar values of activity of the *lacZ* fusions. Results indicate that uORFs are active and that expression of uORFs mirrors that of the main ORF

Mutations of *HAP4* uORFs Affect the Expression of the Downstream Main Open Reading Frame

To determine whether uORFs affect the activity of the main *HAP4* ORF, I created *HAP4p-lacZ* fusions that contained a mutation in the start codon of either uORF1 (uORF1m), uORF2 (uORF2m), or both (uORF1,2m). The uORF mutants were created via site-directed mutagenesis using the PCR SOEing technique (see Materials and Methods). Schematic diagrams of constructs are presented in Figure 3. The mutations were confirmed via sequencing at the W.M. Keck Conservation and Molecular Genetics Laboratory using the HAP4.5'.-360 primer (Table 2). Constructs were transformed into PSY142 and BY4741 cells. Transformants were grown in both YNBcas5%D and YNBcasRaff media, and β-galactosidase activities were determined.

Figure 4A shows that *HAP4p-lacZ* activity in PSY142 cells grown in repressing (dextrose) medium is ~135 nmols/min/mg protein and a mutation in uORF1 increases *HAP4p-lacZ* expression by 1.4-fold; a mutation in uORF2 as well as mutations in both uORFs increase *HAP4p-lacZ* by 1.7-fold. Under derepressing conditions, induced by growing cells in raffinose medium, wild-type *HAP4p-lacZ* activity is ~1300 nmols/min/mg protein; uORF1, uORF2, and the double mutations increased *HAP4p-lacZ* expression by 1.4-, 1.6-, and 1.9-fold, respectively. Similar results were obtained in BY4741 background strain (Figure4B). Figure 4 also shows that uORF mutant effects are largely carbon-source independent, suggesting that *HAP4* uORFs play a house-keeping role rather than a regulatory role in response to changes in carbon sources. These data also suggest that *HAP4* uORFs negatively impact the activity of the *HAP4* main ORF and their effects are additive.

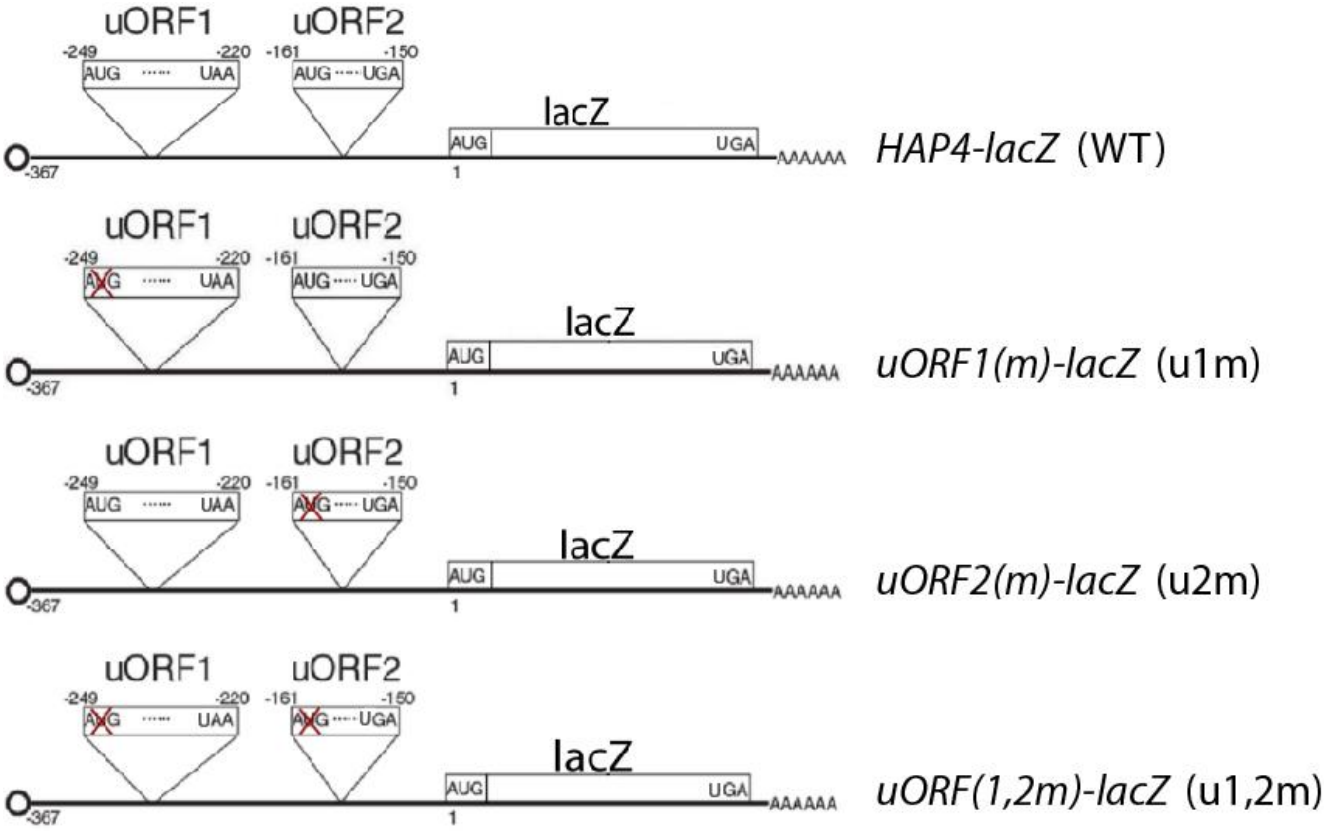

Figure 3: Four constructs used to determine whether uORF mutations affect the activity of the main ORF

From top, WT shows no mutations; uORF1 was mutated (uORF1m); uORF2 was mutated (uORF2m); both uORF1 and uORF2 were mutated (uORF1,2m). All constructs have *HAP4* start codon fused to the *E. coli lacZ* reporter gene to test promoter activity of *HAP4*.

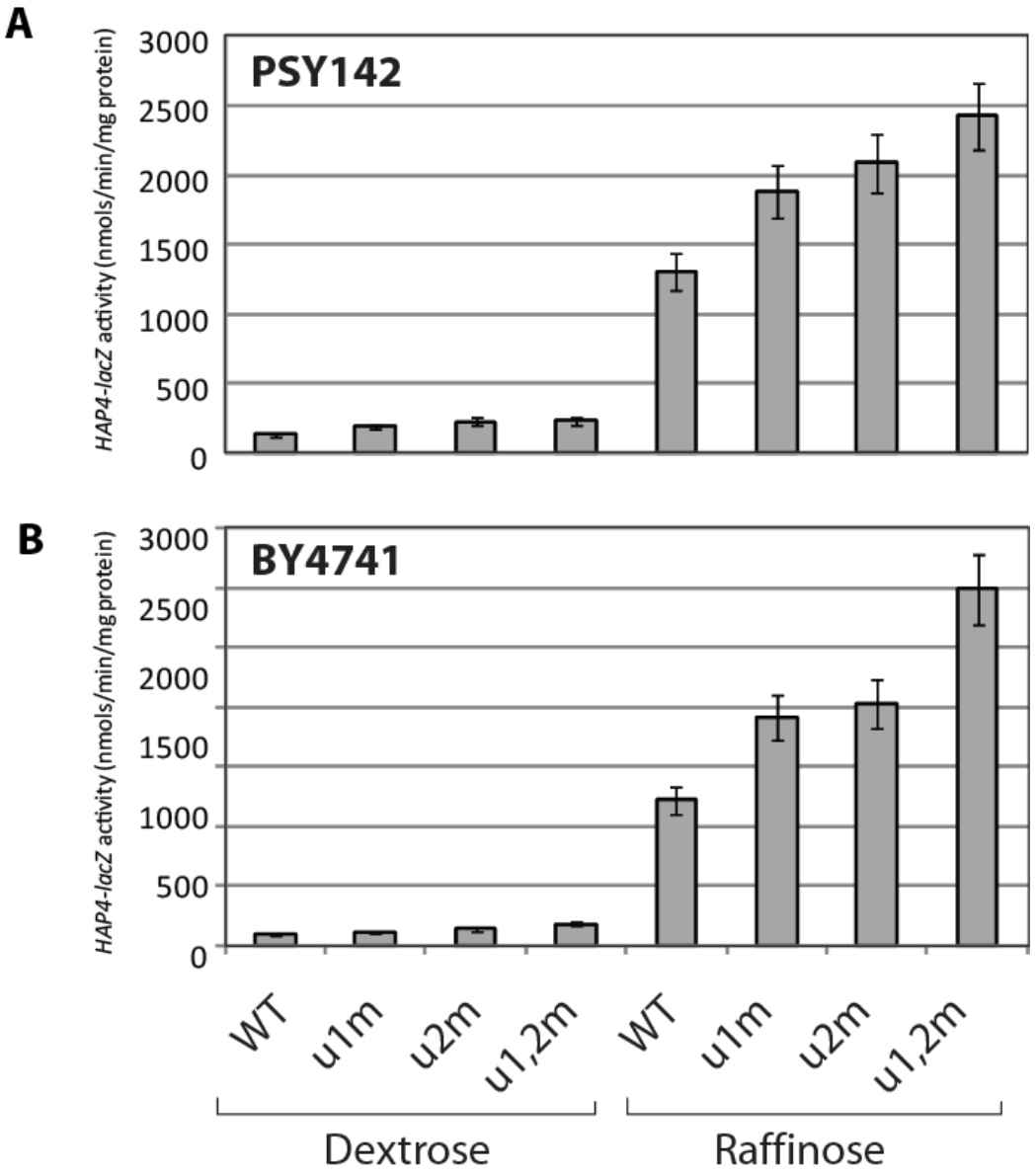

Figure 4: Mutations in *HAP4* uORFs increase *HAP4p-lacZ* activity
β-galactosidase activity of *HAP4p-lacZ* (*HAP4p-lacZ* or WT); *HAP4p-lacZ* which contains a mutation in the uORF start codon (uORF1m); *HAP4p-lacZ* which contains a mutation in the uORF start codon (uORF2m); and *HAP4p-lacZ* which contains mutations in the start codon of both uORFs (uORF1,2m).

3-AT Treatment Has Little Effect on *HAP4p-lacZ* Expression

As an initial step to uncover the mechanism by which uORFs inhibit *HAP4* activity, I first studied literature of *GCN4* regulation by its four uORFs. This system represents the most thoroughly researched mechanism of translational control by uORFs. The uORF repression of *GCN4* translation is alleviated during times of nutrient starvation because of a limiting amount of eIF-2-GTP-Met-$tRNA_i^{Met}$ ternary complexes (Hinnebusch 2005). Gcn2 protein kinase is responsible for phosphorylating eIF-2α during nutrient starvation and renders the eIF2 complex unable to undergo the change from the GDP form to the GTP form. Currently, Gcn2 is only known to regulate the expression of *GCN4* in yeast (Hinnebusch 2005). However, nutrient sensing pathways are inextricably linked and Gcn2 is also responsive to glucose starvation (Yang, Wek et al. 2000). Thus, I wanted to investigate whether *HAP4* uORFs present a translational barrier that is similar to the regulation of *GCN4*.

3-aminotrizole (3-AT) is a drug that inhibits activity of *HIS3*, an enzyme in the histidine biosynthesis pathway in yeast. Thus, treatment of cells with 3-AT leads to an increase in uncharged tRNA levels and activates Gcn2-dependent phosphorylation of eIF-2α. To understand whether Gcn2 is implicated in *HAP4* uORFs regulation, I assayed *HAP4p-lacZ* activity with and without 3-AT treatment. I grew PSY142 cells carrying a plasmid encoding *HAP4p-lacZ* (WT), *HAP4p*(u1m)-*lacZ*, or *HAP4p*(u2m)-*lacZ* plasmids in S5%D or SR medium supplemented with leucine and lysine, with and without 3-AT. Cells were grown overnight to mid-logarithmic phase and harvested at OD_{600} 0.55 - 0.8. Cellular extracts were prepared and assayed for β-galactosidase activity as described in Materials and Methods. Figure 5 shows that histidine

starvation has little effect on any of the three *lacZ* reporter genes. Thus, our data indicate that a Gcn2-mediated decrease in the level of eIF-2-GTP-Met-$tRNA_i^{Met}$ ternary complexes is not implicated in uORF inhibition of *HAP4* expression.

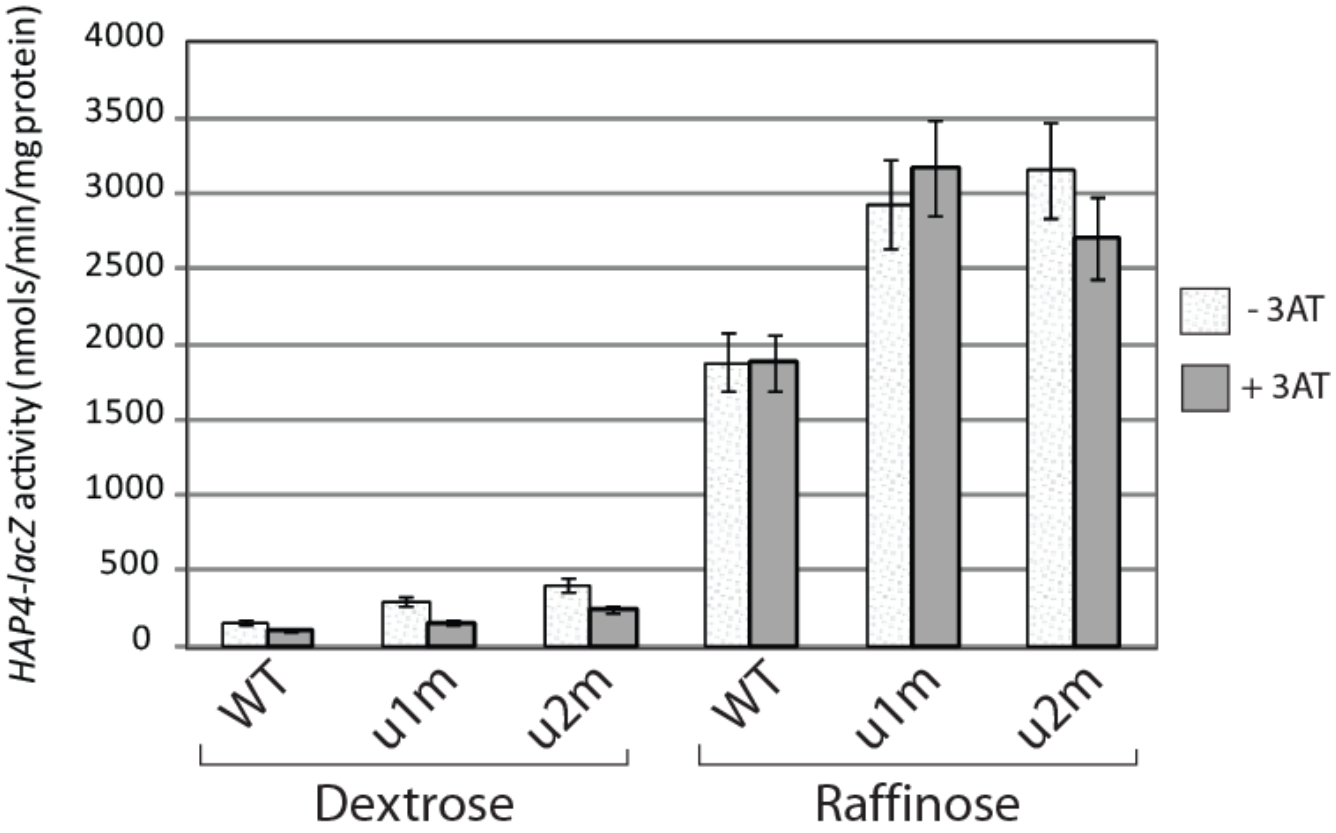

Figure 5: 3-AT effect on *HAP4p-lacZ* expression
β-galactosidase activity of *HAP4p-lacZ* containing either its wild-type promoter (WT), a promoter with a mutation in uORF1 (u1m), or a mutation in uORF2 (u2m). Cells were grown in S5%D or SR medium, with or without 3-AT treatment. White bars indicate no 3-AT treatment, and grey bars indicate 3-AT treatment.

Mutations in *UPF1/2/3* Do Not Increase *HAP4p-lacZ* Expression

The nonsense mediated decay (NMD) pathway is responsible for recognizing aberrant stop codons and mediates the degradation of mRNAs. NMD targets mRNAs with aberrant stop codons that arise through mutations and naturally occurring transcripts that have built-in features that target them for accelerated decay (error-free mRNAs) (Isken and Maquat 2007). In another attempt to uncover the mechanism of uORF regulation of *HAP4* expression, I wanted to determine whether *HAP4* uORFs, with their stop codons upstream of the main ORF, rely on the NMD pathway to regulate the activity of the *HAP4* main ORF. The *UPF1-3* (up frameshift) genes encode proteins that recognize premature stop codons in mRNA and signal them for degradation; thus Upf1/2/3 are required for activity of the NMD pathway (Culbertson and Neeno-Eckwall 2005). I transformed *upf1*Δ*, upf2*Δ*, and upf3*Δ single deletion mutants with a plasmid encoding *HAP4p-lacZ* and assayed for β-galactosidase activity. If the uORFs stimulate the NMD pathway, then a deletion in any of the *UPF* genes should increase the activity of *HAP4p-lacZ.* Surprisingly, all *upf*Δ mutations reduce *HAP4p-lacZ* expression, suggesting that the NMD pathway is unlikely to be involved in uORF mediated degradation of *HAP4* transcripts.

The unexpected effect of *upf*Δ mutations on reducing *HAP4p-lacZ* expression prompted us to determine whether *upf*Δ mutations also affect *KGD1-lacZ* expression, which is under the control of Hap2-5. To that end, we transformed a plasmid encoding *KGD1-lacZ* into wild-type and isogenic *upf*Δ mutant cells and assayed for β-galactosidase activity. Figure 6B shows that *upf1*Δ*, upf2*Δ*,* and *upf3*Δ mutations all reduce *KGD1-lacZ* expression, consistent with reduced *HAP4* expression in these mutant cells. Together, these data indicate that the NMD pathway is unlikely to be implicated in uORF inhibition of *HAP4* expression.

