A Study on the Regulation of Amino Acids and Glucose Sensing Pathways in Saccharomyces cerevisiae

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A Study on the Regulation of Amino Acids and Glucose Sensing Pathways 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

Mengying Chiang
B.S. University of New Orleans, 2010

August, 2013
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Challenges in graduate school are not what I had ever experienced, especially in research. 90 percent frustration and 10 percent exciting results made up my experience in research, but this 10 percent joy is the motivation to keep me going. However, I could not ever imagine accomplishing what I have achieved in this thesis without all the amazing support I received from the lab, my family, and my friends.

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ABSTRACT

Nutrient availability regulates eukaryotic cell growth. This study focuses on two signaling pathways involved in sensing amino acids and carbon sources, which allow cells to respond appropriately to their presence. The first part of this study shows that Ssy1, a plasma membrane localized sensor in the Ssy1-Ptr3-Ssy5 (SPS) amino acid sensing pathway, can detect 19 common L-amino acids with different potencies and affinities based on the physiochemical structure of amino acids. Substituents around alpha carbon are critical for amino acid sensing by Ssy1. Furthermore, a high concentration of cysteine is toxic to cells. Inactivation of SPS signaling confers resistance to cysteine. The second part focuses on the regulation of Hap4, the regulatory subunit of the Hap2/3/4/5 transcriptional factor complex. Many components of the 25-subunit Mediator complex negatively regulate HAP4 expression. Srb8 undergoes post-translational modification in response to changes of the carbon source, Gal11 and Med3 positively regulate HAP4 expression.

Keywords: SPS, Ssy1, Amino Acid Sensing, Cysteine Toxicity, Hap4, Saccharomyces cerevisiae, the Mediator complex, Srb8, Gal11, Med3
CHAPTER 1: GENERAL INTRODUCTION

*Saccharomyces cerevisiae*, commonly known as Baker’s yeast, requires nutrients to survive. There are several different environmental nutrient sources, such as carbon sources and nitrogen sources, utilized by yeast for cellular metabolic processes. Two signal transduction pathways in yeast that sense amino acids and glucose respectively are studied here.

**The SPS Amino Acid Sensing Pathway in Yeast**

Amino acids are nutrients for cellular metabolic processes. In the presence of environmental amino acids, the Ssy1-Ptr3-Ssy5 (SPS) amino acid signaling pathway is activated and enables yeast cells to import extracellular amino acids [1-3]. Ssy1 is a homolog of functional amino acid permeases and consisted of 12 transmembrane helices and an N-terminal signaling domain (Figure 1) [4]. Amino acids binding to Ssy1 initiate the signaling pathway [1, 5]. After sensing amino acids, the N-terminal signaling domain of Ssy1 has been proposed to recruit two casein kinase I proteins, Yck1 and Yck2, resulting in hyperphosphorylation of the peripheral plasma membrane protein Ptr3 [6-8]. Ssy5, a peripheral membrane protein and a chymotrypsin-like serine protease, associates with Ptr3 to form a complex [3]. Ssy5 undergoes autocatalytic processing to generate an N-terminal prodomain and a C-terminal catalytic domain, which remain associated. When the N-terminal prodomain is phosphorylated, it is degraded through the ubiquitin/proteasome system, resulting in the activation of the C-terminal catalytic domain. The active catalytic domain removes an N-terminal inhibitory domain from two zinc finger transcription factors, Stp1 and Stp2, resulting in their activation [6, 9, 10]. Processed Stp1 and Stp2 then translocate from the cytoplasm into the nucleus to activate target gene expression (Figure 1) [11]. The yeast genome encodes about 24 amino acid permeases [12, 13]. Expression of seven amino acid
permeases, Bap2, Bap3, Agp1, Gnp1, Tat1, Tat2, and Mup1, is induced by extracellular amino acids through the activation of the SPS signaling pathway [1, 4, 14-16]. Expression of these amino acid permeases on the plasma membrane allows amino acid uptake.

Other proteins are also involved in SPS signaling. The effect of Yck1/2 can be reversed by the Rts1-containing subcomplex of the trimeric protein phosphatase type 2A [7]. Grr1 is the F box-containing component of the SCF Grr1 E3 ubiquitin ligase complex, which is required for degradation of the N-terminal prodomain of Ssy5 and activation of the C-terminal catalytic domain of Ssy5 [4, 7]. Dal81 is a pleiotropic transcription factor that is essential for full activation of Stp1/2-target gene expression [17]. Shr3, an amino acid permease-specific packaging chaperone in the membrane of the endoplasmic reticulum (ER), regulates the SPS signaling pathway by mediating ER exit of amino acid permeases and possibly Ssy1 [3, 18].

Previously, amino acid sensing by Ssy1 was studied using fixed amino acid concentrations [4, 19, 20]. It is unclear as to the relative affinity and potency of 20 amino acids on Ssy1. In this study, I aim to address this question.
Figure 1: Illustration of Ssy1-Ptr3-Ssy5 amino acid sensing pathway

Hap2/3/4/5 Complex

The second signal transduction pathway addressed in this study is related to mitochondrial biogenesis. Mitochondria are the cellular power plants where ATP is produced. Mitochondrial biogenesis in S. cerevisiae is regulated in part by the CCAAT-binding factors, the Hap2/3/4/5 complex. Eukaryotic messenger RNA synthesis in response to changing environments can be initiated by the binding of RNA Polymerase II, along with general transcription factors (GTFs such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), to the promoter sequences of target genes. Transcription factors are sequence-specific DNA-binding proteins that are needed for transcriptional regulation [21]. CCAAT-binding proteins are transcriptional activators that promote expression of
target genes by interacting with CCAAT sequence motifs in target gene promoters [22]. *S. cerevisiae* can grow in either fermentative (glucose) or nonfermentative (ethanol, glycerol, lactate, etc.) carbon sources. In cells grown in the presence of a nonfermentative carbon source such as ethanol, the Hap2/3/4/5 complex activates expression of genes required for respiratory metabolism [23-28]. It has been reported that Hap2, Hap3, and Hap5 are constitutively expressed and form a complex at the promoters of target genes. Hap4 expression is at basal levels in cells grown in glucose medium, and its expression is greatly induced in cells grown in non-glucose medium [29].

Hap4 is a protein of 554 amino acids. It contains two transcriptional activation domains: one is a Gcn5-independent at the C-terminus between amino acid residues 359 and 476; the other is Gcn5-dependent at the N-terminus between amino acid residues 124 and 329 [30]. While Hap2/3/5 heterotrimer binds to targeted DNA, Hap4 is the main regulatory subunit of Hap complex by binding and activating the entire complex, and its protein level determines the activity of this complex.

**Mediator Complex**

The Mediator complex is a 25-protein complex that plays important roles in gene expression [31-33]. Its interaction with both transcription factors and RNA Polymerase II can increase gene expression [32]. The Mediator complex is conserved and exists in all eukaryotic organisms [34, 35]. The Mediator complex can be divided into four groups: head, middle, tail, and kinase modules (Figure 2) [36, 37]. The head module consists of eight subunits, Med6, Med8, Med11, Srb4/Med17, Srb5/Med18, Rox3/Med19, Srb2/Med20 and Srb6/Med22 [38]. Electron microscopy shows that the Mediator head module closely associates with the Rpb3/Rpb11 heterodimer of RNA Pol II, the Rpb4/Rpb7 subunits, and TBP [39, 40]. The subcomplex Med18/20 is responsible for
conformational changes of the head module, which allows the interaction between the Mediator complex and RNA Pol II [40].

The middle module, which includes eight subunits, Med1, Med4, Nut1/Med5, Med7, Cse2/Med9, Nut2/Med10, Srb7/Med21 and Soh1/Med31, is elongated and flexible and contributes to one third of the length of the entire mediator complex[41]. The Med7/Med21 interface contains a four-helix bundle domain and a coiled-coil protrusion connected by a flexible hinge, and Med 6 is the bridge connecting the head and middle modules[42]. Med6, Med7, and Med21 are responsible for the mediator complex conformational changes at the interface of the head and middle modules upon binding of RNA Pol II.

The tail module contains five subunits, Med2, Pgd1/Med3, Rgr1/Med14, Gal11/Med15 and Sin4/Med16 [43]. Two-hybrid and glutathione S-transferase interaction assays showed that the Med3 subunit negatively regulates transcription by associating directly with the Cyc8-Tup1 transcription co-repressor [44]. It was shown that Med2, Med3, and Gal11 form a triad that can be recruited to transcription activator Gcn4p independently without interfering with Sin4 [45].