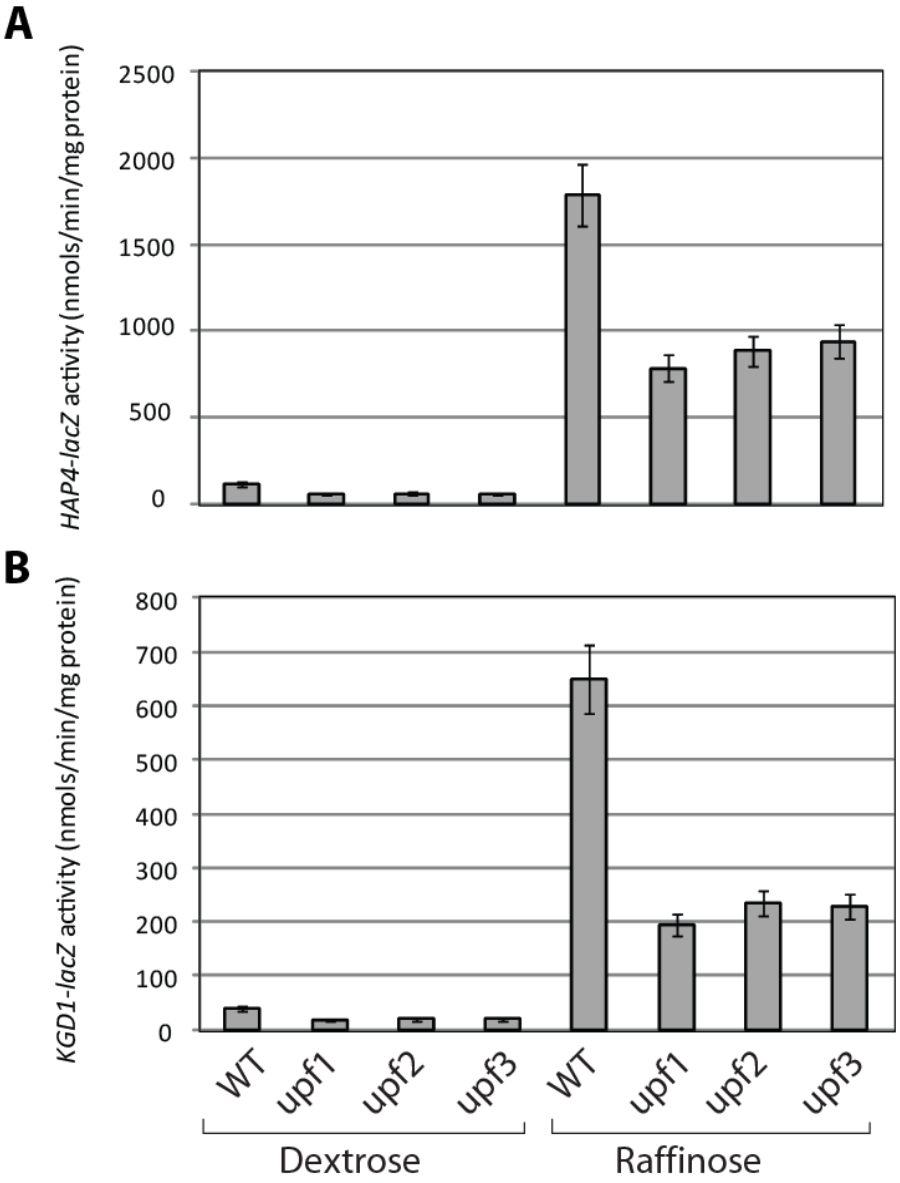

Figure 6: The effect of *upf1*Δ, *upf2*Δ, and *upf3*Δ mutations on expression of *HAP4p-lacZ* and *KGD1-lacZ* reporter genes

HAP4p-lacZ (A) and *KGD1-lacZ* (B) activity in wild-type (WT), *upf1*Δ, *upf2*Δ, and *upf3*Δ mutants grown in dextrose or raffinose medium. *upf*Δ mutations decrease the expression of both *lacZ* reporter genes.

Regulation of *HAP4* by Mitochondrial Functional State

Loss of mtDNA affects expression of nucleus-encoding mitochondrial proteins. When a cell loses its mtDNA and becomes *rho*^{0}, the expression of genes involved in respiratory functions is repressed (Liu and Butow 1999; Bourges, Mucchielli et al. 2009). It has been suggested that the Hap2-5 complex senses the signal generated in respiration-impaired cells and that Hap4 is specifically involved in linking mitochondrial dysfunction with decreased expression of these respiratory genes. As such, the way that the state of the mitochondria is implicated in a signaling pathway has yet to be fully understood. It has been reported that Hap4 protein levels are reduced in *rho*^{0} cells (Bourges, Mucchielli et al. 2009). However, how Hap4 expression is altered in response to changes in mitochondrial respiratory function is unclear. First, I needed to establish the effect of mtDNA loss on *HAP4* expression.

HAP4p-lacZ Activity in *rho*^{0} Petite Cells

I used previously constructed PSY142 and BY4741 *rho*^{0} strains and transformed them with a plasmid encoding full-length *HAP4p-lacZ* or *HAP4p-lacZ* with various uORF mutations and followed with assays of β-galactosidase activity of the transformants. Figure 7 shows *HAP4p-lacZ* activity with or without uORF mutations in PSY142 *rho*$^{+}$ and *rho*^{0} cells grown in dextrose or raffinose medium. In YNBcas5%D-grown cells, loss of mtDNA results in a ~2-fold decrease in *HAP4p-lacZ* expression, when compared to *rho*$^{+}$ cells. Expression of *HAP4p-lacZ* carrying uORF1, uORF2, and a double uORF mutation is 2.9-, 1.8-, and 2.0-fold lower in *rho*^{0} cells compared to that in *rho*$^{+}$ cells, respectively. In cells grown in YNBcasRaff medium, loss of mtDNA results in a 2.7-fold decrease in wild-type *HAP4p-lacZ* expression. Expression of *HAP4p-lacZ* carrying a mutation in uORF1, uORF2, or a double uORF mutation is 2.9-, 2.4-,

and 1.7-fold lower in *rho*^{0} cells compared to *rho*$^+$ cells, respectively. Similar results were obtained in BY4741 background cells (data not shown). These data suggest that *HAP4* expression is repressed in response to a loss of mitochondrial respiratory activity and that uORFs play little role in *HAP4* expression in response to mtDNA loss.

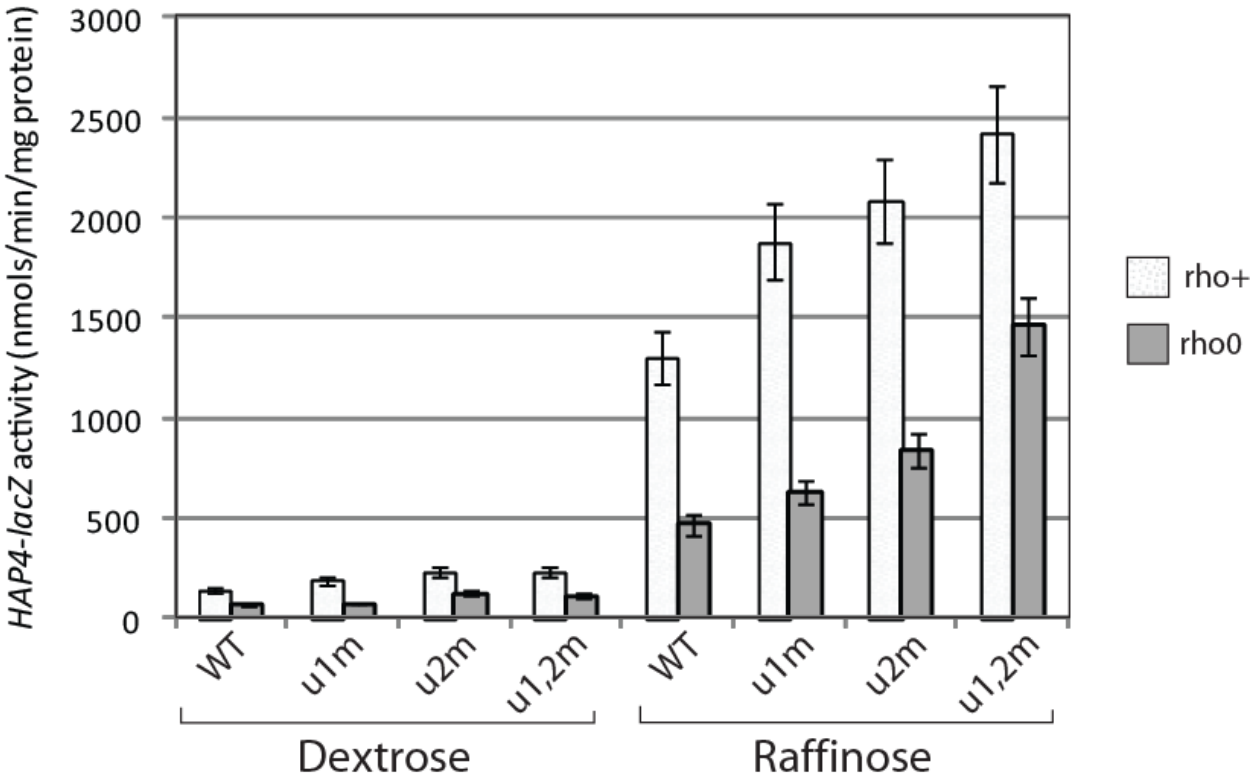

Figure 7: The effect of uORF mutations on *HAP4p-lacZ* expression in both *rho*$^{+}$ and *rho*^{0} cells

β-galactosidase activity of *HAP4p-lacZ* in PSY142 *rho*$^{+}$ (white bars) versus *rho*^{0} (grey bars) cells grown in dextrose or raffinose medium. *HAP4p-lacZ* is either wild-type (WT) or contains a mutation in uORF1 (u1m), uORF2 (u2m), or both uORFs (u1,2m). Data presented is in PSY142 background strain.

uORF Mutations Affect Hap4 Protein Levels

To test whether Hap4 protein levels are affected due to uORF mutations, I created constructs to express Hap4 with a C-terminal 3xHA tag (Table 2). Each contained the full-length *HAP4* ORF with none, one, or both uORFs mutated (Figure 8). Plasmids were transformed into both *rho*$^{+}$ and *rho*^{0} *hap4*Δ mutant cells and selected on YNBcasD medium. *rho*$^{+}$ transformants were able to grow on nonfermentable carbon sources (i.e., YPGlycerol), indicating that the HA epitope did not grossly interfere with Hap4 function, since *hap4*Δ mutant cells cannot grow on YPGlycerol medium (data not shown).

The effect of uORF mutations on *lacZ* expression is maximal in *rho*^{0} cells grown in raffinose medium (Figure 7). Thus, I chose to study the effect of uORF mutations on Hap4 protein levels in *rho*^{0} cells grown in raffinose. Cells carrying a plasmid encoding *HAP4-3xHA* under the control of wild-type *HAP4* promoter, or promoters with various uORF mutations, were grown in liquid YNBcasRaff medium overnight to an optical density of OD_{600} 0.6 - 0.8. Total cellular extracts were prepared as described in Materials and Methods. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was carried out as described in Materials and Methods. Figure 9 shows that uORF1 and uORF2 mutations have an additive effect on increasing Hap4 protein levels which is consistent with the uORF mutant effects on *HAP4p-lacZ* expression (Figure 7). As a control for protein loading, the membrane was deprobed and reprobed for Ilv5 (isouleucine biosynthesis), which is constitutively expressed.

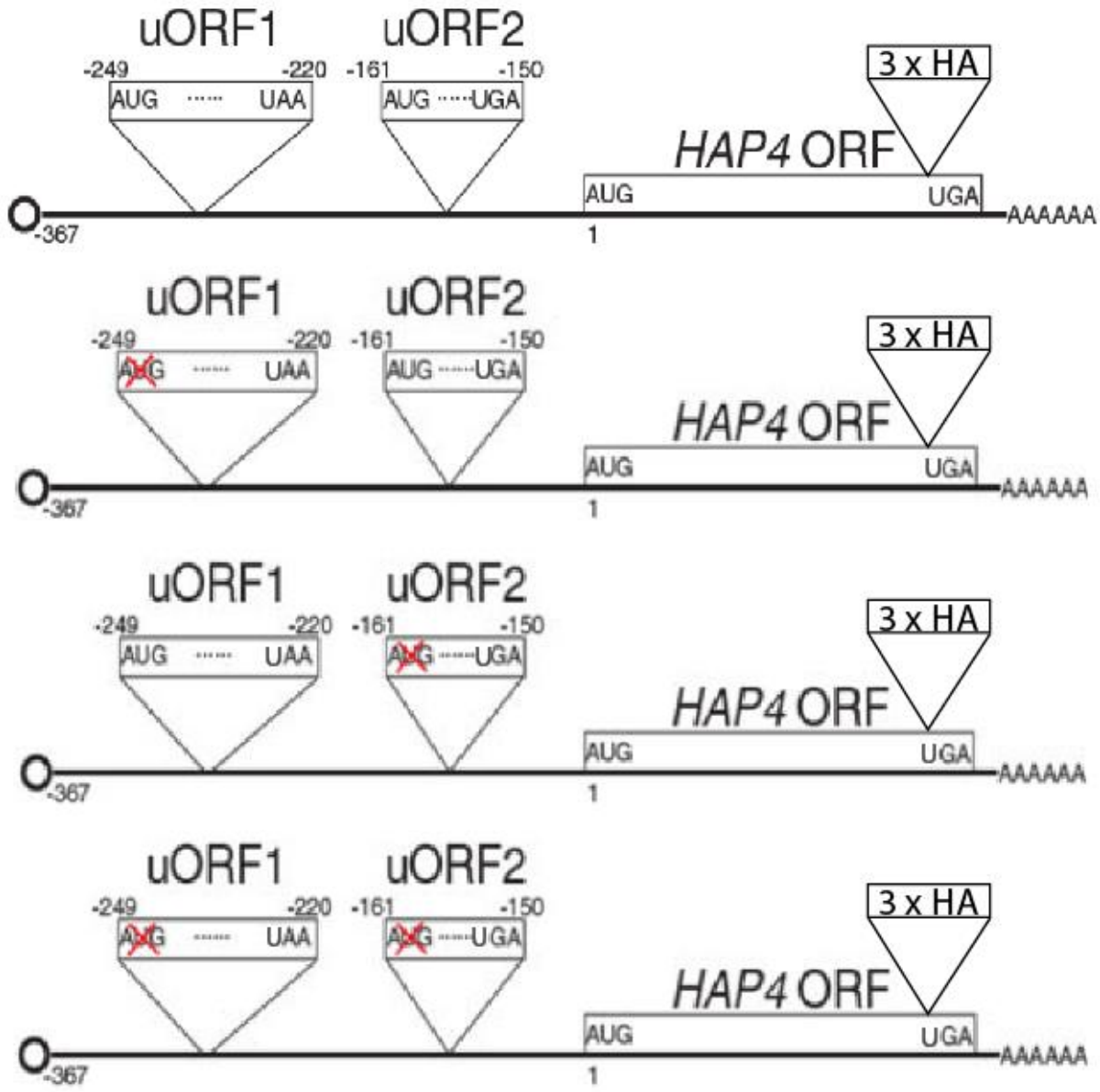

Figure 8: *HAP4-HA* constructs
Diagram of transcript of *HAP4* with a C-terminal 3xHA tag. From top, the WT transcript without mutations in the *HAP4* promoter; *HAP4-HA* transcripts with uORF1 mutated, uORF2 mutated, or both uORFs mutated. These constructs were cloned into pRS416 *CEN* plasmid.

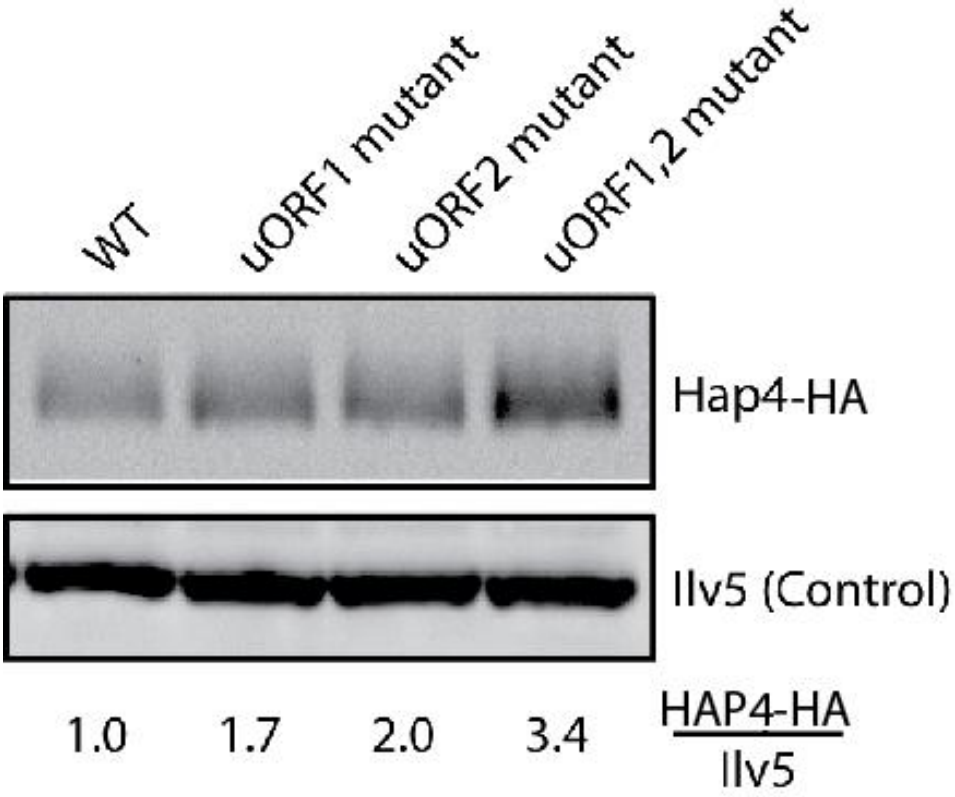

Figure 9: Increased Hap4 protein levels due to uORF mutations
Strains carry a centromeric plasmid encoding *HAP4-HA* under the control of the wild-type promoter (WT) or a promoter with a mutation in uORF1, uORF2, or both uORFs (see Figure 8 for construct diagram). Cells were grown in raffinose medium. Western blots were probed with anti-HA antibody to detect Hap4-HA. The membrane was deprobed and reprobed for Ilv5, a loading control. Quantification of the ratio of Hap4-HA:Ilv5 is presented at the bottom.

uORF Mutations of *HAP4* Affect Expression of *KGD1-lacZ*

After determining that uORF mutations increase Hap4 protein levels, I wanted to determine whether an increased Hap4 protein amount due to uORF mutations increases expression of *KGD1,* which encodes the mitochondrial α-ketoglutarate dehydrogenase and is a known target of the Hap2-5 complex (Repetto and Tzagoloff 1989; DeRisi, Iyer et al. 1997). Therefore, I utilized *KGD1* expression to monitor Hap2-5 activity. First, I created a *KGD1-lacZ* reporter gene (Table 2). In a wild-type strain grown in repressing medium, *KGD1-lacZ* activity is ~35 nmols/min/mg protein. In the wild-type strain grown in derepressing medium, *KGD1-lacZ* activity is ~370 nmols/min/mg protein, but when *HAP4* is deleted, activity decreases to ~70 nmols/min/mg protein in cells grown in YNBcasRaff medium (Figure 10), indicating that *KGD1* is under Hap2-5 control, as previously reported.