The kinase module is composed of four subunits, Med12/Srb8, Med13/Srb9, Srb11 and Cdk8/Srb10 [46, 47]. In an early study, it was suggested that Srb10 repressed activation of genes that enable cells to survive under nutrient deprivation, and Srb10 expression is greatly reduced when cells enter the diauxic shift [48]. By observation of strong defects in Gal4-activated transcription in an srb9 mutation strains, it was shown that the kinase module, Srb8-Srb11, is directly involved in GAL1 transcription. The binding of Srb9 at the GAL1 gene promoter region induces the recruitment of TBP [49].
HAP4 expression is increased approximately ten-fold when yeast cells are grown in raffinose medium compared to dextrose media. Despite the importance of mitochondria in cellular metabolism and the essential role of Hap4 in mitochondrial biogenesis, the regulatory mechanism of HAP4 expression is still unclear. The Mediator complex component Sin4 and Cyc8, a general transcriptional co-repressor, have been shown in our lab to be negative regulators of HAP4 expression. However, how the Mediator complex regulates HAP4 expression is still unknown.

Figure 2: A structure model of the Mediator complex
Research Aims

The Ssy1 amino acid sensor of the SPS signaling pathway is a functional homolog of amino acid permeases. Ssy1 has been shown to have a broad specificity for amino acids [4]. However, there has not been a systematic study done to address the binding affinity of activity of individual amino acids on Ssy1. I hope to address the issue in this study. In the glucose sensing pathway, the expression of \textit{HAP4} is not well understood. I aim to address the role of individual Mediator complex components on \textit{HAP4} expression.
CHAPTER 2: AMINO ACID STRUCTURE DIFFERENTIALLY AFFECTS THE SSY1-PTR3-SSY5 AMINO ACID SENSING PATHWAY

Introduction

The Ssy1-Ptr3-Ssy5 (SPS) amino acid signaling pathway in *S. cerevisiae* responds to and induces the uptake of extracellular amino acids [1-3]. When amino acids bind to the Ssy1 sensor on the plasma membrane, Yck1/2 are recruited and phosphorylates Ptr3 and Ssy5, leading to activation of the C-terminal catalytic domain of Ssy5. Activation of Ssy5 enables processing of Stp1 and Stp2 and their subsequent translocation into the nucleus to activate target gene expression [1, 3, 6-9, 11]. There have been no systematic amino acid titration studies performed to determine the affinity and potency of individual amino acids on Ssy1. There has also been no careful analysis of whether the physical characteristics of amino acids affect Ssy1 sensing. In this study I subjected yeast cells to titrations of individual amino acids and determined the activity of the SPS amino acid signaling pathway using an AGP-lacZ reporter gene. Both L- and D-isoforms of the amino acids were used as well as structural isoforms that have alterations on substituents around the alpha carbon in an effort to characterize the effect of physiochemical differences on SPS amino acid sensing.

Materials and Methods

*Strains, Primers, Plasmids, Growth Media and Growth Conditions*

Yeast strains, primers, and plasmids used in this study are listed in Table 1, Table 2, and Table 3, respectively. Yeast cells were grown at 30°C in SD medium (0.67% yeast nitrogen base and 2% D-glucose) or YPD (2% peptone, 1% Bacto-yeast extract, and 2% D-glucose). The amino acids L-leucine, L-lysine, L-histidine, L-methionine, L-
tryptophan, and/or uracil were added to growth media to a final concentration of 30 mg/L to meet auxotrophic requirements [50]. Solid media contained 2% agar in addition to the components described above when needed.

**Yeast Transformations and β-galactosidase Activity Assays**

Yeast cells were transformed [51]. Liquid cultures were inoculated with a pool of several independent transformants and grown overnight in SD medium with specified amino acids to an optical density at 600 nm of ~0.6. The cell extracts were prepared by using glass bead lysis method. 10 – 90 mL of clear cell extract of independent samples were objected to β-galactosidase assays, hydrolyzing o-nitrophenyl-β-D-galactopyranoside (ONPG) [50]. Protein concentration of individual sample was carried out by performing Bradford assay, using bovine serum albumin as the standard. Reporter gene activity was calculated in nmoles of hydrolyzed ONPG per minute per mg of protein Assays were conducted in duplicate and independent experiments were carried out two to three times.

**Amino Acid Titration Assay**

Amino acid titration assays were done by supplementing growth media with various concentrations of indicated amino acids as listed in figures and figure legends. Cells were analyzed as described for β-galactosidase activity assays.

**EMS Mutagenesis**

Yeast cells (ZLY043) were grown overnight at 30°C in 10 mL YPD liquid media to saturation. 1 mL of the saturated culture was pelleted and subjected to EMS mutagenesis as described in Methods Enzymology [52]. After EMS mutagenesis, cysteine resistant colonies were selected for on SD + 5 mM cysteine plate media. Cysteine resistant cells were subject to confirmation by β-galactosidase assays as described above.
Mating

MATa and MATα haploid yeast cells were cross streaked and mated on YPD plate media for 4 hrs. Diploids were selected for on SD plate media supplemented with amino acids to cover auxotrophic markers.

Results

The Effects of 20 Common L-amino Acids on AGP1-lacZ Expression

It has been shown previously that, with the exception of proline, the presence of a single amino acid in the growth medium induces the expression of the lacZ gene under the control of the promoter of high-Affinity Glutamine Permease 1 (AGP1), a target gene of SPS signaling. Leucine and tryptophan were found to be strong inducers of expression of the AGP1-lacZ reporter. Ssy1-dependent AGP1-lacZ expression can also be triggered by nitrogenous compounds other than common amino acids, such as citrulline, GABA, and ornithine [14]. However previous experiments were carried out using a fixed amino acid concentration. In my study, systematic amino acid titration assays were performed in order to obtain information on the affinity and potency of individual amino acids on Ssy1.

First, I performed amino acid titration assays of 20 L-amino acids individually at eight different concentrations from 0 to 1.6 mM on the activity of an AGP1-lacZ reporter. RBY98661 strain yeast cells containing an integrated AGP1-lacZ reporter gene were grown in minimal SD medium overnight in the presence of different concentrations of individual amino acids. Cells were collected at OD<sub>600</sub> 0.6-0.8 and β-galactosidase assays were performed to measure the activity of the AGP1-lacZ reporter gene as described in Material and Method.
Overall, the results showed that \textit{AGP1-lacZ} expression was induced to various levels by 19 L-amino acids, except proline (Fig. 3). This suggests that the Ssy1 sensor has a broad substrate binding specificity; furthermore, Figure 3 shows that expression of \textit{AGP1-lacZ} gradually increases in cells grown in the presence of increasing amounts of amino acids until reaching a maximum, and its activity slowly decreases.

Figure 4 represents a single response curve selected from figure 3. It shows a typical response of \textit{AGP1-lacZ} expression in cells grown in SD medium supplemented with titrating amounts of an amino acid, in the case of phenylalanine. \textit{AGP1} expression starts at zero in the absence of phenylalanine in the growth medium, then peaks, then gradually decreases with increasing amounts of phenylalanine. The \((x, y)\) coordinate of the peak point provides two important parameters: the value on the X-axis provides information on the affinity and the value on the Y axis on the potency of phenylalanine on Ssy1. The concentration of amino acid that gives the maximum \textit{AGP1-lacZ} expression provides information on how tightly it binds to Ssy1: the lower the concentration of that amino acid when reaches its maximal activity, the higher the affinity of the Ssy1 sensor toward that amino acid. The peak value on the Y axis reveals the potency of this amino acid in activating Ssy1: the higher the value on the Y axis of the peak point, the more potent that amino acid in activating Ssy1.
Figure 3: AGP1-lacZ expression in response to titrating levels of 20 L-amino acids individually. AGP1-lacZ expression was determined in RBY98661 strain grown in minimal SD medium with titrating concentrations (0-1.6 mM) of 20 different L-amino acids individually using β-galactosidase assays.
Figure 4: The AGP1-lacZ expression curve in response to increasing levels of phenylalanine provides information of its activity and potency on Ssy1. β-galactosidase assays of AGP1-lacZ expression were conducted for RBY98661 strain cells grown in minimal SD medium with titrating concentrations (0~1.6 mM) of phenylalanine. The interception of maximal AGP1 expression at the X-axis reveals the affinity of Ssy1 for phenylalanine. The interception of maximal AGP1 expression at the Y-axis shows the potency of phenylalanine in activating Ssy1.