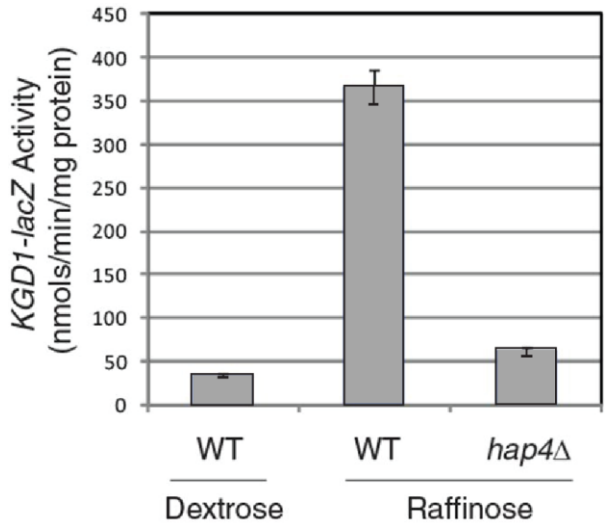

Figure 10: *KGD1-lacZ* expression is Hap4 dependent
KGD1-lacZ activity in wild-type (WT) PSY142 and in *hap4*Δ mutant cells grown in dextrose or raffinose medium.

To determine whether increased Hap4 protein levels due to uORF mutations affect target gene expression, I assayed *KGD1-lacZ* activity in *rho*$^{+}$ and *rho*^{0} *hap4*Δ mutants expressing Hap4-HA with mutations in one, both, or none of its uORFs. First, we created new yeast strains: I integrated *KGD1-lacZ* into the genome of PSY142 wild-type. To do this, I amplified the *KGD1* promoter from genomic DNA, cloned it into an integrative plasmid YIp356 and then digested with NcoI and transformed into PSY142 cells. The *URA3* marker was then mutated using a ura3::kanMX4 cassette. In the end, *HAP4* was deleted using a hap4::LYS2 cassette (Table 2). Selected strains were then induced to lose their mtDNA via treatment with ethidium bromide (see Materials and Methods). Confirmation of loss of mtDNA was confirmed by fluorescence microscopic analysis following DAPI staining (see Materials and Methods [data not shown]).

These two *rho*$^{+}$ and *rho*^{0} *hap4*Δ mutant strains with integrative *KGD1-lacZ* were transformed with the series of *URA3* based plasmids encoding HA-tagged *HAP4* with no mutations in its uORFs (WT), with a mutation in uORF1 (u1m), a mutation in uORF2 (u2m), or mutations in both uORF1 and uORF2 (u1,2m), as seen in Figure 8. Transformants were selected on YNBcasD medium. β-galactosidase activity was assayed and results in Figure 11 show that mutations in *HAP4* uORFs have little effect on *KGD1-lacZ* expression in *rho*$^{+}$ cells and in *rho*^{0} cells grown in YNBcas5%D medium. The effect of a double uORF deletion mutation on *KGD1-lacZ* expression is most pronounced in *rho*^{0} cells grown in YNBcasRaff medium, consistent with a more pronounced uORF1,2 mutant effect on *HAP4* expression (Figure 5 and Figure 7).

Since there is increased *HAP4* expression due to uORF mutations, results in Figure 7 and Figure 11 suggest that, in dextrose-grown *rho*$^{+}$ and *rho*^{0} cells, there is a threshold effect because the mild effect of uORF mutation on *HAP4* expression is not translated into any significant increases in *KGD1-lacZ* expression. Results also show that, in *rho*$^{+}$ cells grown in raffinose medium, *KGD1-lacZ* expression is roughly proportional to *HAP4* expression levels. In *rho*^{0} cells grown in raffinose medium, a significant increase in *HAP4* expression due to a double uORF mutation is translated into a significant increase in *KGD1-lacZ* expression, consistent with Western blotting results showing a 3.4-fold increase in Hap4 protein levels due to double uORF mutations (Figure 9). Together, these data show that *HAP4* uORFs contribute to the regulation of Hap2-5 target gene expression.

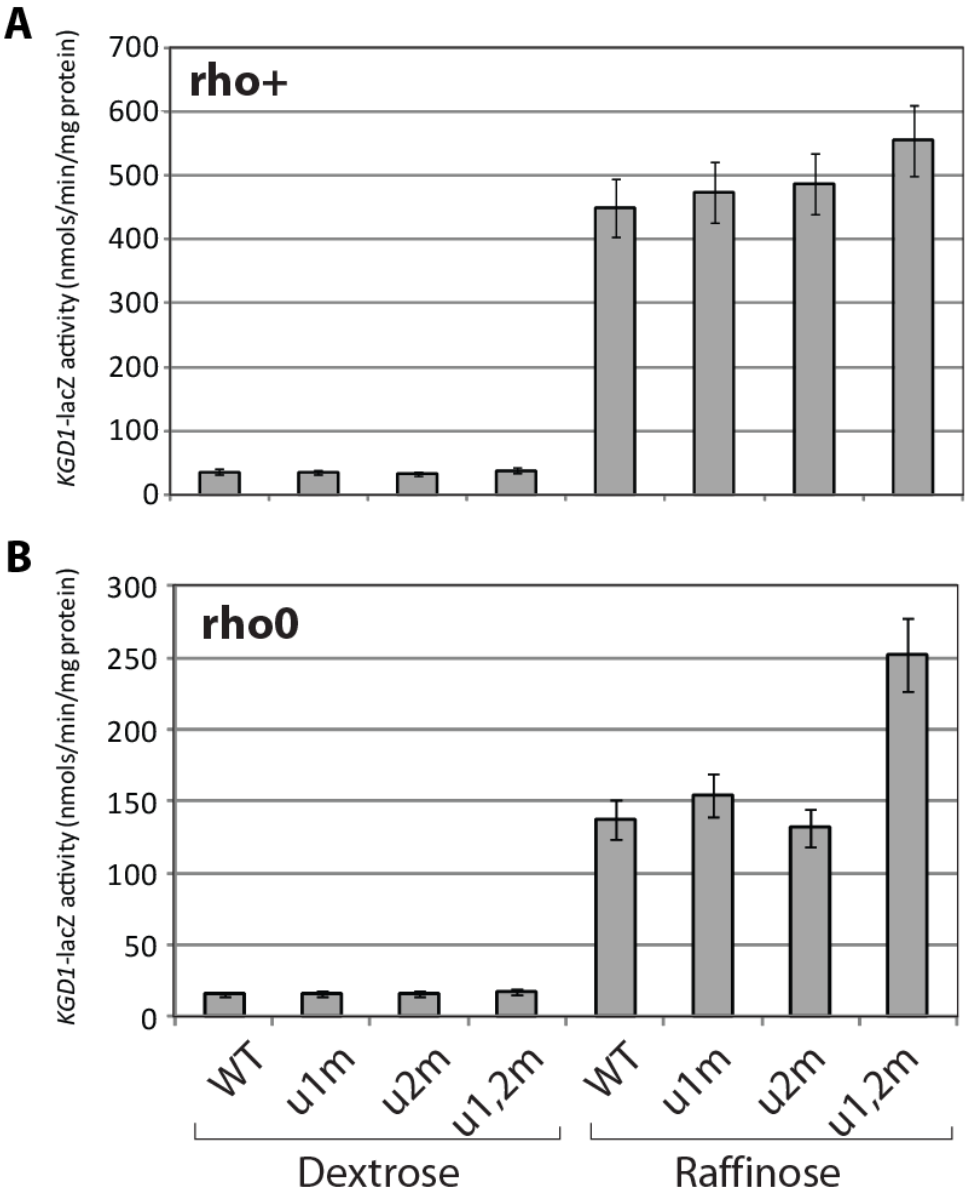

Figure 11: The effect of *HAP4* uORF mutations on *KGD1-lacZ* activity
β-galactosidase activity of integrated *KGD1-lacZ* in PSY142 *rho*$^{+}$ and *rho*^{0} *hap4*Δ mutants containing a centromeric plasmid encoding full-length *HAP4* under the control of the wild-type promoter (WT), or a promoter with a mutation in uORF1 (u1m), a mutation in uORF2 (u2m), or mutations in both uORFs (u1,2m). Cells were grown in YNBcas5%D or YNBcasRaff medium and *KDG1-lacZ* activities were determined.

Hap4 Protein Levels are Reduced in *rho^0* Cells

Since there is disproportionate expression of *HAP4p-lacZ* and *KGD1-lacZ* expression in *rho$^+$* and *rho^0* cells (Figure 7 and Figure 11), I wanted to determine Hap4 protein levels in these cells. I transformed *rho$^+$* and *rho^0* BY4741 *hap4*Δ mutants with Hap4-HA. Transformants were selected on YNBcasD plates and were grown in YNBcas5%D and YNBcasRaff media overnight to OD_{600} 0.6 - 0.8. Cellular extracts were prepared, proteins were separated by SDS-PAGE, and then transferred to a nitrocellulose membrane for Western blotting. Hap4-HA was probed with high-affinity anti-HA antibody. As a loading control, blots were deprobed and reprobed for isoleucine biosynthesis enzyme Ilv5. Figure 12 shows that in dextrose-grown cells, Hap4-HA protein levels are 2-fold lower in *rho^0* cells, compared to *rho$^+$* cells. In raffinose-grown cells, Hap4-HA levels are 3-fold lower in *rho^0* cells, compared to *rho$^+$* cells. Since *HAP4p-lacZ* expression is only reduced 3-fold in *rho^0* cells compared to *rho$^+$* cells (Figure 7), our data in Figure 12 suggest that *HAP4* expression is subject to post-transcriptional regulation.

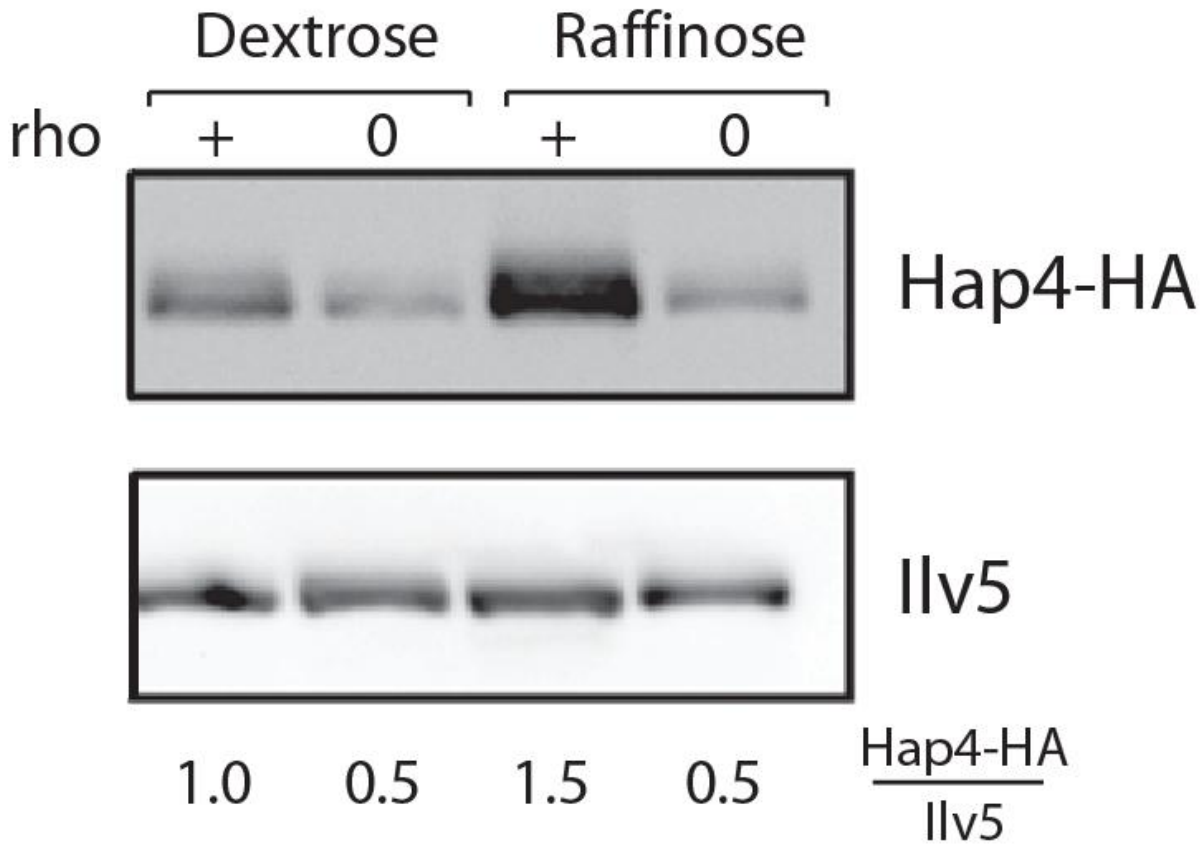

Figure 12: Hap4 protein levels in dextrose- and raffinose-grown *rho*$^{+}$ or *rho*^{0} cells
Hap4 protein levels were investigated with Western blotting. *rho*$^{+}$ and *rho*^{0} BY4741 *hap4*Δ mutants carrying a plasmid encoding Hap4-HA were grown in dextrose or raffinose medium and assayed for relative amounts of Hap4 protein. Dextrose-grown *rho*^{0} cells have a 2-fold reduction in Hap4 protein; raffinose-grown *rho*^{0}cells have a 5-fold reduction in Hap4 protein.

Hap4 Has a Shorter Half-life in *rho*^{0} Cells, Compared to *rho*$^{+}$ Cells

The apparent lower levels of Hap4 protein in *rho*^{0} cells prompted a comparative analysis of protein turnover rates of Hap4 in cells with or without mtDNA. Hap4 activity is needed only when the cell has a functional mitochondria. Therefore, if the cell becomes *rho*^{0}, it may be advantageous for cells to suppress Hap4 activity. Clearly, this is partly achieved by transcriptional regulation of *HAP4* expression (Figure 7). Bourges, Mucchielli and others (2009) postulated that decreased target gene activity due to loss of mtDNA could be resultant from increased turnover of Hap4. However, they did not test this hypothesis.

To investigate whether there is differential stability of Hap4 in *rho*$^{+}$ versus *rho*^{0} cells, I performed a cycloheximide chase assay in *rho*$^{+}$ and *rho*^{0} cells (PSY142 *hap4*Δ) containing a centromeric plasmid expressing Hap4-HA under the control of the strong *GPD* promoter, grown in both repressing and derepressing medium. After addition of cycloheximide, samples were collected every five minutes. After the assay, total cellular extracts were prepared (see Materials and Methods), proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting. Figure 13 shows that loss of mtDNA leads to increased Hap4 turnover. Quantification of band intensities in the Western blots seen in Figure 13 revealed the degradation rate of Hap4 under various conditions. In dextrose-grown cells, the half-life of Hap4-HA in *rho*$^{+}$ and *rho*^{0} cells is 5.8 min. and 3.9 min., respectively. In raffinose-grown cells, the half-life of Hap4-HA in *rho*$^{+}$ and *rho*^{0} cells is 6.9 min. and 2.9 min., respectively. These data suggest that *S. cerevisiae* can sense the functional state of the mitochondria and regulate Hap4 stability accordingly. These results also suggest that lower levels of Hap2-5 target gene

expression in *rho*^{0} cells are due not only to transcriptional regulation of *HAP4*, but also a post-translational control of Hap4.

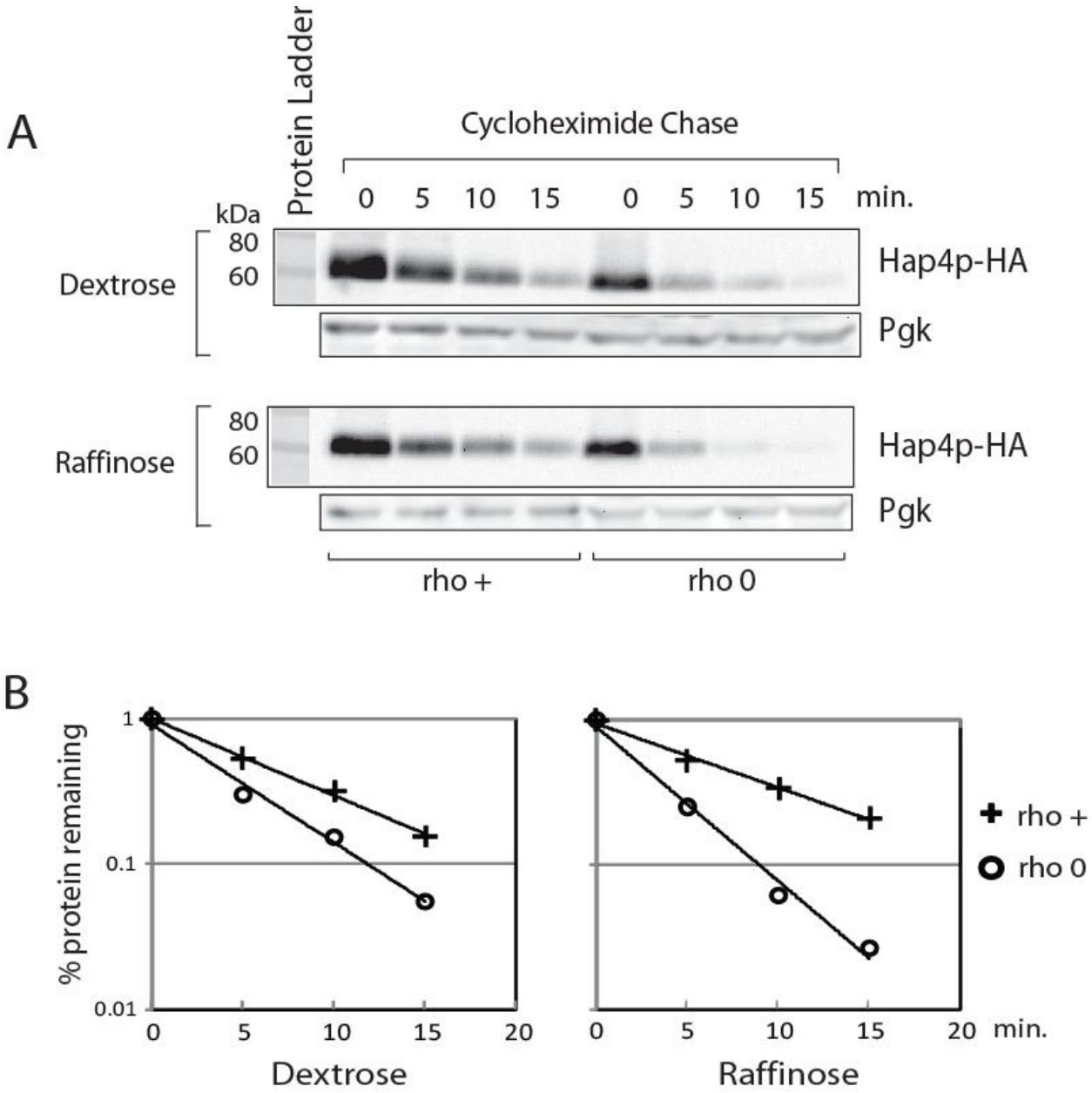

Figure 13: Hap4 stability in *rho*$^{+}$ and *rho*^{0}cells grown in dextrose or raffinose medium
(A) Cycloheximide chase assay of Hap4 protein stability in *rho*$^{+}$ and *rho*^{0} cells grown in dextrose and raffinose medium. Pgk is used as a loading control. (B) Quantification of Hap4-HA levels seen in panel (A).