Amino Acids with Aromatic R Groups Have High Affinity for Ssy1 and High Potency in Activating AGP1-lacZ Expression

Based on the response curve of AGP1-lacZ expression in the presence of titrating levels of individual amino acids, Ssy1 has various binding affinities towards different amino acids (Figure 3). The potency of different amino acids, represented by the
maximum activation of *AGP1-lacZ* expression in the presence of amino acids, vary as well. Therefore, I grouped amino acids based on the physio-biochemical property of their R groups and looked separately at the binding affinity and the potency of the Ssy1 sensor to these amino acids.

Among the 20 L-amino acids, phenylalanine, tryptophan, and tyrosine possess an aromatic R group. *AGP1-lacZ* expression in the presence of very low amounts of these three amino acids (between 25 μM to 100 μM) was highly induced (between 900 to 1100 nmoles/min/mg protein) (Fig. 5) suggesting the Ssy1 amino acid sensor has a high binding affinity for amino acids with aromatic R groups and these amino acids have a high potency for activating Ssy1.
Figure 5. *AGP1-lacZ* expression in response to stimulation with amino acids with aromatic R groups.

β-galactosidase activity assays on *AGP1-lacZ* expression were conducted for RBY98661 strain cells grown in minimal SD medium with titrating concentrations (0-1.6 mM) of tyrosine, tryptophan, and phenylalanine.

**Amino Acids with Hydrophobic R Groups Have Medium Affinity and medium potency on Ssy1**

Alanine, isoleucine, leucine, methionine, valine, and proline contain hydrophobic R groups. With the exception of proline, both the binding affinity of Ssy1 towards this group of amino acids and the potency of these amino acids in activating fall into the middle range (Fig. 6). The *AGP1-lacZ* reporter gene is activated in cells by these amino acids at concentrations between 100 μM to 400 μM, and *AGP1-lacZ* expression is
induced to a level between 400 to ~700 nmoles/min/mg proteins. Consistent with previous studies, proline did not activate expression of the AGP1-lacZ reporter gene [4]. This may be due to the distinctive, rigid cyclic structure of proline’s side chain.

Figure 6: AGP1-lacZ expression in response to stimulation with amino acids with hydrophobic R groups

β-galactosidase activity assays on AGP1-lacZ expression was conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations (0-1.6 mM) of isoleucine, leucine, valine, alanine, methionine, and proline.
Amino Acids with Uncharged Hydrophilic R Groups Have Medium to Low Affinity and Potency on Ssy1

Glutamine, asparagine, cysteine, serine, threonine, and glycine contain uncharged hydrophilic R groups. AGP1-lacZ expression is only fully activated by relatively high concentrations of these amino acids (>200 µM) and AGP1-lacZ expression reaches maximum between 250 to 700 nmoles/min/mg proteins (Fig. 7). These data suggest that amino acids with uncharged hydrophilic R groups have medium to low binding affinity on Ssy1, and medium to low potency in activating Ssy1. Interestingly, expression of AGP1-lacZ did not reach its peak in the presence of 1.6 mM cysteine or glycine, the highest concentration tested in our assays. Threonine had a higher affinity and potency on Ssy1 as compared to other amino acids of this group, which is similar to amino acids with non-aromatic hydrophobic R groups. This may be due to the presence of a methyl group on the side chain of threonine.
Figure 7: AGP1-lacZ expression in response to stimulation with amino acids with uncharged hydrophilic R groups

β-galactosidase activity assays on AGP1-lacZ expression were conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations (0-1.6 mM) of threonine, asparagine, cysteine, glutamine, serine, and glycine.

Effect of Amino Acids with Charged R Groups on AGP1-lacZ Expression

Arginine, histidine and lysine contain positively charged R groups, while glutamic acid and aspartic acid contain negatively charged R groups. Figure 8 shows that in the presence of 400 µM glutamic acid, AGP1-lacZ expression reached a maximum of 600 nmoles/min/mg proteins; and in the presence of 800 µM aspartic acid, AGP1-lacZ expression reached a maximum of 350 nmoles/min/mg protein. Compared to the negatively charged amino acids, cells grown in the presence of positively charged
amino acids did not fully activate AGP1-lacZ expression at the highest concentration 1.6 mM, suggesting that the binding affinity of Ssy1 for positively charged amino acids is extremely low.

Figure 8: AGP1-lacZ expression in response to stimulation with amino acids with charged R groups

β-galactosidase activity assays of AGP1-lacZ expression were conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations (0-1.6 mM) of glutamic acid, aspartic acid, arginine, histidine, and lysine.
The Effect of Very High Concentrations of Glycine, Histidine, Arginine and Lysine on AGP1-lacZ Expression

Out of twenty L-amino acids, glycine, histidine, lysine, arginine, and cysteine did not lead to maximum induction of AGP1-lacZ expression at a concentration of 1.6 mM. Hence, further titration assays were performed on these amino acids to examine their effect on AGP1-lacZ expression at very high concentrations, up to 12.8 mM. Remarkably, Figure 9 shows that AGP1-lacZ expression is highly induced by 12.8 mM glycine or histidine, but the induction still did not reach its maximum. AGP1-lacZ expression appears to be fully induced in cells grown in the presence of 400 μM arginine, and it plateaus in the presence of higher amounts of arginine. In the case of lysine, AGP1-lacZ expression appears to reach maximum at 6.4 mM lysine (Figure 9). Based on the generation time of cells grown in the presence of 1.6 mM individual amino acids, cysteine is the most toxic. In the presence of 1.6 mM cysteine, yeast cells have severe growth defects. Therefore, no further analysis was done on cysteine beyond 1.6 mM concentration.
Figure 9: AGP1-lacZ expression in response to stimulation with very high levels of glycine, histidine, lysine, and arginine.

β-galactosidase activity assays on AGP1-lacZ expression were conducted on RBY98661 cells grown in minimal SD medium with titrating concentrations (0-12.8 mM) of glycine, histidine, lysine, and arginine.
Position of Alanine’s R-Group is Critical for Sensing via Ssy1

Each common amino acid has four substituents around the Cα carbon: the R group, the carboxyl group, the amino group, and the hydrogen atom. To determine whether the position of the R-group around the Cα carbon affects sensing by Ssy1, I compared AGP1-lacZ expression in cells grown in the presence of L-alanine versus β-alanine, in which the amino group linked to the Cβ carbon. Figure 10 shows that β-alanine only weakly activates AGP-lacZ expression, suggesting that the position of the R group of common amino acids is important for amino acid sensing by Ssy1.

Figure 10: AGP1-lacZ expression is affected by the position of alanine’s R-group

β-galactosidase activity assays on AGP1-lacZ expression were conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations (0-1.6 mM) of L-alanine versus β-alanine.
The Hydrogen Atom Linked to the Ca Carbon is Critical for Sensing via Ssy1

Next we examined whether the hydrogen atom attached to the Ca carbon is important for amino acid sensing by Ssy1. We performed amino acid titration assays on AGP1-lacZ expression using L-phenylalanine and L-α-methyl-phenylalanine, in which the hydrogen atom is replaced with a methyl group at the Ca carbon. Figure 11 shows that AGP1-lacZ expression is highly induced in the presence 50 µM phenylalanine, but L-α-methyl-phenylalanine has no effect on AGP1-lacZ expression even at 0.8 mM concentration, suggesting that the hydrogen atom around the Ca carbon of amino acids is critical for amino acid binding to and/or activation of Ssy1.

Figure 11: AGP1-lacZ expression induced by L-phenylalanine is abolished by the substitution of the hydrogen atom linked to the Ca carbon with a methyl group.

β-galactosidase activity assays of AGP1-lacZ expression were conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations (0-900 µM) of L-α-methyl-phenylalanine versus L-phenylalanine.
D-Amino Acids Have Variable Effects on Ssy1 Activation

L-amino acids are the building blocks of proteins, metabolic fuels for cells, and precursors for biosynthesis [53]. D-amino acids are not as common, but they can be found in bacterial cell walls, in antibiotics produced in bacteria and fungi, and in small peptides found in both vertebrates and invertebrates [54, 55]. Since Ssy1 has a broad binding specificity for L-amino acids and other nitrogen compounds [14], I wondered whether a change in the entire configuration of an amino acid around the \( \text{C}_\alpha \) carbon affects Ssy1 sensing. To test this, I compared the effects of 14 pairs of D- and L-amino acids on \( \text{AGP1-lacZ} \) expression using \( \beta \)-galactosidase activity assays. The results showed that the D-isoforms of L-amino acids have variable effects on Ssy1 activation. Based on relative ability of D-amino acids in activating Ssy1 as compared to their L-amino acid counterparts, I divided these 14 pairs of L- and D-amino acids into three groups.