Hap4 Degradation is Proteasome-Dependent

One mechanism by which eukaryotic cells achieve protein degradation is the ubiquitin/proteasome system. In this pathway, substrates are first marked for degradation by the conjugation of multiple molecules of ubiquitin. The ubiquitinated substrate is then degraded by the 26S proteasome. This pathway is involved in degradation of a variety of cellular proteins of different functions (Varshavsky 2005).

N-acetyl-leucinyl-leucinyl-norleucinal (MG132) is a specific, potent, reversible proteasome inhibitor. It reduces the degradation of ubiquitin-conjugated proteins by inhibiting the 26S complex and has proven useful in implicating the proteasome in a variety of proteolytic processes (Lee and Goldberg 1996). MG132 is not normally permeable to the *S. cerevisiae* cell membrane; however, deletion of the ergosterol biosynthesis gene *ERG6* confers cell membrane permeability to MG132 (Lee and Goldberg 1998). Thus, treatment of *erg6*Δ mutants with or without MG132 prior to a cycloheximide chase assay enables the determination of whether protein degradation is proteasome-dependent.

Cells (*rho*$^+$ *erg6*Δ) containing a plasmid expressing *HAP4-HA* under control of its own promoter were grown in YNBcas5%D and YNBcasRaff medium. When cultures reached OD_{600} 0.4, cells were treated with either MG132 or DMSO (DMSO was solvent for MG132) until the cells reached an OD of at least 0.6. I then performed a cycloheximide chase assay (see Materials and Methods). Samples were collected every ten minutes. After the assay, I prepared total cellular extracts by TCA precipitation, separated the proteins by SDS-PAGE, and transferred proteins to a nitrocellulose membrane for immunoblotting. Hap4-HA was probed with the high-

affinity anti-HA antibody 3F10. For a loading control, blots were stripped using a stripping buffer containing β-mercaptoethanol, and the blots were reprobed with antibodies against isoleucine biosynthesis enzyme Ilv5 (see Materials and Methods).

Figure 14 shows that MG132 treatment leads to increased stability of Hap4-HA in both dextrose- and raffinose-grown cells. In cells with only DMSO, degradation of Hap4 is pronounced. However, after treatment with MG132, Hap4 is more stabilized. It was previously hypothesized that, in cells with respiratory deficiency (as in *rho*^{0} cells or in Δ*oxa1* mutants), Hap4 is degraded in the vacuole (Bourges, Mucchielli et al. 2009). However, results in Figure 14 indicate that Hap4 is degraded via a proteasome-dependent mechanism and thus some degradation would occur in the cytoplasm.

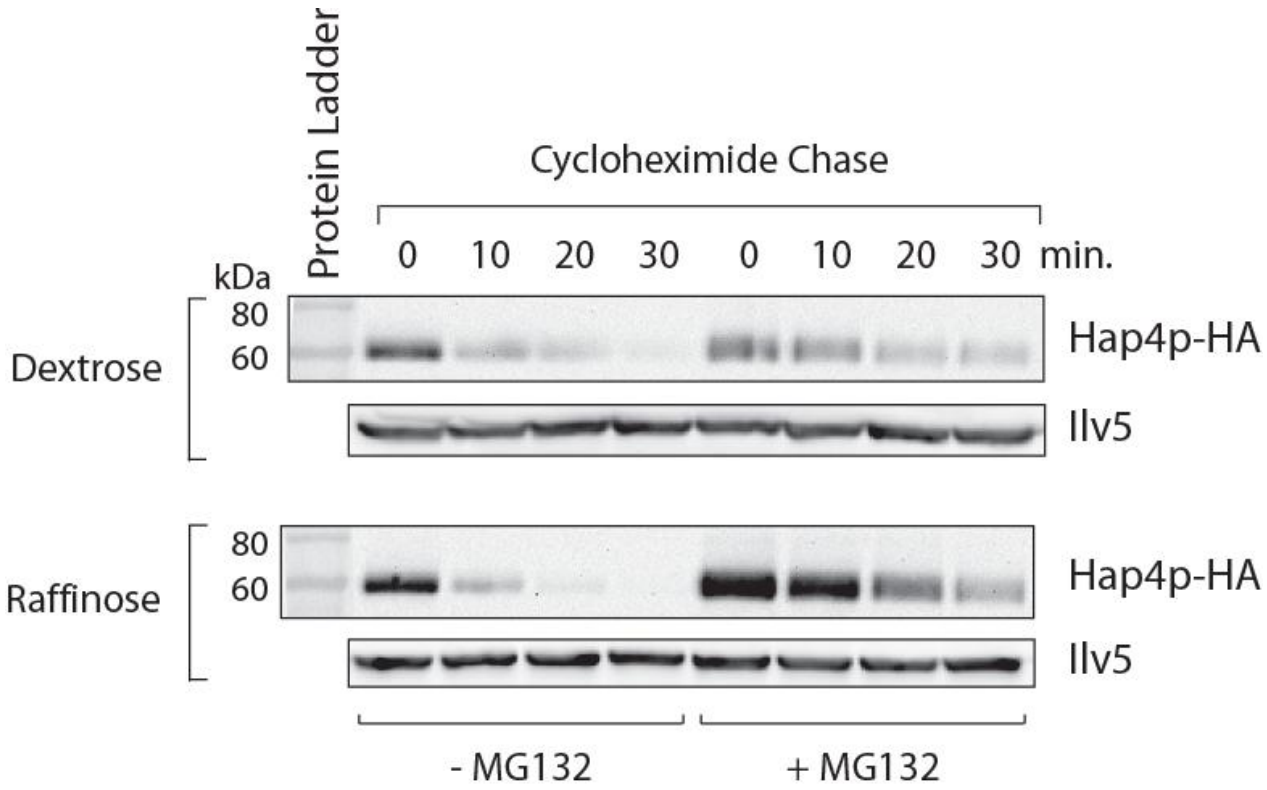

Figure 14: Hap4 degradation is proteasome dependent
Hap4 degradation was investigated by using a cycloheximide chase assay in cells with or without the treatment of the proteasome inhibitor MG132. *rho*$^{+}$ *erg6*Δ mutant cells were grown in dextrose and raffinose medium. Ilv5 (isoleucine biosynthesis) is used as the loading control.

rho^0 Effects Not Mediated Through Respiratory Deficiency: Analysis of TCA Cycle Gene Mutations on *HAP4p-lacZ* Expression

To understand whether decreased *HAP4p-lacZ* expression of *rho*^{0} cells is due to respiratory deficiency, I transformed nine TCA cycle gene mutants (*aco1*Δ; *cit1/2/3*Δ; *fum1*Δ; *idh1*Δ; *kgd1*Δ; *kgd2*Δ; *lsc1*Δ; *mdh1*Δ; *sdh1*Δ) with the *HAP4p-lacZ* reporter gene plasmid and assayed β-galactosidase activity in transformants grown in YNBcasRaff medium. If *rho*^{0}-mediated decrease in *HAP4p-lacZ* expression is due to respiratory deficiency, we would predict that all TCA cycle mutants would reduce *HAP4p-lacZ* expression similar to what is observed in *rho*^{0} cells. Figure 15 shows that mutations in *ACO1, IDH1, LSCI, MDH1,* and *SDH1* have no significant effect on *HAP4p-lacZ* expression, while mutations in *CIT1/2/3, FUM1, KGD1,* and *KGD2* reduce *HAP4p-lacZ* expression by ~2-fold. These data indicate that *rho*^{0}-induced repression of *HAP4p-lacZ* expression is not simply due to respiratory deficiency since all of the TCA cycle mutants are respiratory-deficient and are unable to grow on a nonfermentable carbon source (data not shown).

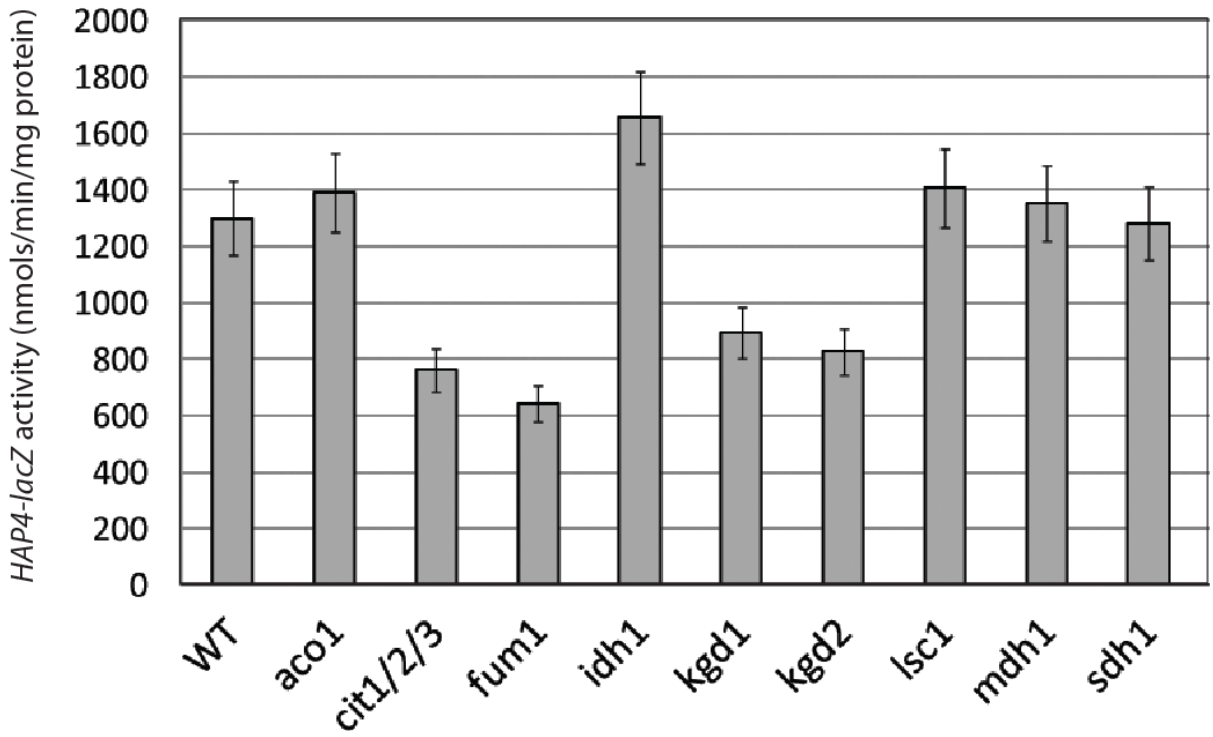

Figure 15: Differential effect of mutations in TCA cycle enzyme-encoding genes on *HAP4p-lacZ* activity

Wild-type and isogenic TCA cycle enzyme-encoding gene mutants carrying a plasmid encoding *HAP4p-lacZ* reporter gene were grown in YNBcasRaff and β-galactosidase activities were determined.

HAP4 Overexpression is Not Sufficient to Fully Activate *KGD1-lacZ* Expression

To determine whether changes in Hap4 protein levels are fully responsible for regulating the Hap2-5 complex in response to changes in carbon sources, I sought to express *HAP4* under the control of strong, constitutive, heterologous promoters. This would allow further characterization of the effect of Hap4 levels on Hap2-5 target gene expression. I aim to determine whether overexpression of *HAP4* under heterologous promoters can lead to full activation of *KGD1-lacZ* expression in glucose-grown *rho*$^+$ cells.

The strong, constitutive active promoter of *GPD* or *TEF2* was fused to the *HAP4* open reading frame and cloned into a centromeric plasmid pRS416 in-frame with an HA epitope tag that was already encoded by the plasmid. I first determined whether *HAP4* is overexpressed under the control of *TEF2* or *GPD* promoter. I used Western Blotting to assay Hap4 protein levels in PSY142 *hap4*Δ mutants containing a plasmid encoding HAP4p-HAP4-HA, TEF2p-HAP4-HA, or GPDp-HAP4-HA. Cells were grown in either YNBcas5%D or YNBcasRaff medium; total cellular protein extracts were prepared and separated by SDS-PAGE, and then transferred to a nitrocellulose membrane and probed with anti-HA antibody. Figure 16 shows that when *HAP4* is under the control of its own promoter, as expected, there is a large increase in Hap4 protein levels in cells grown in raffinose medium, compared to dextrose-grown cells. In contrast, when Hap4 is under control of heterologous promoters *TEF2* or *GPD*, Hap4 protein levels are similar in dextrose- and raffinose-grown cells. These results indicate that the use of

these two heterologous promoters leads to overexpression of *HAP4* and abolishes the differential expression of Hap4 in dextrose versus raffinose medium.

To test downstream target gene activity, I transformed *rho*$^{+}$ PSY142 *hap4*Δ mutants containing an integrated *KGD1-lacZ* reporter gene with a plasmid encoding HAP4p-HAP4-HA, GPDp-HAP4-HA or TEF2p-HAP4-HA. Cells were grown in YNBcas5%D or YNBcasRaff medium and *KGD1-lacZ* activities were determined. Figure 17 shows that in cells grown in dextrose medium, *KGD1-lacZ* activity is increased by 6- and 3.5-fold when *HAP4* is expressed under the control of the *GPD* or *TEF2* promoter, respectively. In cells grown in raffinose medium, *KGD1-lacZ* expression is increased by 1.3- and 1.4-fold in cells expressing *HAP4* under the control of the *GPD* or *TEF2* promoter, compared to cells expressing *HAP4-HA* under its endogenous promoter. These data show that when *HAP4* expression is driven by a stronger promoter, *KGD1-lacZ* expression is greater than when *HAP4* is under control of its own promoter. Significantly, these data also show that *KGD1-lacZ* expression is increased by ~12-fold in cells grown in raffinose medium compared to cells grown in dextrose medium. However, in cells overexpressing *HAP4* under the *GPD* or *TEF2* promoter, *KGD1-lacZ* expression is increased 2.4- and 4.3-fold in raffinose-grown cells compared to glucose-grown cells, even though Hap4-HA levels are almost equal in dextrose versus raffinose-grown cells. Together, these data demonstrate that an increase in Hap4 protein levels is not solely responsible for Hap2-5 activation under glucose derepressing conditions.

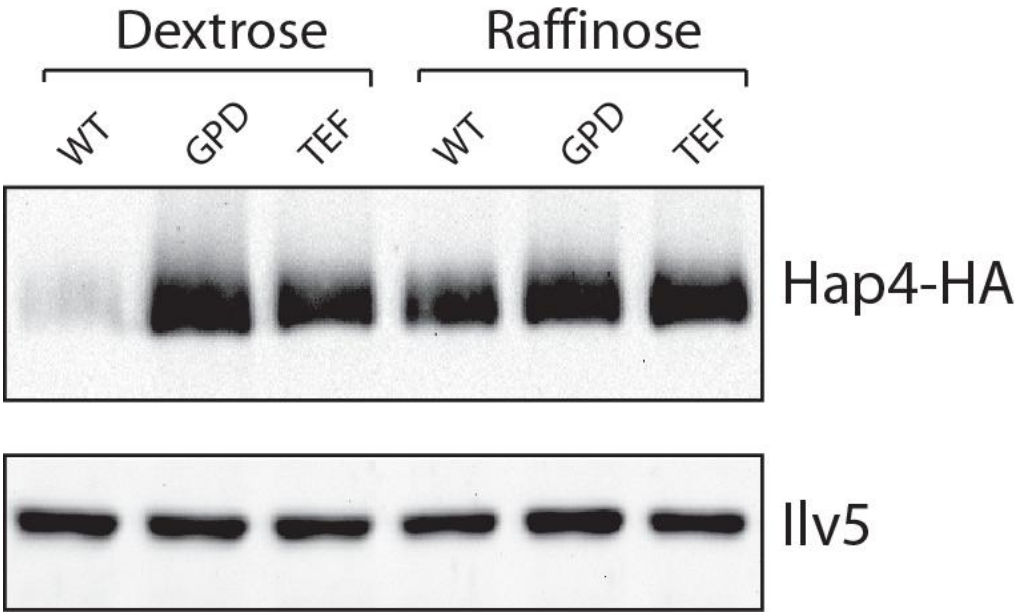

Figure 16: Hap4 is overexpressed when under the control of heterologous promoters
Hap4-HA protein levels were determined by Western blotting in *hap4*Δ mutant cells carrying a plasmid encoding *HAP4-HA* under the control of its own promoter (WT), *GPD* promoter (GPD), or *TEF2* promoter (TEF). Cells were grown in YNBcas5%D (Dextrose) or YNBcasRaff (Raffinose) medium. Ilv5 was included as a loading control.