Group 1, including leucine and aspartic acid, showed that the D-isoform appeared to have the same affinity but higher potency for activating Ssy1 as compared to its L-isoform counterpart (Figure 12).
Figure 12: The D- isoforms of Group 1 amino acids have the same affinity and higher potency on Ssy1 activation.

β-galactosidase activity assays of AGP1-lacZ expression were conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations of (A) D- and L-leucine and (B) D-and L-aspartic acid.
Group 2 amino acids, including alanine, phenylalanine, serine, tyrosine, asparagine, methionine, and arginine showed that the D-isoforms had much lower affinity yet similar or greater potency on Ssy1 as compared to their L-isoform counterparts (Fig. 13). Interestingly, although L-arginine is a weaker activator of Ssy1 (Fig. 9), D-arginine induced \( AGP1-lacZ \) expression to a level similar to that achieved by the most potent L-aromatic amino acids. Group 3 amino acids, lysine, glutamic acid, glutamine, valine, and proline, the D-isoforms had little or no activity on Ssy1.
Figure 13: The D-isoforms of Group 2 amino acids have lower affinity but similar or higher potency for Ssy1.

β-galactosidase activity assays of AGP1-lacZ expression were conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations (0-12.8 mM) of the D-
and L-isoforms of (A) serine, (B) tyrosine, (C) alanine, (D) phenylalanine, (E) methionine, (F) arginine, or (G) asparagine.

Figure 14: The D-isoforms of Group 3 amino acids had little or no activity in activating Ssy1.
β-galactosidase activity assays of AGP1-lacZ expression were conducted for RBY98661 cells grown in minimal SD medium with titrating concentrations (0-12.8 mM) of the D- or L-isoforms of (A) glutamine, (B) glutamic acid, (C) lysine, (D) valine, or (E) proline.

**Cysteine Toxicity**

As I mentioned previously, excess amounts of amino acids inhibit yeast cell growth. Cells started to die in response to increased amounts of amino acids present in the growth medium (data not shown). Among the twenty essential L-amino acids I assayed, cysteine appeared to be the most toxic to the cells. It took 48 hours to grow 6 generations of yeast cells in 1.6 mM cysteine while the wild type cells took less than 24 hours to grow 6 generations. Growth of yeast cells was not observed at cysteine concentrations beyond 1.6 mM. Hence I questioned why is cysteine, among all other amino acids, the most toxic? Also, how does cysteine cause growth deficiency in cells? To answer these questions it was necessary to isolate cysteine resistant mutants.

Cysteine is part of the methionine biosynthesis superpathway; it can be produced from methionine degradation (Fig 15). I suspected that cells grown in high concentration of cysteine results in accumulation of intracellular cysteine, the end product of methionine degradation, which triggers negative feedback that inhibits breakdown or usage of methionine in the cell. To that end, I grew PLY126K1 cells in the presence of 1.6 mM cysteine, and in the presence of 0.2 mM methionine or/and 0.2 mM threonine respectively. By comparing the growth of cells in the absence or presence of cysteine, the additional methionine or threonine did not improve the growth defect of cells in the presence of cysteine (data not shown). The result suggests that cysteine toxicity in cells may not due to inhibition of methionine degradation, but may inhibit other pathways which cysteine is involved in.
Figure 15: Methionine metabolic pathway

Isolation Cysteine Resistant Mutants

Since the presence of cysteine activates *AGP1-lacZ* expression through the SPS amino acid signaling pathway [4], I first sought to determine whether mutations to *SSY1*, *PTR3*, or *SSY5*, which block the SPS signaling pathway and uptake of cysteine, could confer resistance to cysteine toxicity. To that end, I tested the growth of ssy1Δ, ptr3Δ, ssy5Δ strains generated previously in Liu’s lab and the WT cells on SD versus SD + 5mM cysteine plates. Figure 16 shows that the deletion of *SSY1*, *PTR3*, or *SSY5* can confer resistance to cysteine toxicity, indicating that toxicity of cysteine involves, at the very least, sensing it in the extracellular environment.
Figure 16: ssy1Δ, ptr3Δ, and ssy5Δ mutations confer resistance to cysteine toxicity. WT, ssy1Δ, ptr3Δ, and ssy5Δ strains were grown on SD plates without or with 5 mM cysteine. WT strains were not able to grow on medium contain high concentration of cysteine, but ssy1Δ, ptr3Δ, and ssy5Δ strains showed resistance to cysteine toxicity.

The discovery that deleting SSY1, PTR3, or SSY5 confers resistance to cysteine toxicity is not surprising since the SPS amino acid sensing pathway requires these proteins to sense and respond to extracellular amino acid [2]. This information however, does not provide enough information to answer the question of how or why cysteine is toxic to cells. To that end I conducted a genetic screen to identify mutations that can confer resistance to high concentrations of cysteine other than mutations in Ssy1, Ptr3, and Ssy5. In order to do that, I transformed PLY126K1 cells, integrated with the AGP1-lacZ reporter gene, with plasmids pRS416-STP1p-STP1C and pRS417-GPD1-STP1-myc3-2. It has been shown that overexpressing STP1 activation domain leads to constitutive activation of transcription in SPS signaling pathway, meaning
transformants could bypass Ssy1, Ptr3, and Ssy5 to constitutively express AGP1-lacZ reporter gene [6]. I intentionally increased the WT cell’s ability to uptake extracellular cysteine, which would theoretically prevent isolation of mutation in Ssy1, Ptr3 and Ssy5. Then the transformants were mutagenized with ethyl methanesulfonate (EMS), which produces mutations in the DNA via guanine alkylation which generates random G-C nucleotide substitution [52]. EMS treated cells were diluted and plated directly on solid SD + 5 mM cysteine plate. Single colonies that were resistant to cysteine toxicity were isolated and assayed on solid SD versus SD + 5 mM cysteine plates for their ability to grow normally compared to the growth deficiency of the WT cells (Table 1). 10 colonies were further confirmed to be cysteine resistant.

These 10 colonies were grown in SD + 0.02% leucine to activate SPS amino acid sensing and analyzed via β-galactosidase activity assays, which allowed me to phenotypically analyze different mutations. Figure 17 shows that of the 10 cysteine resistant mutants, 7 showed no AGP1-lacZ reporter gene activity. This observation indicates that these 7 mutations were due to mutation in the SPS signaling pathway leading to defects in activation of AGP1 reporter gene, even though I had intentionally minimized the possibility of isolating SPS pathway defect mutations.

Mutants 1 and 2 expressed the AGP1-lacZ reporter gene to a similar level to WT cells, and mutant 10 had a 0.5 fold increase in AGP1-lacZ activity than WT cells. Because these three mutants have expression of the AGP1-lacZ gene, amino acids appears to be transported into yeast cells normally in these mutants; hence, this observation suggests that cysteine resistance in these three mutants are not due to deficiency in uptake of extracellular cysteine caused by mutation in SPS signaling pathway, but may be due to the intracellular processing of cysteine.
Table 1. Mutants generated from EMS mutagenesis are resistant to high levels of cysteine. Wild type and mutant strains were streaked onto SD plates with or without 5 mM cysteine and incubated at 30 C for 3-4 days.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Relative growth rate in SD Medium</th>
<th>Relative growth rate in SD + Cys</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mut 1</td>
<td>4</td>
<td>4</td>
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<td>Mut 4</td>
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<td>Mut 5</td>
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<td>Mut 6</td>
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<td>Mut 8</td>
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<td>Mut 10</td>
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<td>Mut 11</td>
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<td>Mut 14</td>
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<tr>
<td>Mut 16</td>
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Identification of Cysteine Resistant Mutants