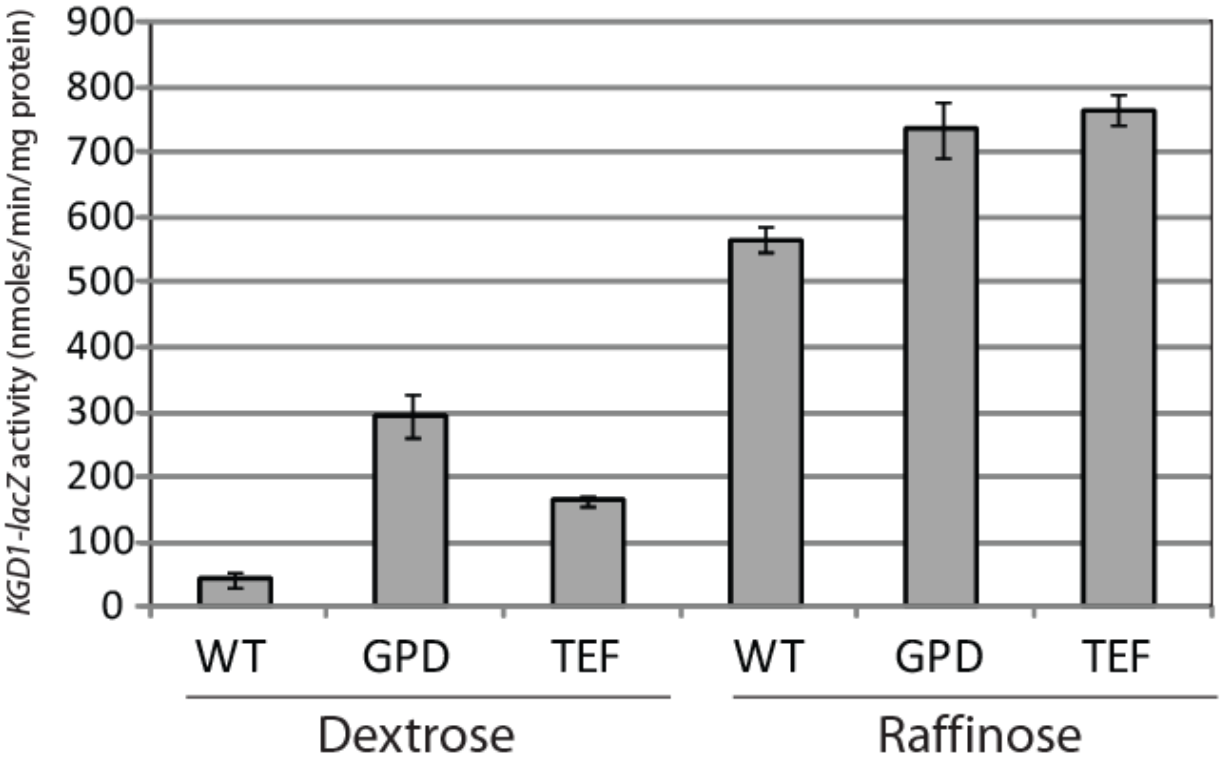

Figure 17: The effect of *HAP4* expression on *KGD1-lacZ* activity
Cells in the Figure 16 legend carrying an integrated *KGD1-lacZ* reporter gene were grown in YNBcas5%D (Dextrose) or YNBcasRaff (Raffinose) medium and analyzed for β-galactosidase activity.

Lower Levels of Hap4-HA Expression from the *TEF2* or *GPD* Promoter in *rho^0* Cells

Since Hap4 degradation is induced in *rho^0* cells, I analyzed the Hap4 protein levels expressed from the strong *GPD* promoter in *rho$^+$* versus *rho^0* cells to determine whether the *rho^0* effect also takes place in cells overexpressing Hap4. To this end, PSY142 *rho$^+$* or *rho^0 hap4*Δ mutant strains were transformed with plasmid pRS416-GPDp-HAP4-HA and transformants were grown overnight in YNBcas5%D or YNBcasRaff medium to reach OD_{600} 0.6 - 0.7. Total cellular extracts were prepared and separated by SDS-PAGE, and then transferred to a nitrocellulose membrane and probed with anti-HA antibody. Figure 18 shows that the steady state levels of Hap4 is lower in *rho^0* cells compared to *rho$^+$* cells, consistent with our previous observations showing degradation of Hap4 in *rho^0* cells (Figure 13).

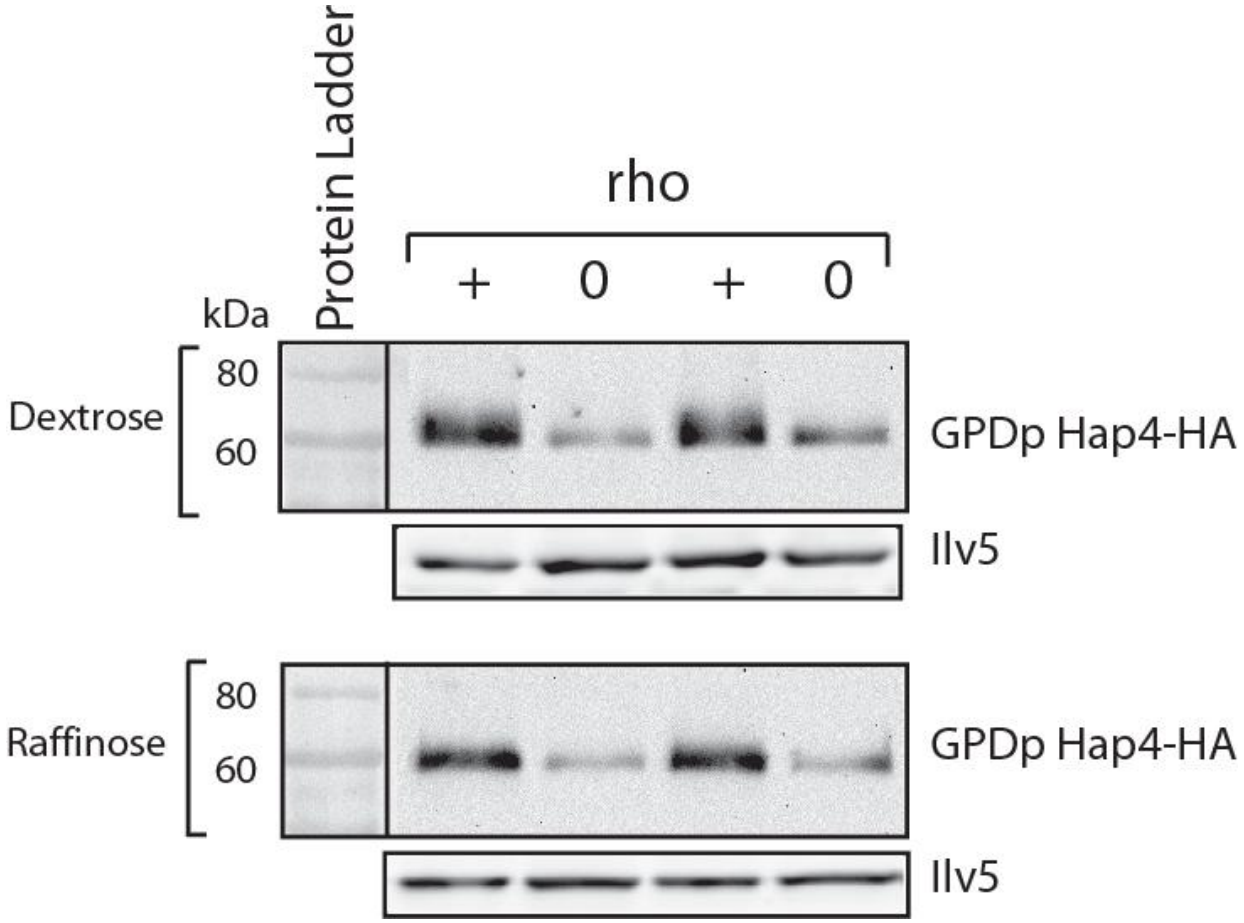

Figure 18: Hap4 protein levels in *rho*$^{+}$ and *rho*^{0} overexpressing Hap4-HA under the control of *GPD* promoter

Ilv5, an isoleucine biosynthesis enzyme, is used as a loading control.

Localization of Hap4 in Vivo

I was interested in determining the localization of Hap4 under repressing and derepressing conditions. It has been hypothesized that Hap4 is shuttled from the cytoplasm to the nucleus when switched from repressing to derepressing conditions. A recent study has failed to detect Hap4-GFP localization when *HAP4* was expressed from a genomic copy under its own promoter. In cells overexpressing *HAP4*, Hap4-GFP was detected in the vacuole, leading the researchers to believe that only a small portion of Hap4, undetectable in their experiments, is present in the nucleus and that excess Hap4 is removed and degraded in the vacuole (Bourges, Mucchielli et al. 2009). Thus, I was not only interested in determining subcellular localization of Hap4-GFP, but I also wanted to assess whether localization of Hap4 was part of the regulatory process.

In Vivo Localization of GFP Tagged Hap4

I first determined the localization of Hap4-GFP expressed under its native promoter from a centromeric plasmid. To help visualize its localization, I introduced a GFP tag at its C-terminus. The GFP tagged Hap4 was able to complement a *hap4*Δ mutant enabling growth on glycerol medium, indicating the Hap4-GFP fusion was functional (data not shown). I then transformed both rho^{+} and rho^{0} PSY142 *hap4*Δ mutants with a plasmid encoding a Hap4-GFP fusion and grew transformants overnight in repressing (S5%D) or derepressing (SR) medium. Synthetic minimal medium was used rather than YNBcas because of the inherent fluorescence of YNBcas medium that is significant enough to mask the GFP signal. Log-phase grown cells were pelleted and viewed under a fluorescence microscope as indicated in Materials and Methods. Since minimal medium was used for localization experiments, these conditions were tested using

β-galactosidase assays and Western blotting for Hap4-HA expressing and I found that minimal medium mirrors YNBcas5%D/R medium (data not shown).

For all GFP localization images presented in Figure 19, picture intensity levels were set to be the same. DIC images were not controlled for levels. All images were taken on the same day and captured within 5 min. of cell collection. Experiments conducted on different days produced similar data. Since the Hap4-GFP images in Figure 19 are controlled for levels, the relative intensity of color is indicative of relative levels of Hap4-GFP. These results indicate that in *rho*$^{+}$ cells, there are more Hap4-GFP proteins in cells grown in raffinose compared to cells grown in dextrose medium, which is indicated by stronger fluorescence signals in raffinose-grown cells. Hap4-GFP is concentrated in the nucleus when the cells are growing in a derepressing carbon source; when glucose is the sole carbon source, there appears to be less nuclear localization and more cytoplasmic localization. Although the levels are low in dextrose-grown cells, Hap4-GFP signals are clearly visible. In *rho*^{0} petites grown in both dextrose and raffinose medium, Hap4-GFP signals are low, although they can still be detected, which is consistent with my Western blotting data showing that Hap4 is quickly degraded in *rho*^{0} cells (Figure 13). In *rho*^{0} cells, I failed to detect nuclear concentration of Hap4-GFP. Similar data were obtained in BY4741 (data not shown). These data suggest that during activation of the Hap2-5 complex under derepressing conditions, nuclear localization of Hap4 likely constitutes an activation mechanism, which is largely due to increased Hap4 protein.

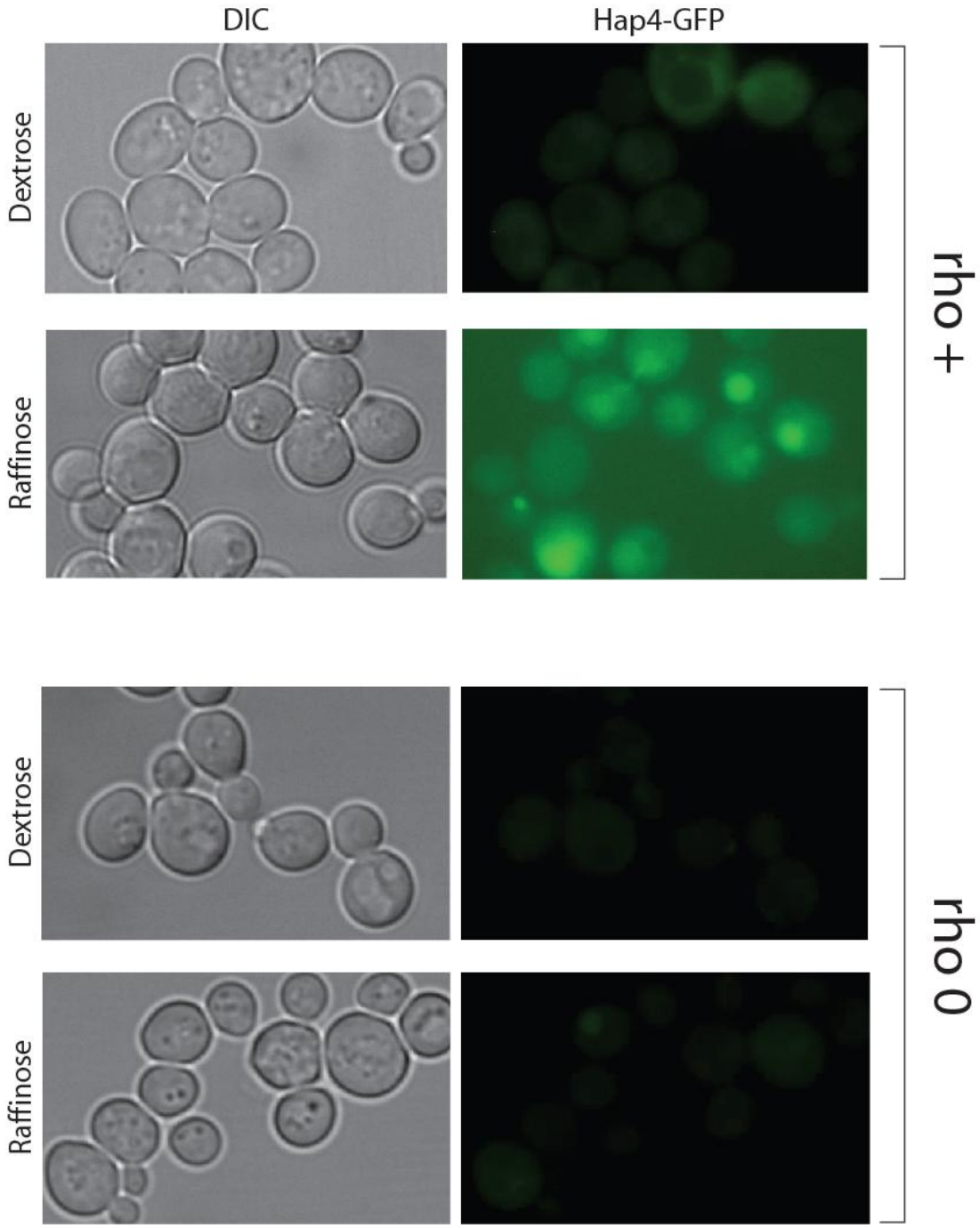

Figure 19: In vivo localization of Hap4-GFP expressed from a centromeric plasmid
Fluorescence microscopic analysis of Hap4-GFP under repressing (Dextrose) or derepressing (Raffinose) conditions. Images were edited using ImageJ software and levels were controlled so that qualitative comparisons could be made across all Hap4-GFP images.

Hap4-GFP Localizes in the Nucleus in *rho*$^+$, Raffinose-grown Cells

To confirm that Hap4-GFP signals are concentrated in the nucleus in raffinose-grown *rho*$^+$ cells, I decided to conduct colocalization experiments in Hap4-GFP expressing cells that are also stained with DAPI, a fluorescent dye which binds to DNA. To that end, *hap4*Δ mutant cells expressing Hap4-GFP from a centromeric plasmid were grown in SR medium to log-phase and treated with DAPI at a concentration of 1 µg/mL for three hours. Cells were pelleted and viewed under a fluorescence microscope. Images were obtained of the GFP fluorescence and the DAPI staining. ImageJ software was used to color GFP pictures green and DAPI images red (even though DAPI fluorescence is actually blue). I then merged the images so that the colocalization would appear as yellow-orange. Figure 20 shows the Hap4-GFP signal colocalized with DAPI fluorescence in raffinose-grown *rho*$^+$ cells, confirming that Hap4-GFP is localized in the nucleus. DAPI fluorescence images also showed punctuate cytoplasmic structures, which are mitochondrial nucleoids. Together, my data indicate that under conditions where Hap2-5 is active, Hap4 is localized in the nucleus.

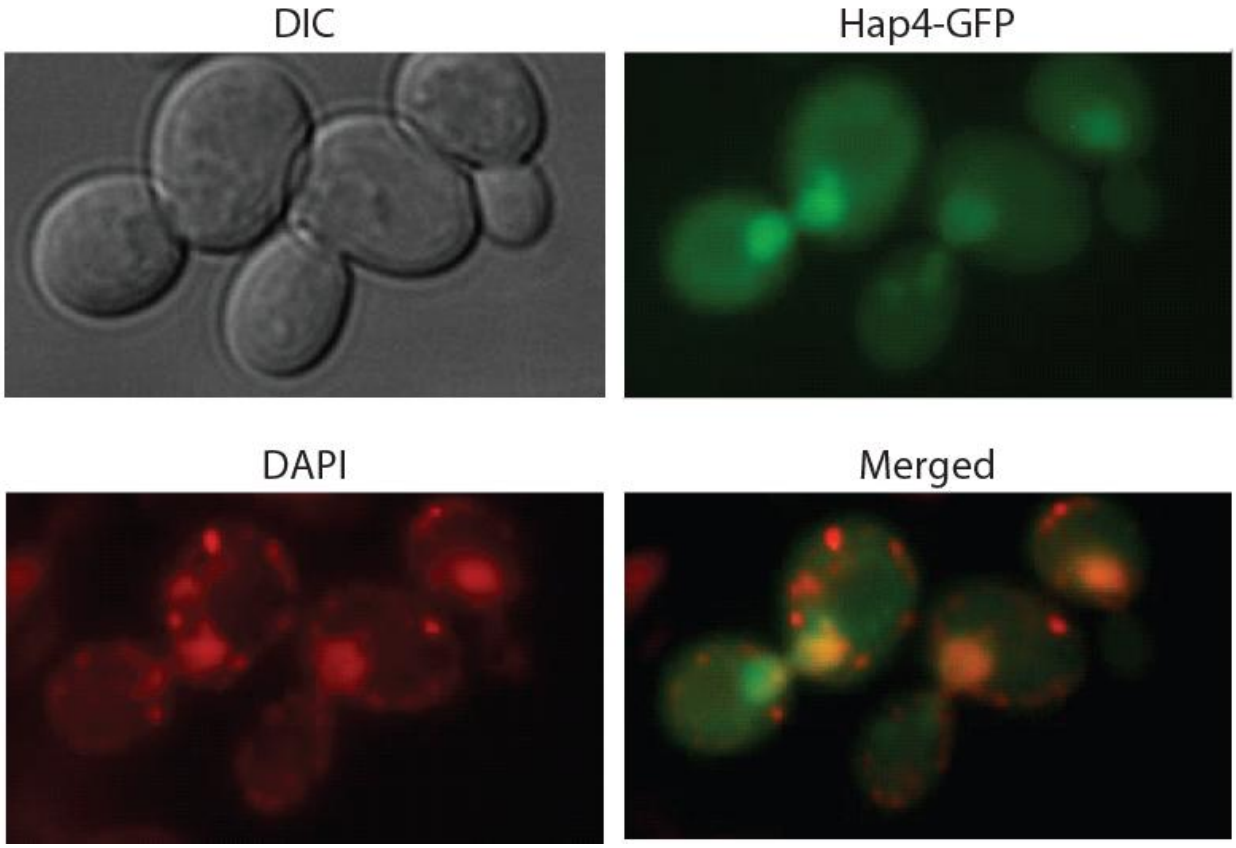

Figure 20: Hap4-GFP colocalizes with nuclear DAPI staining in raffinose-grown *rho*$^{+}$ cells
*hap4*Δ *rho*$^{+}$ cells expressing Hap4-GFP from a centromeric plasmid were grown in raffinose medium. DAPI was added to cell cultures at a concentration of 1 µg/mL to help visualize the nucleus. GFP and DAPI fluorescence images were captured then processed using ImageJ software, and merged in Adobe Photoshop.