As shown previously, cysteine resistance can be caused by mutations in components involved in the SPS amino acid signaling pathway. These mutations likely block extracellular cysteine uptake into yeast cells. Even though I tried to generate mutants that bypass SSY1, PTR3, and SSY5, β−galatosidase results in Figure 17 showed some mutants were defective in expressing AGP1-lacZ reporter gene. To identify the mutations responsible for cysteine resistance, especially the ones which were defective in SPS signaling pathway, I first sought to identify whether some of the cysteine resistant colonies were still due to mutations in SSY1, PTR3, or SSY5. To that end, I first streaked mutants on YPD plate to lose their plasmids, then transformed MATα WT and the 10 cysteine resistant mutants with pRS416 empty vector containing the URA3 marker. I also transformed MATα WT, ssy1Δ, ptr3Δ ssy5Δ, ssy5Δ, dal81Δ, and shr3Δ strains containing the AGP1-lacZ reporter gene, which all are essential components in activating the SPS amino acid signaling pathway, with the pRS417 empty vector containing the LYS2 marker for selection purposes. I mated MATα transformants to the MATα deletion strains and then assayed the expression of AGP1-lacZ in the resulting
diploids. Of the 10 mutants, mutant 1 failed to mate indicating that mutant 1 may have a mutation in a gene involved in the mating process. Diploid cells from the remaining 9 mutant groups were grown in SD media + 0.02% leucine to activate SPS amino acid sensing and assayed individually via β-galatosidase assays. Figure 18A shows that diploids resulting from mating with WT cells showed activation of AGP1-lacZ expression indicating mutations leading to cysteine resistance are recessive. When cysteine mutants mated with ssy1Δ strain, AGP1-lacZ activities of mutants 5, 11, 13, 14, and 16 were completely abolished, indicating that these mutants are resistant to cysteine due to the mutation in the gene encoding the Ssy1 sensor (Fig.18 B). Similarly, AGP1-lacZ activity was abolished in diploids from mutant 6 mated with ssy5Δ cells indicated that mutant 6 is resistant to cysteine because of a mutation in the gene encoding the Ssy5 protease (Fig.18 D).

Mutant 8 mated to the double ptr3Δ ssy5Δ double mutant had no AGP1-lacZ activity (Fig. 18C); yet, mating mutant 8 with the single ssy5Δ mutant had AGP1-lacZ expression similar to WT (Fig. 18D). This suggests that mutant 8’s cysteine resistance is likely due to a mutation in the gene encoding the Ptr3 protease.

Diploids resulting from mutants 4 and 10 showed varying AGP1-lacZ activities compared to WT and none showed abolishment of AGP1-lacZ activity (Fig.18) indicating that these mutations are in ssy1Δ, ptr3Δ ssy5Δ, ssy5Δ, dal81Δ, or shr3Δ. Specific mutated sites of mutant 4 and 10 are still unknown.

Overall, seven mutants which abolished AGP1-lacZ activity were found to be due to mutations in components that lead to malfunctions of the Ssy1-Ptr3-Ssy5 amino acid signaling pathway. This observation confirms that defective SPS amino acid signaling pathway can block uptake of extracellular cysteine and result in cell resistance of cysteine toxicity.
Figure 17: *AGP1-lacZ* gene expression in 10 cysteine resistant mutants

Cultures of 10 cysteine resistant mutant cells grown in SD medium + 1.5mM leucine were assayed for *AGP1-lacZ* activity via β-galactosidase assay. 7 out of 10 mutant cysteine resistant strains are due to SPS signaling pathway defects, but the 3 mutations are not.
Figure 18: Identification of cysteine resistant mutants

AGP1-lacZ activity in diploids of 9 haploids mutated cells mated to (A) WT cells, (B) ssy1Δ mutant cells, (C) ptr3Δ ssy5Δ double mutant cells, (D) ssy5Δ mutant cells, (E) dal81Δ mutant cells, and (F) shr3Δ mutant cells. Diploid cells were grown in SD media + 0.02% leucine and assayed via β-galactosidase assay.
**Mutant 8 is Resistant to Cysteine Because of a Mutation in PTR3**

Mutant 8’s cysteine resistance is likely due to a mutation in the gene encoding the Ptr3 protease. (Fig. 18 C and D). To confirm this, I transformed wildtype PTR3 encoded on a centromeric plasmid into mutant 8. If mutant 8 is due to a *ptr3Δ* mutation, the WT copy of PTR3 gene transformed in plasmid will complement its mutation and restore AGP1-*lacZ* activity. Figure 19 shows that a WT copy of PTR3 complements the mutation of mutant 8 and restores AGP1-*lacZ* confirming that mutant 8 is resistant to cysteine toxicity because of a mutation in PTR3.

![Cell activity graph](image)

**Figure 19: Mutant 8 contains a mutation in PTR3**

β-galactosidase assay of AGP1-*lacZ* expression in WT, mutant 8, and mutant 8 transformed with Ptr3 plasmid grown in SD media + 0.02% leucine respectively were analyzed.
Discussion

In this study, I show that the Ssy1 sensor has different binding affinity and sensitivity to 20 common L-isoform amino acids, with the exception of proline. Also, positioning of the R group and hydrogen atom around the alpha carbon of amino acids is crucial to amino acid sensing. Remarkably, the Ssy1 sensor can also sense D-amino acids to various degrees. The fact that the Ssy1 sensor can detect the most common amino acids in both L- and D- isoforms suggest that the Ssy1 sensor binding pocket is likely very large and flexible. The Ssy1 sensor shows strongest affinity and sensitivity to amino acids with aromatic groups followed by those with hydrophobic groups, next to those with neutral hydrophilic group, then to negative charged group, and lastly has the lowest affinity and sensitivity to positively charged amino acids. Based on this observation, the Ssy1 sensor more likely contains more hydrophobic amino acids residues, and more negatively charged amino acid residues in its binding pocket that may play an important role in its sensing activity.

Among 20 common amino acids, L-cysteine is an amino acid containing a thiol group that is nucleophilic and easily oxidized. Cysteine is the precursor of the antioxidant glutathione whose thiol group of cysteine undergoes redox reactions and forms a peptide linkage to carboxyl group of the glutamate side chain [56, 57]. Oxidation of the cysteine thiol group generates a disulfide bond, playing an important role in the folding and stability of some proteins. It is also a very important precursor of iron-sulfur cluster proteins, which play important roles in oxidation-reduction reaction in mitochondria electron transport.

Like any other living organism, cysteine is essential for cellular metabolism in yeast. In S. cerevisiae, cysteine can be synthesized from methionine or be taken up from the surroundings. However, high concentrations of cysteine are the most toxic amino...
acid to cells. Its cysteine toxicity was examined in yeast cells grown in minimal medium with up to 5 mM cysteine, and cysteine inhibits growth of yeast in a dose-dependent manner [58]. It has been shown that all 7 permeases targeted by the SPS signaling pathway (Bap2, Bap3, Agp1, Gnp1, Tat1, Tat2, and Mup1) are able to uptake extracellular cysteine in either non-repressing nitrogen source or ammonium-based source [19, 59, 60]. High level of expression of cysteine uptake permeases possibly explains why cysteine at high concentration is the most toxic to yeast cells compared to other amino acids. My results show that mutations in the SPS signaling pathway lead to resistance to cysteine toxicity in cells, likely because these mutations inhibit expression of cysteine transporter genes and block cysteine uptake from the environment. Interestingly, three cysteine resistant mutants were still able to express the **AGP1-lacZ** reporter gene; therefore, cysteine appears to be transported normally into these mutants. This observation suggests that cysteine resistance in these three mutants may be due to either mutation in an unknown signaling pathway expressing permeases that also transport cysteine into the cells, or, more likely, the intracellular processing of cysteine.

The intracellular mechanism that causes cysteine cytotoxicity in yeast has not been well studied; therefore, it is difficult to speculate why these three mutants are cysteine resistant. One possible explanation of intracellular cysteine resistance could be due to mutations in methionine-cysteine biosynthesis. Many enzymes involved in nitrogen anabolism are inhibited and their synthesis is repressed upon accumulation of the end or intermediate products of biosynthetic pathways [61]. Homocysteine is the intermediate in the ubiquitous methionine cycle to synthesize cysteine, and it is essential to generate one-carbon methyl groups for transmethylation reactions [62]. Cysteine toxicity effects studied in human shown that elevated level of homocysteine increases the risk of having cardiovascular diseases [63] and associates with other
diseases. A high level of cysteine shows similar toxic effects. Changes in plasma
cysteine correlates with changes in BMI, cholesterol, and diastolic blood pressure [64].
As predicted, total plasma cysteine is also a risk factor for vascular diseases, and
concentrations of cysteine present in plasma show a U-shape correlation with
peripheral and cerebrovascular disease [65]. \textit{S. cerevisiae}, which shares a considerable
similarity with higher eukaryotic cellular organization and function, has been used as a
model to study the impacts of elevated level of homocysteine and cysteine on cellular
physiology and pathology. Kumar and his colleagues observed that additional thiols
from homocysteine or cysteine down-regulated genes coding for antioxidant enzyme,
but upregulated genes responsible for ER stress and glycolysis pathway. They
concluded that cytotoxicity is probably due to ER stress [58]. It is very likely that
cysteine resistance observed in mutants that could still express \textit{AGP1} may be due to
mutations in genes involved in a methionine signaling pathway (Fig.19), which allows
bypass of detection and signaling of excess thiol groups in cells.
CHAPTER 3: HAP4 EXPRESSION IS REGULATED BY THE MEDIATOR COMPLEX

Introduction

CCAAT-binding proteins (CBPs) are transcription activators that promote expression of protein-coding genes by interacting with CCAAT consensus sequence motifs within promoters [22]. Under fermentative conditions (in the presence of glucose), the CBP of yeast, termed the Hap2/Hap3/Hap4/Hap5 complex, binds to CCAAT-box containing upstream activation sites (UASs) to initiate gene responses to metabolize non-fermentable carbon sources (galactose/raffinose) [23-28]. Hap2, Hap3, and Hap5 form a heterotrimer which is required for CCAAT DNA binding at the promoter region [24], however activation of transcription can only be triggered when the Hap4 completes the complex [26]. The regulatory subunit, Hap4, is known to be transcriptionally controlled by carbon sources, and contains the activation domain of the complex [29]. Increased Hap4 levels results in switching from fermentation to respiration in yeast, termed the diauxic shift. However, regulation of \textit{HAP4} expression has been poorly studied. Previously the Liu Lab observed \textit{SIN4}, a component of the mediator tail region, and \textit{CYC8}, a general transcription co-repressor with \textit{TUP1}, showed as negative regulators of HAP4 expression. This prompted me to study the mechanism underlying HAP4 regulation by the Mediator complex.
Materials and Methods

Strains, Primers, Plasmids, Growth Media and Growth Conditions

Yeast strains, primers, and plasmids used in this study are listed in Table 1, Table 2, and Table 3, respectively. Yeast cells were grown at 30 °C in 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). The amino acids L-leucine, L-lysine, L-histidine, L-methionine, L-tryptophan, and/or uracil were added to growth media to a final concentration of 30 mg/L to meet auxotrophic requirements [50]. Solid media contained 2% agar in addition to the components described above when needed. MG132 (50 µM) was added to the growth media to inhibit proteasome activity [66] for experiments testing proteasome dependent protein degradation.

Yeast Transformations and β-galactosidase Activity Assays

Yeast cells were transformed [51]. Transformants carrying the desired plasmids were selected on YNBcasD plates. Liquid cultures were inoculated with a pool of several independent transformants and grown overnight in YNBcas5%D or into YNBcasR medium to an optical density at 600 nm of ~0.6. In glutamate repression analysis, liquid S5%D and SR media were used. The cell extracts were prepared by using glass bead lysis method. 10 – 90 mL of clear cell extract of independent samples were objected to β-galactosidase assays, hydrolyzing o-nitrophenyl-β-d-galactopyranoside (ONPG) [50]. Protein concentration of individual sample was carried out by performing Bradford assay, using bovine serum albumin as the standard.
Reporter lacZ activity was calculated in nmoles of hydrolyzed ONPG per minute per mg of protein. For each plasmid-strain combination, assays were conducted in duplicate and independent experiments were carried out two to three times.

**Cellular Extract Preparation and Immunoblotting**

Total cellular protein extracts were prepared by lysing yeast cells in extraction buffer (1.85 N NaOH – 7.5% β-mercaptoethanol) followed by precipitation with trichloroacetic acid (TCA) [67]. TCA was neutralized and total cellular proteins were structurally linearized in 1M un-buffered Tris, 1M dithiothreitol, and 1X SDS-PAGE loading buffer followed by heat treatment. Proteins were separated by SDS-PAGE gel, and transfer onto nitrocellulose membrane via Western blotting.

myc- and HA- tagged proteins were probed with anti-myc primary antibody and anti-HA primary antibody (3F10, Roche), respectively. Primary antibodies were detected by HRP conjugated goat anti-mouse secondary antibody and HRP conjugated goat anti-rat secondary antibody, respectively. Chemiluminescence was induced by an ECL reagent. Chemiluminescence images of Western blots were captured using the Bio-Rad Chemi-Doc photo documentation system. (Bio-Rad Laboratories, inc., Hercules, CA). For loading control visualization, blots were deprobed in stripping buffer (2%SDS-0.08% β-mercaptoethanol) for 45 min at 60 °C with shaking, and then be reprobed with anti-PGK1 (3-phosphoglycerate kinase) primary antibody, followed by HRP conjugated goat anti-rabbit secondary antibody. Chemiluminescence was induced by an ECL reagent. Images of reprobed blots were captured using the Bio-Rad Chemi-Doc photo documentation system.

**Library Complementation of a HAP4 Expression Activating Mutation**

One highly activating HAP4 expression mutant (DCY125-13) was transformed with myc-tagged LEU2 CEN mediator plasmids, which were constructed in this study.
Transformants were selected for on SD plate media supplemented with histidine and methionine (SD+HM). After 2-3 days, pools of colonies from each transformation were grown in 10mL SD+HM liquid media. Potential candidates containing a complementing plasmid were selected by their growth on plates and further phenotype confirmation via β-galactosidase assay.

**Cycloheximide Chase Assay**

Cells expressing indicated myc-tagged proteins were grown in liquid 55%D+HM or SR+HM medium to OD600 0.6 – 0.8. Protein synthesis was inhibited by addition of 50 µg/mL cycloheximide. Every 30 minutes, a 1 mL sample of the cell culture was withdrawn and immediately subjected to TCA precipitation. Total cellular extracts were prepared, and myc-tagged protein levels were determined by probing Western blots as described above. Loading controls were obtained by stripping the membrane and reprobed with anti-PGK1 (3-phosphoglycerate kinase) primary antibody.

**Determination of Srb8 Half-life**

*Erg6Δ* mutant cells, expressing HA-tagged Srb8 were treated with 50 mM and 100 mM MG132 and no treatment, and grown in liquid YNBcas5%D or YNBcasRaff medium. Once cells reached OD600 between 0.6 and 0.8 they were subjected to cycloheximide chase assay. Srb8-HA protein levels were detected by Western blot, and Srb8 protein half-life was estimated from observation of different protein levels expressed in every 30 minutes.

**Mating and Tetrad Dissection**

MAT a and MAT α haploid yeast cells were mated on YPD plate media for 4 hrs. Diploids were selected for on SD plate media supplemented with amino acids to cover auxotrophic markers. Diploids were induced to undergo sporulation by growing in
sporulation media (1% potassium acetate, 0.1% Bacto-yeast extract, 0.05% raffinose) for 6-10 days. Tetrads were dissected on YPD plates using a micromanipulator.

Results

Hap4 Expression is Regulated by the Mediator Complex

HAP4 expression increases approximately ten-fold when yeast cells are grown in raffinose media compared to dextrose media. Despite the importance of mitochondria in cellular metabolism and the essential role of hap4 in mitochondrial biogenesis, the regulatory mechanism of HAP4 expression is still unclear. The mediator complex of yeast has been shown in Liu’s lab to be involved in negative regulation of HAP4 expression. Recently, Med8, another component of the Mediator complex, has been implicated in the carbon catabolite repression pathway [68]. Therefore, I sought to characterize the role of each component of the Mediator complex in Hap4 regulation.

HAP4 expression was analyzed in cells lacking 15 non-essential mediator components. These mutant strains, transformed with plasmid carrying HAP4-lacZ reporter gene, were already made in the Liu lab. Transformants were grown in S5%D or SR liquid medium overnight to OD\text{\textsubscript{600}} 0.6-0.8, and assayed for β-galactosidase activity. Figure 20 shows that in S5%D grown cells, deletion of mediator components results in increased HAP4-lacZ gene expression, up to 10 fold compared to basal HAP4-lacZ activity in WT cells. However, two of the 14 non-essential mediator complex component mutations did not increase HAP4-lacZ activity, a med3Δ resulted in HAP4-lacZ activity comparable to WT and deletion of GAL11 resulted in a 0.5 fold decrease in HAP4-lacZ activity. In SR liquid medium, deletion of mediator components results in up to a 5 fold increase in HAP4-lacZ activity. Similar to dextrose grown cells, the exception to this is that a med19Δ mutation results in HAP4-lacZ activity comparable to WT and gal11Δ,
med3Δ, hxx2Δ, and med20Δ mutations result in a decrease in HAP4-lacZ activity. These data suggest that some of the non-essential mediator components negatively regulate HAP4-lacZ expression while others may positively regulate it.
Figure 20: The Mediator complex regulates expression of HAP4-lacZ

HAP4-lacZ activity in non-essential mediator component deletion mutants compared to WT cells. Cells were grown in 55%D medium (A) or SR medium (B) and assayed using β-galactosidase assays.
Myc-tagged Mediator Components are Functional

The non-essential mediator components were shown to regulate $HAP4$-lacZ expression to various degrees (Fig. 20). It is possible that posttranslational modifications or protein levels of the mediator complex components may vary depending on growth conditions and affect $HAP4$ expression. To test this possibility, I first constructed centromeric LEU2 plasmids encoding myc-tagged mediator components individually and tested their functionality (see Table 2).