In vivo Localization of Hap4-GFP Expressed under the Control of the *TEF2* Promoter

When Hap4 is overexpressed under the control of the *GPD* or *TEF2* promoter, there are similar levels of Hap4 protein in *rho*$^+$ cells grown under repressing and derepressing conditions (Figure 16). However, there is a differential activation of *KGD1-lacZ* expression in Hap4-overexpressed cells in dextrose versus raffinose medium, suggesting that a mechanism other than Hap4 protein levels affects *KGD1-lacZ* expression. One facile possibility is that overexpressed Hap4 is not targeted to the nucleus as efficiently in glucose-grown cells as in raffinose-grown cells.

To determine whether glucose may keep Hap4 in the cytoplasm even though Hap4 is overexpressed under the control of *TEF2* promoter, I constructed a centromeric plasmid encoding TEF2p-HAP4-GFP (Table 2). The resultant plasmid was then transformed into PSY142 *hap4*Δ *rho*$^+$ and *rho*^{0} cells and transformants were grown in YNBcas5%D or YNBcasRaff medium. Cells were collected for microscopic analysis. Figure 21 shows that overexpressed Hap4-GFP is concentrated in the nucleus in both glucose- and raffinose-grown *rho*$^+$ cells. I confirmed equal levels of Hap4-GFP expressed in glucose- and raffinose-grown cells using Western blotting (Figure 22). I also confirmed that expression of Hap4-GFP under the control of the *TEF2* promoter in a *hap4*Δ *rho*$^+$ mutant results in lower levels of *KGD1-lacZ* expression in glucose-grown cells, in comparison to raffinose-grown cells (data not shown). In *rho*^{0} cells, GFP fluorescence is lower compared to *rho*$^+$ cells, reflecting increased degradation of Hap4 in *rho*^{0} cells. Since equal levels of overexpressed Hap4 in glucose- and raffinose-grown *rho*$^+$ cells localizes to the nucleus to the same extent but leads to differential activation of *KGD1-*

lacZ expression, another mechanism other than Hap4 amount and cellular localization is likely to be responsible for glucose repression of the Hap2-5 complex.

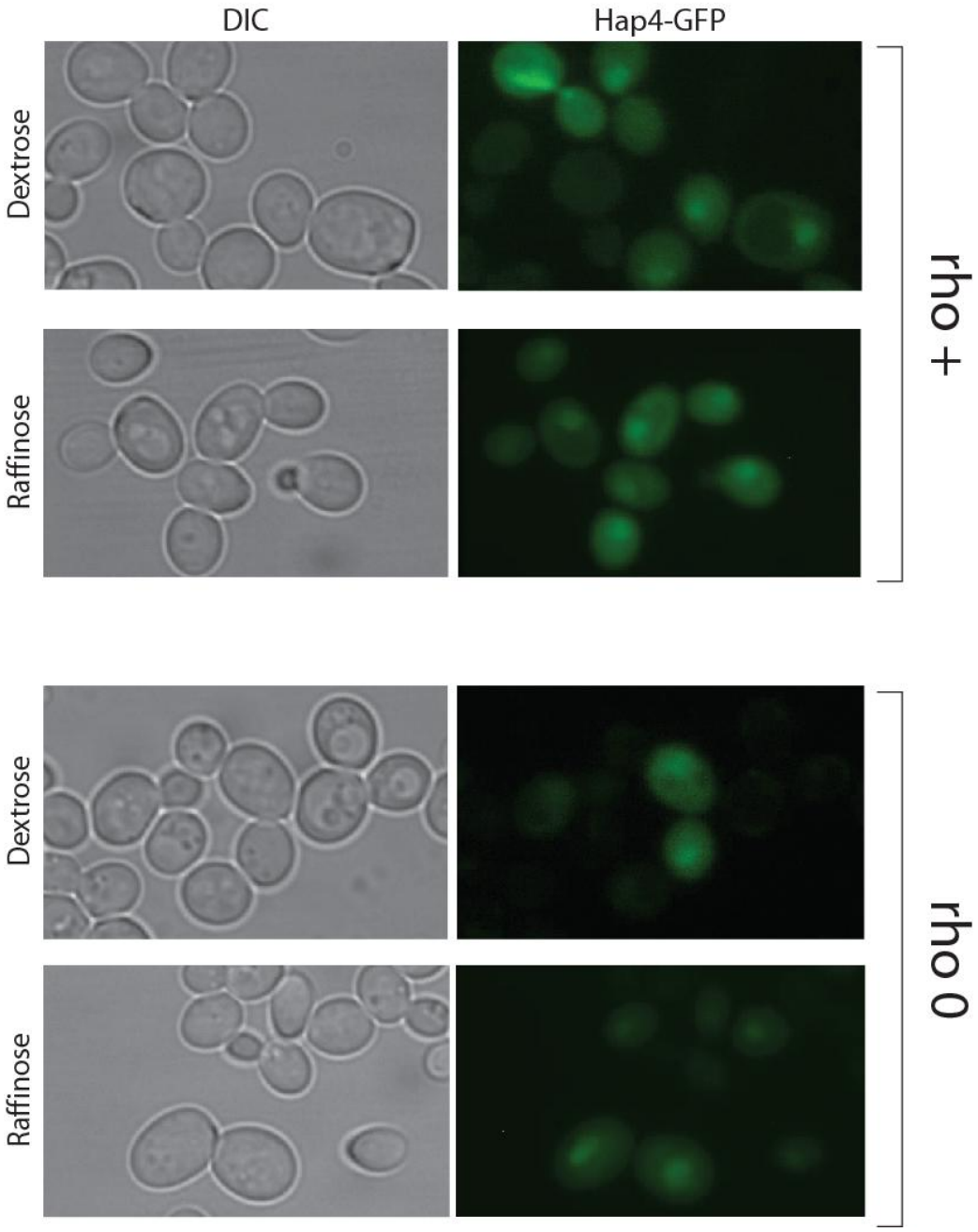

Figure 21: Subcellular localization of overexpressed Hap4-GFP expressed from the TEF2 promoter

rho^{+} or *rho*^{0} *hap4*Δ mutant cells carrying a centromeric plasmid encoding TEF2p-HAP4-GFP were grown in YNBcas5%D (Dextrose) or YNBcasRaff (Raffinose) medium. GFP fluorescence images were captured under a fluorescence microscope under the same exposure time and processed with ImageJ software within the same parameters.

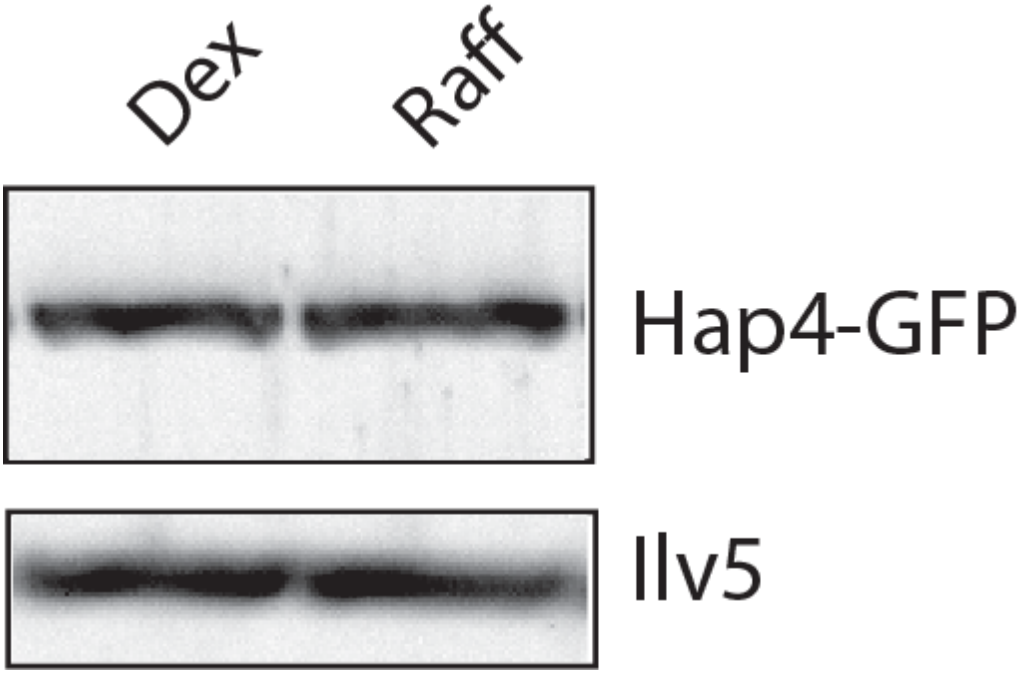

Figure 22: Equal amounts of Hap4-GFP expressed from the *TEF2* promoter in glucose- and raffinose-grown *rho*$^+$ cells
Cells described in the Figure 21 legend were analyzed for Hap4-GFP expression by Western blotting. Rabbit anti-GFP polyclonal antibody was used to probe for Hap4-GFP. Ilv5 was used as a loading control.

Isolation and Cloning of *HAP4* Constitutive Activating Mutants

A Genetic Screen to Identify Mutants with Increased *HAP4* Expression

To identify genes which are involved in *HAP4* regulation, I conducted a genetic screen to isolate mutants with increased *HAP4* expression. Four different strains, DCY122 (rho^+), DCY125 (rho^+), DCY140 (rho^0), and DCY170 (rho^0), each containing plasmids encoding a *HAP4p-lacZ* reporter gene, were subjected to EMS mutagenesis as described in Materials and Methods.

Briefly, the cells were mutagenized with ethyl methanesulfonate (EMS), which produces mutations in the DNA via G-C nucleotide substitution. EMS treated cells were plated directly on solid YNBcasD medium containing X-Gal. Since *HAP4p-lacZ* has a low-level expression in cells grown on glucose medium, wild-type cells form colonies with a light-blue or white color on X-gal plates. I selected colonies which appeared dark blue. These dark blue colonies were selected for further analysis by subjecting them to β-galactosidase activity assays, which gave more reliable results than visual inspection. Of the dark blue colonies assayed, 38 showed increased *HAP4p-lacZ* expression when grown in glucose medium (Figure 23 and data not shown).

I confirmed that increased *HAP4p-lacZ* expression in these mutants was due to a nuclear gene mutation rather than mutations on the pWCJ-HAP4p-lacZ plasmid since, after crossing to a wild-type strain of the opposite mating type, all resultant diploids had low levels of *HAP4p-lacZ* expression (data not shown). These data also suggest that all mutations are recessive. Several mutant yeast strains were further confirmed to have a genomic mutation by first inducing the

cells to lose their reporter plasmid followed by retransformation with a fresh plasmid and subsequent β-galactosidase activity assays (data not shown).

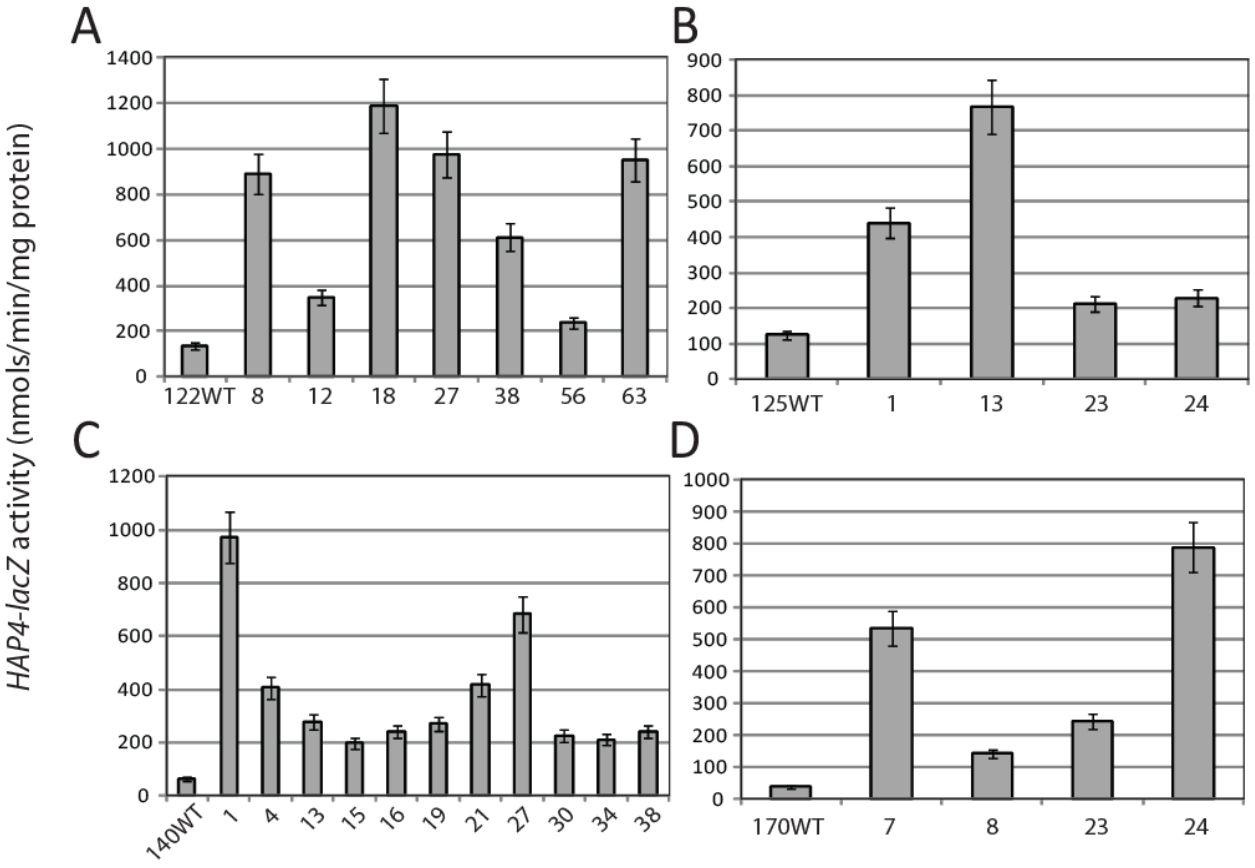

Figure 23: Increased *HAP4p-lacZ* expression in EMS mutagenized cells
After treatment with EMS, 38 (26 shown here) dark-blue colonies were confirmed to have high *HAP4p-lacZ* activity when grown in YNBcas5%D medium. (A) DCY122 is a PSY142 (*MAT*α) *rho*$^{+}$ strain; (B) DCY125 is a BY4741 (*MAT***a**) *rho*$^{+}$ strain; (C) DCY140 is a PSY142 *rho*^{0} strain; (D) DCY170 is a BY4741 *rho*^{0} strain.

Complementation Group Analysis

To determine whether any of the mutant cells have mutations in the same gene, mutants of the opposite mating type were crossed and β-galactosidase activity was determined in the resultant diploid strains. Any diploids which retained high activity indicated that the parent haploids had mutations on the same gene; when two mutations do not complement, high β-galactosidase activity ensues. To maximize the number of the mutants for complementation group analysis, it was important to examine a mutant with the same mating type. However, since strains with the same mating type cannot mate, I switched the mating type of some of the mutants. To do this, I transformed cells with a plasmid encoding the HO endonuclease that enables the cells to switch mating type, under the control of a galactose inducible promoter. To induce the mating-type switch, I inoculated cells in YNBcasRaff with 2% galactose. 600 cells were plated after ≥8 hours of galactose induction and individual colonies were picked. To confirm a mating type switch, individual colonies were mixed with either a *MAT**a*** or a *MATα* tester strain to determine their mating type. However, after many crossings, β-galactosidase activity assays showed that all haploid mutants that I tested complemented each other. Thus, I did not succeed in placing mutants into complementation groups.

HAP4-activating Mutants Are Due To Single Gene Mutations

To determine whether the *HAP4* activating mutants contained a single gene mutation, several mutants were crossed to a wild-type strain and diploids were sporulated and dissected by other people in the lab. Data in Table 1 and Figure 24 show that the tetrads have two-to-two segregation of high:low *HAP4p-lacZ* expression, indicating that all of the four mutant yeast strains tested contain a single gene mutation that confers high *HAP4p-lacZ* activity.