Centromeric LEU2 plasmids containing non-essential myc-tagged mediator components were transformed in their corresponding mediator component knockout strains, containing plasmid of $HAP4$-lacZ reporter gene, and analyzed for β-galactosidase activity. Figure 21 shows that myc-tagged proteins restored derepressed Hap4 activity to wild type $HAP4$-lacZ level; in term, non-essential myc-tagged mediator component were functional. Non-essential myc-tagged mediator components in their corresponding mediator component deletion strains complemented the effect a mediator component deletion mutation has on the expression of $HAP4$-lacZ.
Figure 21: Non-essential myc-tagged mediator components are functional.

$HAP4-lacZ$ activities in non-essential mediator component deletion strains without or with respective myc-tagged mediator component confirm the functionality of non-essential myc-tagged mediator component plasmids. Cells were grown in YNBcas5%D or S5%D medium and assayed via β-galactosidase assay.

Deletion of essential mediator components is lethal to yeast cells. To examine the functionality of the constructed essential myc-tagged mediator component plasmids, I transformed these plasmids into their respective diploid MED/medΔ::kanMX4 strain. Transformants were subjected to sporulation, and yeast tetrads were dissected onto YPD plates by Tammy Pracheil and tetrad analysis was done by undergraduate Adrienne McGihn. Tetrads that showed viability of all four spores were selected for
further analysis on the basis that two spores must contain the wildtype MED gene and two must have the medΔ with a plasmid based wildtype myc-tagged MED gene. Viability of all four spores already indicates functionality of the myc-tagged essential mediator component.

Confirmed essential myc-tagged mediator component plasmids were transformed into WT cells with the plasmid based HAP4-lacZ reporter gene. Transformants were then subjected to β-galatosidase assay to examine whether mild overexpression of essential MED genes from centromeric plasmids affects Hap4 promoter activity. Figure 22 shows that expression of essential myc-tagged mediator components did not grossly affect HAP4-lacZ gene expression.
Figure 22: Overexpression of essential myc-tagged mediator components do not effect HAP4 expression.

HAP4-lacZ activities in essential mediator component deletion strains with respective myc-tagged mediator components were not significantly affected compared to HAP4 expression in WT cells. Cells were grown in S5%D medium and assayed via β-galactosidase assay.

**Carbon Source Affects Myc-Tagged Mediator Components Expression and Processing**

It is possible that regulation of HAP4 expression by the mediator complex involves posttranslational modification or proteolytic processing of mediator components depending on carbon source availability. To test this, non-essential
mediator deletion strains with corresponding myc-tagged plasmids and wild type strains with myc-tagged essential mediator gene used in earlier experiments were grown in S5% D and SR medium overnight to OD$_{600}$ 0.6-0.8. Cell extracts were prepared and proteins were separated by SDS-PAGE and visualized by Western-blotting. Figure 23 shows there were seven MED genes that showed a difference in protein expression in cells grown in dextrose versus raffinose medium. Protein expression of Med4, Med10, Med20, and Med31 genes were at least twice as strong in dextrose versus raffinose grown cells. Conversely, protein expression of Gal11 and Srb9 were stronger in raffinose versus dextrose grown cells.
Figure 23: Effect of carbon source on Mediator Complex components
Myc-tagged mediator component protein expressions were detected in dextrose versus raffinose grown cells.

Interestingly, Srb8-myc was processed into truncated fragments in dextrose grown cells as compared to raffinose grown cells. One of the possible explanations is that Srb8 is degraded by ubiquitin dependent proteolytic processing in dextrose grown cells. To examine this possibility, erg6Δ mutant cells expressing Srb8-myc were grown in dextrose medium and treated with 50µM and 100µM MG132 (see Material and Methods). Total cellular proteins with or without treatment of MG132 were precipitated, separated by SDS-PAGE and Srb8-myc protein was visualized by western blotting. An erg6Δ mutation increases MG132 permeability [66]. MG132 inhibits ubiquitin-dependent protein degradation via the proteasome. If Srb8 protein processing is ubiquitin-dependent, treatment of MG132 should be efficient to block the degradation of Srb8 protein as I expected to detect a full length Srb8 protein. However, figure 24 shows no difference in protein expression with or without treatment of MG132 and treatment of MG132 did not inhibit processing of Srb8-myc in dextrose grown cells. This observation suggested that Srb8 protein processing in dextrose grown cells is not due to proteasomal degradation.
Figure 24: Processing of Srb8 in dextrose grown cells is proteasome independent.

Cells expressing in myc-tagged Srb8 were grown in dextrose medium with or without treatment of MG132 proteasome inhibitor.

*Mutant 125-13 is Identified as SRB8 Mutation*

Previously, a former member of Liu’s lab, Denise Capps, isolated an unknown mutant DCY125-13 which fully activates HAP4-lacZ activity in the BY4741 background dextrose-grown cells. I sought to identify the mutation responsible for the phenotype observed in mutant. Therefore, I transformed two known negative transcription regulators, HXK2 or TUP1, or plasmid based myc-tagged mediator components into DCY125-13 to examine whether these genes could complement the mutation in DCY125-13. Figure 25 shows that only introduction of SRB8 significantly reduced the HAP4-lacZ expression in mutant from ~900 nmols/min/mg protein to ~200
nmols/min/mg protein. This approximately 4 fold reduction of HAP4-lacZ activity suggested that the DCT125-13 mutant was caused by mutation in Srb8 gene of mediator complex.

Figure 25: SRB8 mediator component complements HAP4-lacZ activating mutation.

DCY125-13 mutant cells expressing in myc-tagged mediator components were grown in S5%D medium and assayed via β-galactosidase assay.
Since Srb8 is a subunit of kinase module of mediator complex. I wanted to confirm that DCY125-13 mutant is due to srb8 deletion only. To that end, I mated MATα srb8Δ, srb9Δ, srb10Δ and srb11Δ strains containing the plasmid of AGP1-lacZ reporter gene with MATα 125-13 strain and then assayed the expression of AGP1-lacZ in the resulting diploids. Figure 26 showed that high HAP4-lacZ activity in 125-13 mutant was restored to WT cells level by complementing with srb9, srb10, srb11 deletion strain, but not srb8Δ. This observation further confirmed that 125-13 mutant was caused by mutation in Srb8 gene of mediator complex.

Figure 26: High HAP4-lacZ activity remains in srb8 deletion diploid.

DCY125-13 mutant mated with srb8Δ, srb9Δ, srb10Δ, and srb11 Δ diploid cells were grown in YNBcas5%D medium and assayed via β-galactosidase assay.
Effect of Overexpression of Mediator Components on Expression of HAP4

Previously, I showed that the protein levels of Med4, Med10, Med20 and Med31 are stronger in dextrose grown cells as compared to raffinose grown cells; however, no significant impacts of these genes on $HAP4$-lacZ activity were observed in earlier experiments. One explanation for this could be that overexpression of these genes could affect $HAP4$ expression. To test this possibility, I cloned these myc-tagged MED genes into 2 micron plasmids which would greatly induce promoter transcription activity of these genes. I then transformed the 2 micron plasmids containing these MED genes into BY4741 WT background cells and confirmed they were overexpressed. Figure 27 shows that protein expression of the myc-tagged MED genes encoded in 2 micron plasmids resulted in higher protein levels compared to myc-tagged MED genes encoded in centromeric plasmids grown in both dextrose and raffinose medium.
Figure 27: Overexpression of myc-tagged Mediator components.

Protein expression show comparison of centromeric plasmid based myc-tagged mediator components versus 2µ plasmid based myc-tagged mediator components grown in dextrose versus raffinose medium.
Once 2 micron plasmid based myc-tagged MED genes were confirmed to be overexpressed, I analyzed the effect of their overexpression on $HAP4\text{-}lacZ$ expression. Figure 28 shows that overexpression of $MED4$, $MED10$, $MED20$ or $MED31$ did not significantly affect $HAP4\text{-}lacZ$ activity in dextrose grown cells, but overexpression of $MED4$, $MED10$, $MED20$ or $MED31$ did reduce $HAP4\text{-}lacZ$ expression in raffinose grown cells. My previous data showed that Med4, Med10, Med20, and Med31 protein levels were reduced in raffinose grown cells. Therefore, the data suggests that Med4, Med10, Med20, and Med31 might play a negative regulatory role in inducing $HAP4$ promoter activity since an overexpression of these MED genes correlates with a decrease in $HAP4\text{-}lacZ$ activity in raffinose grown cells, and we can infer that the decrease in expression of these MED genes seen in raffinose grown cells correlates with an increase in $HAP4\text{-}lacZ$ activity to a level that matches that found in dextrose grown cells.

Comment [TS]: I may be wrong on my conclusion here, you should probably get Dr. Lius opinion on what it means and why and write that instead...
Figure 28: Effect of overexpression of myc-tagged Mediator components on *HAP4-lacZ* activity.

*HAP4-lacZ* activity in cells overexpressing Med4, Med10, Med20 and Med31 were grown in dextrose (A) versus raffinose (B) medium and were assayed via β-galactosidase assay.
Overexpression of GAL11 Increases HAP4 Expression

Earlier experiment suggested that Gal11 and Med3 might be positive regulators of HAP4 expression. In order to test this hypothesis, I transformed plasmids encoded Gal11-myc and Med3-myc into BY4741 WT cells containing the HAP4-lacZ reporter gene to see whether additional copies of Gal11 and Med3 would increase HAP4-lacZ activity. Figure 29 showed that slight overexpression of GAL11 resulted in a 3-fold increase in HAP4-lacZ activity in dextrose grown cells, and a 0.5-fold increase in HAP4-lacZ expression in raffinose grown cells as compared to WT cells. Surprisingly, slight overexpression of MED3 in WT cells had no effect on HAP4-lacZ activity in cells grown in both dextrose and raffinose medium.

It is possible that Med3 is a positive regulator of HAP4 expression but to a lesser extent than Gal11 and thus would require stronger overexpression to see an effect on HAP4-lacZ activity. To that end, I constructed 2µ plasmids encoding MED3 or GAL11 and transformed them into WT cells containing the HAP4-lacZ reporter gene. Transformants were subjected to β-galatosidase assays. Figure 29 shows that high overexpression of GAL11 further increases HAP4-lacZ activity 10-fold as compared to it is mild overexpression, but high overexpression of MED3 still no significant effect on HAP4-lacZ activity.
Figure 29: Effect of overexpression of GAL11 and MED3 on HAP4-lacZ activity

HAP4-lacZ activity in cells overexpressing GAL11 and MED3 on centromeric or 2μ plasmids grown in dextrose versus raffinose medium.

Med3 and Gal11 Function Together as a Positive Regulator of HAP4 Expression

Neither a MED3 deletion nor overexpression of MED3 affects HAP4-lacZ activity in WT cells; however, it is possible that Med3 might regulate HAP4-lacZ activity through Gal11. Therefore, I constructed a gal11Δ med3Δ double deletion mutant strain. WT, gal11Δ, med3Δ single and double deletion mutant cells were transformed with the HAP4-lacZ reporter gene and grown in YNBcas5%D and YNBcasR media and analyzed for β-galactosidase activity. Figure 30 shows that a double deletion of GAL11 and MED3 does not completely abolishes HAP4-lacZ reporter gene activity but shows a similar regulatory effect on HAP4 expression as it is shown in single gal11Δ mutant strain in figure 20. This observation suggests that the regulatory effect of Med3 is redundant.
There are other factors that regulate HAP4 expression. By forming a complex with Gal11, Med3 may positively regulate *HAP4* expression in conjunction with Gal11.

**Figure 30: HAP4-*lacZ* activity in med3Δ gal11Δ double mutant**

*HAP4-*lacZ activity of WT, med3Δ, gal11Δ, and med3Δ gal11Δ double deletion strains were assayed via β-galactosidase in cells grown in dextrose versus raffinose medium.

**Discussion**

In this study, I report that the mediator complex components either positively or negatively regulate *HAP4-*lacZ activity. Out of 25 mediator components, Med4, Med10, Med20, and Med31 likely play a negative regulatory role in inducing *HAP4* promoter activity. The kinase, Srb8 is processed in response to glucose medium, and its deletion
greatly induced \textit{HAP4-lacZ} activity (Fig. 20, 23). Gal11 appears to be a positive regulator of \textit{HAP4} promoter activity. Sin4 and Hxk2 were previously shown in the Liu lab to negatively regulate \textit{HAP4} promoter activity; my results further suggest that the mediator complex interacts with a \textit{HAP4} transcription factor and each component of mediator have specific regulatory roles in inducing \textit{HAP4} promoter activity. Since the mediator complex is composed of 25 subunits, they often interact with each other in regulating transcription; therefore, deletion of one mediator component may have an effect on neighbor subunits in their regulation of \textit{HAP4} promoter activity.

In an early study, it was suggested that Srb10 repressed activation of genes that enable cells to survive under nutrient deprivation, and that Srb10 expression is greatly reduced when cells enter diauxic shift [48]. However, changes in \textit{SRB10} expression in nutrient depleted medium was not observed in this study which may due to the difference in non-glucose sources I used, raffinose compared to galactose they previously used. Interestingly, I found that another component of kinase complex, Srb8, underwent processing in cells grown in dextrose. However Srb8 processing is not due to ubiquitin-dependent degradation and the type of post-translational modification is still unknown.

Previously, research found that mutation of Srb8 induced invasive growth in haploid strains [69]. Mutation of Srb8 alleviates repression by Cyc8-Tup1 in vivo. Srb8 can positively regulate the function of Srb10, and balance between the activities of Srb8+Srb10 and Srb11 is important for normal growth of the cells [70]. Srb8, 9, 10, and 11 tightly interact with each other and form a Kinase complex that positively or negatively regulates different targeted gene transcription. As their role individually in regulating \textit{HAP4} transcription factor, it seems that Srb8 is a negative regulator of \textit{HAP4};
in glucose grown cells, Srb8 interferes with Srb10 and its expression is repressed in fermentative growth condition.

Another mediator component Gal11 also plays an important role in Hap4 regulation. Gal11/Med15 in the mediator tail module contains four N-terminal activator-binding domains that are crucial for interaction with transcription activators [71, 72]. Gal11, Med2 and Med 3 form a triad which is capable of binding the promoter region of Gcn4 activator as a free subcomplex. The entire tail module is essential for recruitment and stabilization of mediator complex to Gcn4p[73]. Western analyses and column binding assays show that Gal11 directly interacts with gene-specific transcriptional activators[74]. Not only Gal11 required for interaction of mediator complex and gene-specific transcription activator, but it also required for the efficient recruitment of RNA polymerase II holoenzyme to a promoter via activator-specific interactions for transcriptional repression or derepression of many genes[75]. In Hap4 regulation, it is reasonable to believe that the activator-binding domain of Gal11 directly binds to the Hap4 transcription factor and positively regulates HAP4 promoter activity, by interfering with Med3 subunit which is not absolutely required.

Overall, both amino acids signaling pathways for nitrogen-based nutrient sensing and mitochondria biogenesis metabolism for carbon source sensing are very important in yeast growth. By using S. cerevisiae as a model and studying its cellular
mechanism responding to different nutrient sources can help us understand the overall physiological system of sensing and utilizing nutrient in eukaryotes.

**FUTURE WORK**

Genomic DNAs of three unknown cysteine-resistant mutants have been generated and will be sequenced to pinpoint the mutations behind the cysteine resistance phenotype. Once the mutated genes are revealed, studies will be directed to understand the role of these genes in cysteine toxicity. My results have shown that Srb8 processing does not require the 26S proteasome. The underlying mechanism of Srb8 processing will be studied.
REFERENCES


12. Nelissen, B., R. De Wachter, and A. Goffeau, Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the
complete genome of Saccharomyces cerevisiae. FEMS Microbiology Reviews, 1997. 21(2): p. 113-134.


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### Table 2: Plasmids used in this study

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Mengying Chiang, daughter of Lily Y. Saitta and Min Jiang, was born on June 3rd, 1987 in Shanghai, China. She attended Pallotti High School in Belize City, Belize and graduated in 2006. In 2010, she received her B.S. in Biology, with a minor in Chemistry, from University of New Orleans. In 2011, 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 Metairie of Jefferson Parish, Louisiana. In her spare time, she likes to sing, dance, and cook.