A

DCY122-8	Tetrad 1				Tetrad 2			
	A	B	C	D	A	B	C	D
Minus histidine	+	+	-	-	-	+	+	-
Minus methionine	+	-	+	-	-	+	+	-
Minus lysine	+	-	+	-	+	-	-	+
All	+	+	+	+	+	+	+	+
Size on YNBcas5%D	homogenous							
Other note	Flo	Flo			Flo		Flo	
Hap4-lacZ activity	↑	↑	N	N	↑	N	↑	N

B

DCY122-27	Tetrad 1				Tetrad 2			
	A	B	C	D	A	B	C	D
Minus histidine	-	+	-	+	+	-	+	-
Minus methionine	-	+	+	-	+	-	+	-
Minus lysine	-	-	+	+	-	+	-	+
All	+	+	+	+	+	+	+	+
Size on YNBcas5%D	s	b	s	b	b	s	s	b
Other note	Flo		Flo			Flo	Flo	
Hap4-lacZ activity	↑	N	↑	N	N	↑	↑	N

C

DCY125-1	Tetrad 1				Tetrad 2			
	A	B	C	D	A	B	C	D
Minus histidine	-	+	+	-	+	-	-	+
Minus methionine	-	+	+	-	+	-	-	+
Minus lysine	-	+	-	+	-	+	+	-
All	+	+	+	+	+	+	+	+
Size on YNBcas5%D	s	b	s	b	b	b	s	s
Other note								
Hap4-lacZ activity	↑	N	↑	N	N	N	↑	↑

D

DCY125-13	Tetrad 1				Tetrad 2			
	A	B	C	D	A	B	C	D
Minus histidine	-	-	+	+	+	-	+	_
Minus methionine	+	-	+	-	+	-	-	+
Minus lysine	-	-	+	+	-	-	+	+
All	+	+	+	+	+	+	+	+
Size on YNBcas5%D	s	b	b	s	s	s	s	b
Other note	Flo			Flo	Flo	Flo		
Hap4-lacZ activity	↑	N	N	↑	↑	↑	N	N

Table 1: Constitutive *HAP4*-activating mutants due to a single gene mutation
Phenotypic characterization of tetrads resultant from diploids formed from crossings of (A) DCY122-8 x WT; (B) DCY122-27 x WT; (C) DCY125-1 x WT; and (D) DCY125-13 x WT. From each diploid, a tetrad is labeled A-D in the table. Each dissected tetrad was assayed for auxotrophic markers (growth on media lacking histidine, methionine, lysine, or none). The size of resultant ascospores grown on YNBcas5%D plates was scored if possible. Some dissected tetrads resulted in flocculence (indicated by Flo). β-galactosidase activity was either normal (N) or high (↑) in YNBcas5%D medium (see Figure 24).

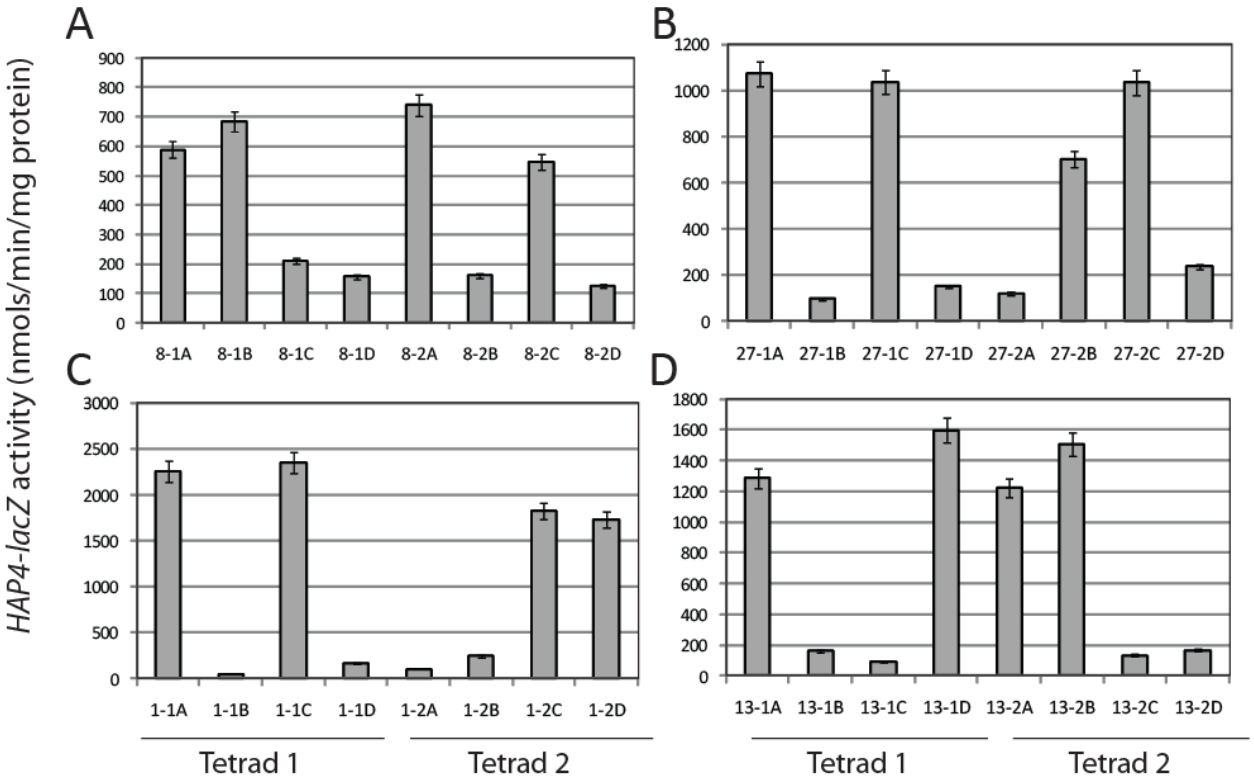

Figure 24: β-galactosidase assays of tetrads dissected from diploids formed between *HAP4* activating mutant and a wild-type strain

HAP4p-lacZ activity of two tetrads each from diploids formed from crossings of (A) DCY122-8 x WT; (B) DCY122-27 x WT; (C) DCY125-1 x WT; and (D) DCY125-13 x WT. Indication of the particular mutant is noted in the first number on the bar labeled (8, 27, 1, or 13). Each tetrad exhibits a two-to-two segregation of high:normal *HAP4p-lacZ* expression.

Library Complementation of Three *HAP4* Activating Mutants

To clone the mutated genes responsible for high levels of *HAP4p-lacZ* expression in glucose-grown cells, we first introduced a genomic copy of the *HAP4p-lacZ* reporter gene into three of the mutants. The mutant strains were then transformed with YCp50, a *CEN URA3* plasmid encoding a genomic DNA library created by Liu, Spirek and others (2005). Ura^+ transformants were replica-plated (via sterile velvet) to YNBcasD plates containing X-gal so that *HAP4p-lacZ* expression in individual colonies could be monitored. If a library plasmid complemented the mutation, the colony would be light blue or white on X-gal plates.

Identification of Constitutive Activating Mutants

After replication, seven light-blue/white colonies from three different mutant strains were obtained and confirmed to have a complementing plasmid. We found three light blue transformants from DCY122-8 (rho^+); DCY122-18 (rho^+) also produced three light blue transformants; 170-24 (rho^0) produced one. Figure 25A shows that after losing the library plasmid, mutant 122-8 and 170-24 had high levels of *HAP4p-lacZ* expression. We then recovered the YCp50 library plasmids from these seven light blue transformants. The recovered plasmids were amplified in *E. coli*, and after recovery from bacteria, they were digested with SalI, EcoRI, ClaI, and SphI restriction endonucleases to create DNA fingerprints (Figure 26). Each of the plasmids recovered from DCY122-8 and DCY170-24 showed overlapping fragments as seen by their similar fingerprint; bands spanned most plasmids at ~8 Kb, ~5 Kb, 1.5 Kb, ~0.7 Kb, and 0.5 Kb. Library plasmids recovered from the DCY122-18 transformants only showed two bands, indicating that the genomic DNA inserts of the library plasmid did not contain the

above-mentioned restriction enzyme sites (data not shown). These plasmids were sent for sequencing using primers flanking the genomic DNA insert site (Table 2).

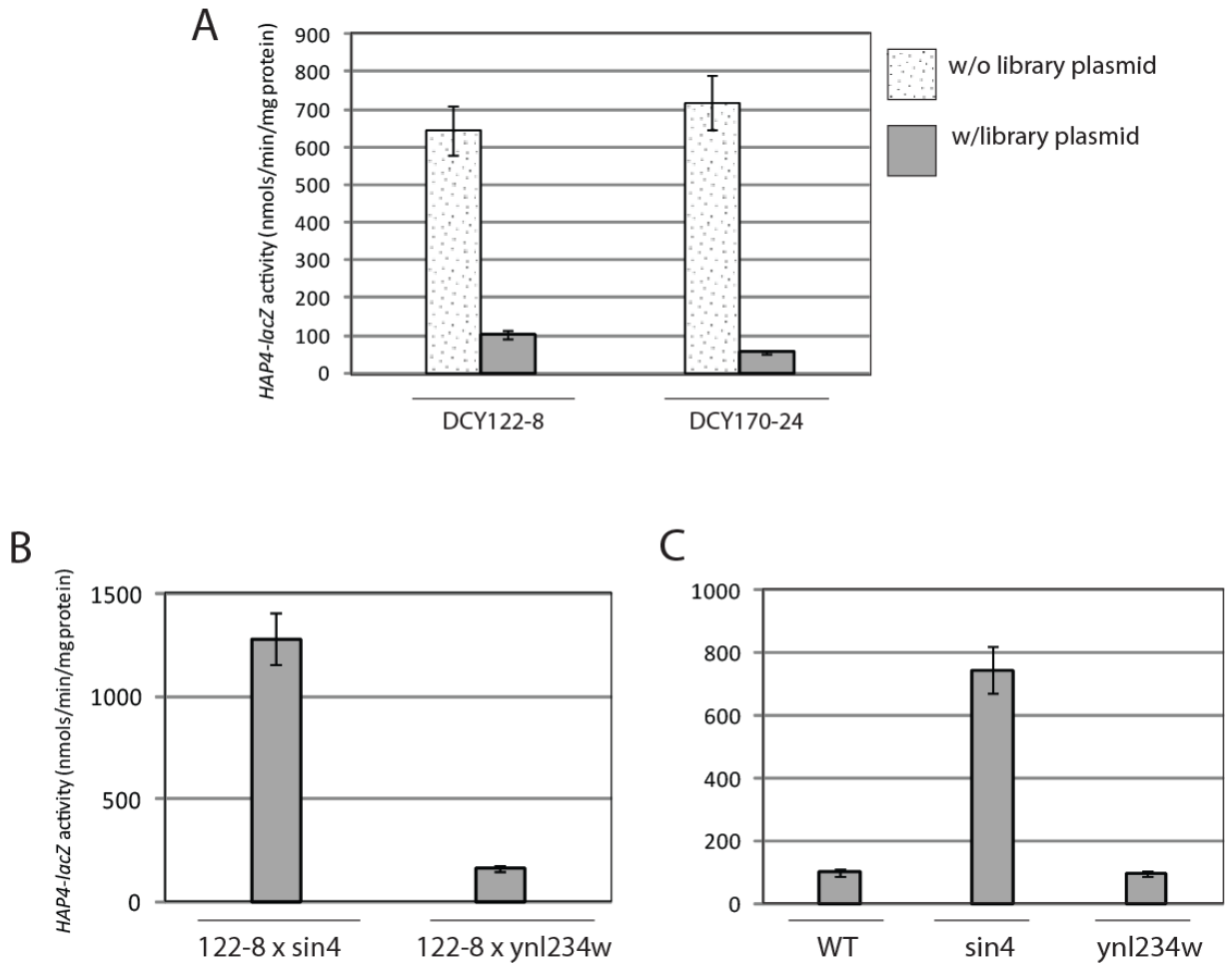

Figure 25: DCY122-8 and DCY170-24 mutant phenotypes are due to mutations in *SIN4*
(A) *HAP4p-lacZ* activity in DCY122-8 and DCY170-24 mutants without (white bars) or with (grey bars) the library plasmid. (B) Crossing of mutant DCY122-8 to *sin4*Δ or *ynl234w*Δ mutants reveal that *SIN4* is likely to be mutated in DCY122-8. (C) A *sin4*Δ mutation increases *HAP4p-lacZ* expression.

Sequencing results showed that plasmids recovered from 122-8 and 170-24 had an overlapping DNA sequence encoding two genes, *SIN4* and *YNL234w*. *SIN4* encodes a subunit of the Srb/Mediator complex (Li, Bjorklund et al. 1995). *YNL234w* encodes an uncharacterized globin-like protein that has a heme-binding domain (Sartori, Aldegheri et al. 1999). To determine which gene was responsible for complementing the mutant phenotype in DCY122-8 and DCY170-24 mutants, the DCY122-8 mutant was crossed to either a *sin4*Δ mutant or a *ynl234w*Δ mutant, and diploids were analyzed for β-galactosidase activity. Figure 25B shows that the diploid between the DCY122-8 mutant and the *sin4*Δ mutant showed high levels of *HAP4p-lacZ* expression, suggesting that DCY122-8 mutant is due to a mutation in *SIN4*. Finally, *sin4*Δ and *ynl234w*Δ mutant strains (from the yeast deletion project) were transformed with pWCJ-HAP4p-lacZ and transformants were assayed for β-galactosidase activity. Figure 25C shows that a *sin4*Δ mutation increases *HAP4p-lacZ* expression. Together, these data indicate that high levels of *HAP4p-lacZ* expression in DCY122-8 and DCY170-24 mutants are due to mutations in the *SIN4* gene.

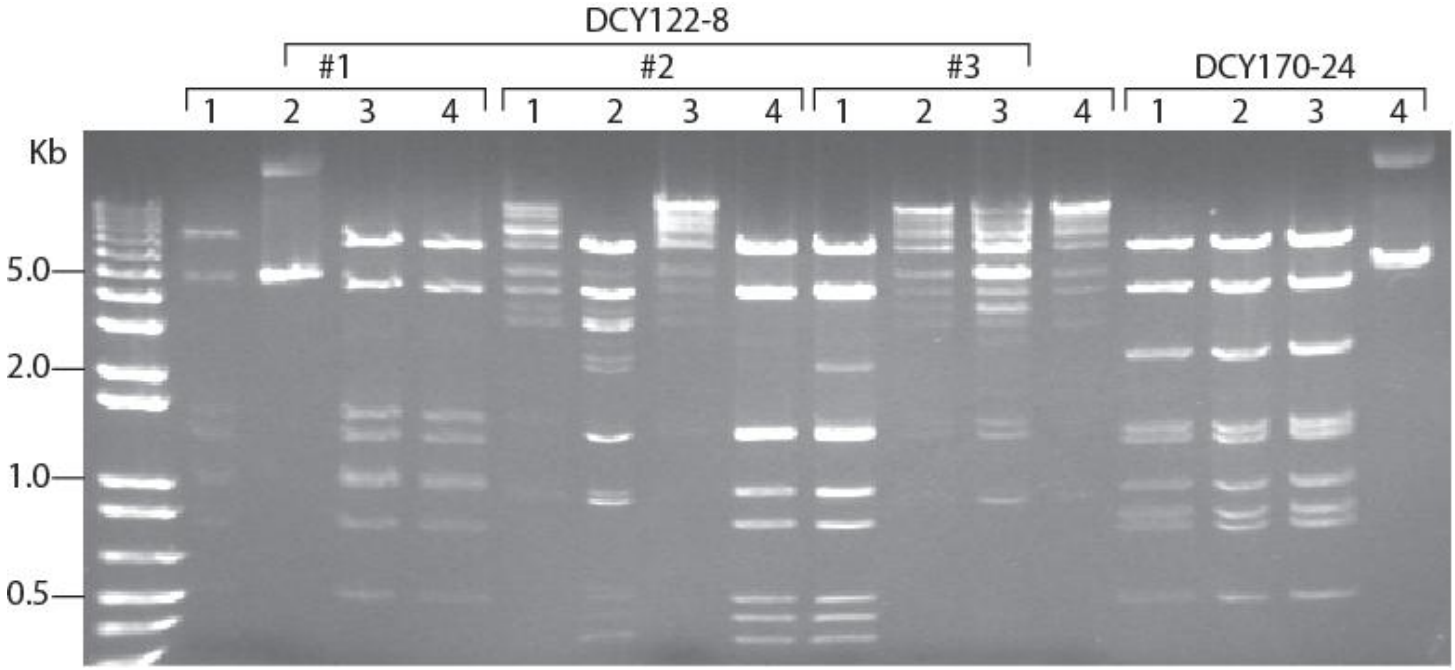

Figure 26: DNA fingerprint of digested library plasmids reveals overlapping sequences
Post-digestion pattern of library plasmids recovered from mutant yeast strains DCY122-8 and DCY170-24. Library transformations of DCY122-8 yielded three light-blue/white complementary colonies and are indicated as #1, #2, #3; DCY170-24 yielded one light-blue colony. For each colony, four individual library plasmids recovered into bacteria and are indicated as 1, 2, 3, and 4.

Library plasmids recovered from DCY122-18 mutants had an overlapping DNA sequence encoding three ORFs: *CYC8*, *SUS1* and *YSA1*. To determine whether *CYC8* was the gene mutated in 122-18, DCY122-18 (which contained an integrative *HAP4p-lacZ* gene) was transformed with a plasmid encoding HA-tagged *CYC8* (Liu lab stock) and assayed for β-galactosidase activity. This brought the *HAP4p-lacZ* activity from ~1600 to ~370 nmols/min/mg protein (Figure 27). To clone *CYC8*, I used restriction endonucleases XbaI and BamHI to isolate a DNA fragment encoding only the *CYC8* gene from one of the recovered library plasmids and inserted the DNA fragment into the plasmid pR416, transformed *E. coli*, and selected positive transformants using blue/white screening. Curiously, positive clones were found to be blue transformants. I then transformed mutant DCY122-18 with the resultant plasmid, pRS416-CYC8, and assayed transformants for *HAP4p-lacZ* expression. Figure 27 shows that the *CYC8* plasmid reduced *HAP4p-lacZ* expression in 122-18 mutant similar to the original complementing plasmid, suggesting that a *CYC8* mutation is responsible for the *HAP4p-lacZ* expression in DCY122-18 mutant cells.

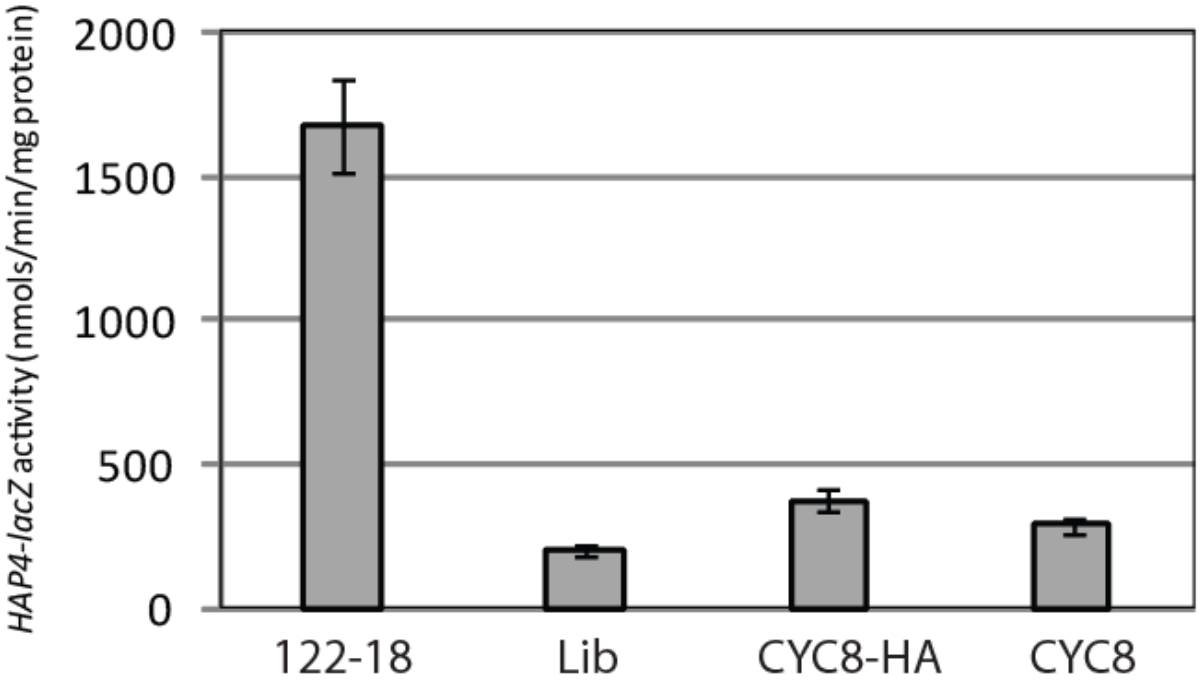

Figure 27: The DCY122-18 mutant phenotype is due to a mutation in *CYC8*
β-galactosidase activity of an integrative *HAP4p-lacZ* reporter gene in DCY122-8, with one of the original library plasmids (Lib), a plasmid encoding CYC8-HA or *CYC8*.

DISCUSSION

In the current literature, little is known about *HAP4* regulation. It has been well documented that *HAP4* transcription is dependent on carbon sources (Forsburg and Guarente 1989; McNabb, Xing et al. 1995; Brons, De Jong et al. 2002; Bourges, Mucchielli et al. 2009). *HXK2,* encoding a protein involved in the first step in glucose metabolism, has also been implicated as a negative regulator of *HAP4* expression (Lascaris, Piwowarski et al. 2004). However, for both of these modes of regulation, the mechanisms remain to be established. Each subunit of the Hap2-5 complex has homologs in other eukaryotes, with Hap2/3/5 having homologs higher organisms including humans. In yeast, the Hap2-5 complex is a global regulator of mitochondrial respiratory functions and Hap4 is the regulatory subunit. Thus, it is important to determine by what means *HAP4* is regulated. This report has detailed the transcriptional and post-translational regulation of *HAP4*. Also, two genes that mediate *HAP4* expression have been discovered.

One aspect of transcriptional regulation of *HAP4* stems from two short upstream open reading frames in the 5' leader of *HAP4* mRNA. Before this study, there have not been any detailed reports focused solely on the activity of *HAP4* uORFs, although several genome-wide studies has identified them as potentially active (Fondrat and Kalogeropoulos 1996; Zhang and Dietrich 2005; Cvijovic, Dalevi et al. 2007). This study has found that the *HAP4* uORFs are active. Also, through mutational analysis, I found that *HAP4* uORFs negatively regulate the downstream main ORF and their effects are additive. The uORFs appear to act by regulating the

stability of *HAP4* transcripts (data not shown, in collaboration with Dr. Mary Clancy). uORFs are becoming increasingly known as an additional layer of genetic modulation that should be taken into account when understanding the genetic landscape of the cell. Although studies regarding *GCN4* uORFs have provided a detailed view on the mechanism by which uORFs can regulate the expression of the downstream main ORF, our experiments designed to uncover how *HAP4* uORFs act have not yielded positive results. However, the negative data presented herein showed that decreased abundance of eIF-2-GTP-Met-tRNAi$_i^{Met}$ ternary complexes via Gcn2 activation does not seem to play a role in uORF regulation of *HAP4*, in contrast to what is seen in *GCN4*. Results in this report also indicate that *HAP4* uORFs do not function via the NMD pathway. Future work will be directed at uncovering the mechanism of *HAP4* uORF's inhibitory effect.

The manipulations of *HAP4* expression and growth conditions have resulted in a graded range of Hap4 levels in the cell. We overexpressed *HAP4* by using two heterologous promoters, *GPD* and *TEF2*, and we found that *HAP4* overexpression only partially abolishes the carbon catabolite repression of *HAP4*. Western analysis revealed that there are equal Hap4 protein levels in glucose- versus raffinose-grown *rho*$^+$ cells when *HAP4* is overexpressed under the control of the *GPD* or *TEF2* promoter. However, Hap2-5 target gene *KGD1* was still differentially expressed in dextrose- versus raffinose-grown cells, which suggests that *HAP4* derepression alone is not sufficient to achieve full activity of the Hap2-5 complex. These findings suggest that other mechanisms exist to regulate Hap2-5 activity.

Throughout the course of my research, results from multiple experiments showed the effect of loss of mtDNA on *HAP4* expression. Several experiments showed that, despite increased Hap4 production due to deletion of *HAP4* uORFs or *HAP4* overexpression under the control of strong, heterologous promoters, *KGD1-lacZ* expression cannot be fully activated in *rho*^{0} cells. This led to the identification that Hap4 protein has a shorter half life in *rho*^{0} cells. In raffinose-grown cells, Hap4 is degraded 2.4 times faster when there is a loss of mtDNA ($t_{1/2}$ in ρ^{+} cells = 6.9 min; $t_{1/2}$ ρ^{0} = 2.9 min). Even in glucose-grown cells, a condition where the cell has little need for mitochondrial respiratory functions, the half-life of Hap4 in *rho*$^{+}$ and *rho*^{0} cells is different ($t_{1/2}$ in *rho*$^{+}$ cells = 5.7 min; $t_{1/2}$ *rho*^{0} cells = 3.8 min). This led us to try to determine how Hap4 is degraded. The cell can degrade proteins in a variety of ways, for example, through the lytic enzymes in the vacuole and ubiquitin/proteasome system in the cytoplasm (Varshavsky 2005). It has been proposed that Hap4 is degraded in the vacuole (Bourges, Mucchielli et al. 2009). However, it was demonstrated in this report that Hap4 degradation is clearly proteasome-dependent and thus Hap4 degradation would occur, at least, in the cytoplasm. To further characterize how Hap4 is degraded, Hap4 stability was assayed in a variety of *ubc*Δ mutants and we found that several *ubc*Δ mutations increased Hap4 stability (data not shown). Despite that the route of Hap4 ubiquitination is still unclear, it is evident that such a high turnover rate of Hap4 may help the cell to adapt to the fast-changing internal and external environments.

It was hypothesized that when the cell switches from a fermentative to a respiring state, Hap4 is shuttled from the cytoplasm to the nucleus. The localization data presented herein support this hypothesis: Hap4-GFP largely localizes to the cytoplasm in glucose-grown cells, and in the nucleus when cells are grown in a derepressing carbon source. In this study, Hap4 was

expressed as a single copy on a centromeric plasmid and the GFP signal was apparent, contrary to results reported previously (Bourges, Mucchielli et al. 2009). By overexpressing Hap4 under the control of a strong, heterologous promoter, I also found that Hap4-GFP localizes to the nucleus, even under repressing conditions. However, nuclear localization of Hap4 still cannot completely derepress the Hap2-5 target gene *KGD1*, which further supports the idea that there are other mechanisms within the cell that regulate Hap2-5.

Through mutational analysis followed by library complementation, we have found that *SIN4* and *CYC8* are negative regulators of *HAP4* expression. The *CYC8* (*SSN6*) gene product is a general transcriptional co-repressor that acts together with Tup1. This complex has been implicated in many pathways including the Mig1-Hxk2 and the Snf1-Rgt2 glucose sensing pathways (Gancedo 2008). A mutation in *CYC8* derepresses the expression of *HXT* genes, encoding hexose transporters (Ozcan and Johnston 1995). Thus, glucose transporters are expressed, which would allow more glucose to be imported into *cyc8* mutant cells. By this argument, *HAP4* expression should be even more derepressed. However, *HAP4* expression shows constitutive derepression in the *cyc8* mutant. It remains to be determined how Cyc8 represses *HAP4* expression.

The *SIN4* gene product is a subunit of the Mediator complex and contributes to both positive and negative transcriptional regulation (Li, Bjorklund et al. 1995). Recently Med8, a component of the Mediator complex, has been implicated in the carbon catabolite repression pathway (Palomino, Herrero et al. 2005). It is likely that Hxk2 (with Mig1, Cyc8, and Tup1) and Med8 bind to the promoters of target genes to repress transcription (Gancedo 2008). Although

the exact link between the Mediator complex and *HAP4* regulation is currently unknown, our data nonetheless provides novel insights into the mechanism of *HAP4* regulation. The identification of *SIN4* and *CYC8* as negative regulators of *HAP4* expression has increased our understanding of how the Hap2-5 complex is regulated both by carbon sources and mitochondrial functional state. From what started as an investigation of peculiar upstream open reading frames, the natural progress of research has led to the unfolding of multiple mechanisms of Hap2-5 regulation.

FUTURE WORK

The recent identification of *CYC8* and *SIN4* as negative regulators of *HAP4* is interesting. However, their discovery arrived late in my academic career so I have not had the opportunity to carefully characterize them in relation to *HAP4* regulation. As such, a great deal of further experimentation is needed to better understand how *CYC8* and *SIN4* regulate *HAP4* expression. One experiment that I believe to be important for their characterization is to use co-immunoprecipitation to determine whether there are direct interactions between Cyc8/Sin4 and Hap4, since Hap4 expression is auto-regulated (data not shown). It would also be important to perform Chromatin Immuniprecipitation (ChIP) analysis to address whether Cyc8 or Sin4 interact with regions of DNA which correspond to the *HAP4* gene. Other experiments to perform should assess the *HAP4p-lacZ* and *KGD1-lacZ* expression in all 14 functionally viable Mediator complex mutants (this research is currently being done in the lab).

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APPENDIX

Table 2: Primers used in this study

Primer Name	Sequence	Use/Strategy
Amplification of uORFs for cloning into plasmid already encoding *lacZ*		
HAP4.5'.-1800.XbaI	GTCATCTAGATTGTCGTACCAAGAGAGGCA	WT *HAP4* promoter
HAP4.3'.19.HindIII	GTCAAAGCTTGAAAAGTCTTTGCGGTCATG	
HAP4.5'.-1800.XbaI	GTCATCTAGATTGTCGTACCAAGAGAGGCA	uORF1
HAP4.3'.-223.HindIII	GTCAAAGCTTGAGTGTAAAGTGGACCTGTAG	
HAP4.5'.-1800.XbaI	GTCATCTAGATTGTCGTACCAAGAGAGGCA	uORF2 (includes uORF1)
HAP4.3'.-53.HindIII	GTCAAAGCTTGGCAACCCATTAAAATGCTCT	
Creation of uORF mutants with *lacZ*		
HAP4.5'.-1800.XbaI	GTCATCTAGATTGTCGTACCAAGAGAGGCA	uORF1(m) left fragment (L)
HAP4.3'.uOR1	AAGTGGACCTGTAGATAGTATATACGATATA AAACTGGG	
HAP4.5'.uOR1	CCCCAGTTTTATATCGTATATACTATCTACAG GTCCACTT	uORF1(m) right fragment (R)
lacZ.3'.236	TCAGGAAGATCGCACTCCAG	
HAP4.5'.-1800.XbaI	GTCATCTAGATTGTCGTACCAAGAGAGGCA	uORF2(m) left fragment (L)
HAP4.3'.uOR2	GGTAAAACAAATCAGCAACCTATTAAAATGC TCTTTGATGT	
HAP4.5'.uOR2	GTACATCAAAGAGCATTTTAATAGGTTGCTG ATTTGTTTTACCC	uORF1(m) right fragment (R)
lacZ.3'.236	TCAGGAAGATCGCACTCCAG	
HAP4.5'.-1800.XbaI	GTCATCTAGATTGTCGTACCAAGAGAGGCA	After 8 cycles of amplification of pure L & R fragments, these outer flanking primers were added to create full-length uORF mutants
lacZ.3'.236	TCAGGAAGATCGCACTCCAG	
HAP4.5'.uOR1	CCCCAGTTTTATATCGTATATACTATCTACAG GTCCACTT	uORF1,2(m) R. Used plasmid based uORF2(m) as the template. After purification of uORF1,2(m)R fragment, it was amplified with uORF1(m)L to create full-length uORF1,2(m)
lacZ.3'.236	TCAGGAAGATCGCACTCCAG	

Creation of full-length Hap4-GFP or Hap4-HA with or without uORF mutations		
HAP4.5'.-1.BamHI.	GTCAGGATCCATGACCGCAAAGACTTTTC	To amplify full *HAP4* ORF. Was cloned with one of the *HAP4* promoter constructs, made as indicated above, into vector already encoding 3xHA. WT *HAP4* was cloned into a vector already encoding GFP. When necessary, the WT *HAP4* promoter was swapped with the *TEF2 or GPD* promoter already in the lab.
HAP4.3'.-S.XhoI	GTCACTCGAGAAATACTTGTACCTTTAAAAAATCGAC	
Creation of *hap4*Δ mutants		
HAP4.5'.-360	TTTCTTCTTGCTTAACCCCG	Template: hap4::kanMX4 gDNA
HAP4.3'.+268.XhoI	GTCACTCGAGAATGGCCTTCCAGACCCATA	
Creation of *KGD1-lacZ* reporter		
KGD1.5'.-696.BamHI	AGAGGGATCCTGGGTTTACG	Amplified *KGD1* promoter plus a few codons and cloned into a plasmid already encoding *lacZ.*
KGD1.3'.20.SphI	GTCAGCATGCCGAACCTTAGCATAACGGTA	
Creation of *KGD1-lacZ* reporter		
YCp50 R	TAGGCGCCAGCAACCGC	To sequence gene encoded by library plasmid YCp50 used in library complementation experiments.
YCp50 F	GCCGCCGCCCAGTCCTG	

Table 3: Plasmids used in this study

Plasmid Description	Source
pWCJ-HAP4p-lacZ	This Study
pWCJ-HAP4uORF1-lacZ	
pWCJ-HAP4uORF2-lacZ	
pWCJ-HAP4uORF1m-lacZ	
pWCJ-HAP4uORF2m-lacZ	
pWCJ-HAP4uORF1,2m-lacZ	
pWCJ-KGD1-lacZ	
pRS416-HAP4uORF1m-HAP4-HA, expressing C-terminal 3x HA tagged Hap4	
pRS416-HAP4uORF2m-HAP4-HA, expressing C-terminal 3x HA tagged Hap4	
pRS416-HAP4uORF1,2m-HAP4-HA, expressing C-terminal 3x HA tagged Hap4	
pRS416-HAP4p-HAP4-HA, expressing C-terminal 3x HA tagged Hap4	
pRS416-GPDp-HAP4-HA, over-expressing C-terminal 3x HA tagged Hap4	
pRS416-TEF2p-HAP4-HA, over-expressing C-terminal 3x HA tagged Hap4	
pRS416-HAP4p-HAP4-GFP, expressing C-terminal GFP-tagged Hap4	
pRS416-TEF2p-HAP4-GFP, over-expressing C-terminal GFP-tagged Hap4	
pGAL-HO	(Jensen and Herskowitz 1984)
YCp50	(Liu, Spirek et al. 2005)

Table 4: Yeast strains used in this study

Strain	Genotype	Source/Reference
BY4741	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15*	(Liu and Butow 1999)
PSY142	MATα *ura3-52, leu2-Δ1, lys2-201*	
ZLY1516	MATα *ura3-52, lys2-201, erg6::kanMX4*	(Tumusiime, Zhang et al.)
ZLY2811	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 hap4*Δ::LYS2	(Liu and Butow 1999)
ZLY3462	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 fum1*Δ	
ZLY3463	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 mdh1*	
ZLY3464	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 sdh1*	
ZLY3465	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 lsc1*Δ	
ZLY3466	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 kgd2*Δ	
ZLY3468	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 idh1*Δ	
ZLY3469	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 kgd1*	
DCY177	MATα *ura3-52, leu2-Δ1, lys2-201 rho^0*	This study
DCY178	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 rho^0*	
DCY202	MATα *ura3-52, leu2-Δ1, lys2-201 hap4::kanMX4 rho^0*	
DCY210	MATα *ura3-52, leu2-Δ1, lys2-201 iKGD1-lacZ*	
DCY214	MATα *ura3-52, leu2-Δ1, lys2-201 iKGD1-lacZ rho^0*	
DCY228	*MATα ura3-52, leu2-Δ1, lys2-201 iKGD1-lacZ hap4::kanMX4*	
DCY230	MATα *ura3-52, leu2-Δ1, lys2-201 iKGD1-lacZ hap4::kanMX4 rho^0*	
DCY237	MATa *ura3-52, leu2-Δ1, his3-Δ1, met15 hap4::kanMX4 rho^0*	
DCY242	MATα *ura3-52, leu2-Δ1, lys2-201 iKGD1-lacZ ura3::kanMX4-2 rho^0*	

DCY247	MATα *ura3-52, leu2-Δ1, lys2-201 iKGD1-lacZ ura3::kanMX4-2* hap4::LYS2-7	
DCY271	MATα *ura3-52, leu2-Δ1, lys2-201 iKGD1-lacZ ura3::kanMX4-2* hap4::LYS2-7 *rho*^{0}	
DCY320	Tester A	(Liu, Spirek et al. 2005)
DCY321	Tester alpha	

VITA

Denise Capps, daughter of Michele B. Allen, LCSW and Stephen J. Capps, MD, was born on October 27, 1983 in Juneau, Alaska. She attended Archbishop Blenk High School in Gretna, LA and graduated in 2001. In 2009, she received her B.S. in Psychology, with minors in Biology and Chemistry, from University of New Orleans. In 2009, she was admitted into the Graduate School in the Department of Biological Sciences at University of New Orleans. She joined the research group of Dr. Zhengchang Liu to pursue a M.S. degree. She currently lives in the Garden District of New Orleans, Louisiana. In her spare time, she likes to garden and cook.