5-2006

Unveiling the Regulatory Network for Di/tri-peptide Utilization in *Saccharomyces cerevisiae*

Houjian Cai
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Houjian Cai entitled "Unveiling the Regulatory Network for Di/tri-peptide Utilization in *Saccharomyces cerevisiae*." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Jeffrey M. Becker, Major Professor

We have read this dissertation and recommend its acceptance:

David A. Brian, Pamela L. C. Small, Todd Reynolds, Bruce McKee

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a dissertation written by Houjian Cai entitled "Unveiling the Regulatory Network for Di/tri-peptide Utilization in *Saccharomyces cerevisiae*." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Jeffrey M. Becker
Major Professor

We have read this dissertation
and recommend its acceptance:

David A. Brian

Pamela L. C. Small

Todd Reynolds

Bruce McKee

Accepted for the Council:

Anne Mayhew
Vice Chancellor and Dean of
Graduate Studies

(Original signatures are on file with official student records.)

Unveiling the Regulatory Network for Di/tri-peptide Utilization in *Saccharomyces cerevisiae*

A Dissertation
Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

Houjian Cai
May 2006

DEDICATION

My faith was never shaken. It took me 15 years from the time when I planned to earn a Ph.D degree. This work is dedicated to my family: To my passed eldest brother, who mistakenly sacrificed himself to the distorted era of his country when he still was not able to think clearly; whose spirit is my everlasting driving force for life. To my passed grandmother, who raised me up on her back, and her mother-image is in my memory forever. It was greatly unfortunate that I could not finish up my study before she passed away. To my wife, who has supported me and always makes me smarter. To my brothers, Houyong and Houren, and sister, Sujuan for caring about me and taking care of the family when I was away from home in the past years. To my mother, who always worries about my living outside the country and never gave up her faith for my success. Finally, to my daughter, Lena, who brings the joy to me. Her sweet smile makes me energetic whenever I am tired.

ACKNOWLEDGEMENTS

Life is really un-predictable. Fifteen years ago I had never known that I would finish up my Ph.D study in Dr. Becker's lab. It is my fortune that I have met so many great people in the journey of reaching this degree. The encouragement and support of these people has made possible the realization of my dream, getting a Ph.D degree. It is more than difficult to adequately express my gratitude for their help. I would like to acknowledge Dr. Dittmar Hahn for providing me an opportunity to study in his lab, which brought me from Europe to the US. He showed me how to work efficiently in being a scientist. I would also like to thank Dr. Gary Stacy for recruiting me to the University of Tennessee, Knoxville.

The dissertation committee members made it possible for me to go smoothly in my projects. I would like to thank Dr. Pamela Small, Dr. David Brian, Dr. Todd Reynolds, and Dr. Bruce McKee, for being interested in my ideas and making my time as a graduate student the most challenging adventure I have yet undertaken. I would also like to thank Dr. Dong Xu provided me a lot of fresh ideas when I started my project. My appreciation goes out to Dr. Fred Naider for providing me the toxic peptides continually, and giving me his faith for whatever I am working on.

If I could attribute any one aspect as having contributed most to my success, it would be the talented and supportive people in Becker's lab. I can not express more of my gratitude to the "lab mom" Dr. Melinda Hauser. I started my lab rotation with her. It was her charming knowledge that became one of reasons to work in the lab. She has been a dictionary for me throughout the time in the lab on everything. My special thanks also

go to Dr. Tom Masi, a king of molecular biology in the lab. I could always find answers from him about the experimental difficulties, and he usually is the person to crack the first version of my terrible writing. I owed him for spending a lot of free time on understanding my writing at home. Special thanks to Sarah Kauffman, a super-technician, who conquered the boring and helped me to transfer a plasmid more than two hundred times. Thanks to Dr. Amy Wiles, who taught me a lot of English, showed me American culture, and discussed American political issues. She shared the Transporter niche with me in the lab and provided a lot of valuable ideas. Appreciation to Dr. Yong-Hun Lee, who always challenged me in science with the final conclusion "it is not enough" and "so what". Thanks to my friend, Dr. Cagdas Son, a king in the protein chemistry and computer knowledge in the lab, who showed me a lot of tricks in working with the protein and solving all kinds of computer problems. I enjoyed his humor and shared a lot of life experience. Thanks to Steve Minkin, who came through many times when I needed a laugh. His enthusiasm to political issues and optimum to the life made me understand more about this country. Thanks to Dr. Byung-Kwon Lee, who gave me a lot of suggestion and advice in the scientific field. Special gratitude to Dr. Agnieszka Janiak for a lot of constructive discussions about my project. Thanks to Heejung Kim, George Umanah, Li-Yin Huang, who showed great passion in the lab. I appreciate the enjoyable moment they have brought to me.

To my mentor, teacher, Dr. Jeffrey Becker, more than anyone else, I am very grateful for the opportunity he gave to me to work in his lab. In Chinese, we said "one day being your mentor, forever being your father". In past five years, Jeff's influence and lessons have permeated my life and instilled me how to face the challenge and conquer

difficulties. He provided me with strength, support and direction, and gave enormous freedom, patience, and trust in my research. His critical thinking, self-confidence, and open-mind influence my research in the future. I am proud to declare my thanks.

ABSTRACT

Dipeptides and tripeptides serve as important sources of amino acids, nitrogen and carbon for the growth of all organisms. To identify genes involved in the regulation of small peptide utilization, I performed a systematic, functional examination of this process in a haploid, non-essential, single-gene deletion mutant library of *Saccharomyces cerevisiae*. In addition, we used high-throughput phenotyping in which we grew yeast cells on 284 different dipeptides or 11 tripeptides as the sole nitrogen source to dissect different mechanisms of di/tri-peptide utilization in seven genetically diverse strains. I have identified 103 candidate genes involved in regulating peptide utilization: 57 genes whose deletion decreased dipeptide utilization and 46 genes whose deletion enhanced dipeptide utilization. Since in *S. cerevisiae* the membrane transport protein Ptr2p, encoded by *PTR2,* mediates the uptake of di/tri-peptides and is a key player in the di/tri-peptide utilization process, I focused our studies on the regulation of *PTR2* expression, Ptr2p-GFP localization, and dipeptide uptake assays. Forty-two genes were ascribed to the regulation of *PTR2* expression, 37 genes were involved in Ptr2p localization, and 24 genes apparently did not affect expression or localization. Together with the high-throughput phenotyping study, we have identified gene components involved in regulation of dipeptide utilization in many cellular processes: 1) proteins involved in up- or down-regulation of *PTR2* transcription including the chromatin remodeling INO80 protein complex (Arp5p, Arp8p, Ies6p and Taf14p), transcription factors (Cup9p, Rpn4p, Stp2p, and Dal81p), polymerase mediators (Ssn3p/Srb10p, Ssn8p/Srb11p, Ssn2p/Srb9p, and Srb8p), and mRNA maturation (Kem1p and Pat1p); 2) proteins involved in the Ptr2p

trafficking system including ESCRT I, II, III protein complex; 3) proteins participating in metabolic processes including the pyruvate dehydrogenase complex (Pdx1p, Lpd1, Pdb1p, and Lat1p), the fatty acid synthase complex (Oar1p, Mct1p, and Etr1p), and the glycine decarboxylase multienzyme complex (Gcv2p, Gcv3p, and Lpd1p); 4) proteins involved in other cellular processes, such as Ybt1p, a protein located at the vacuole membrane likely related to the storage of dipeptides in the cytosol and Dal5p previously identified as an allantoate/ureidosuccinate permease, which facilitates di/tri-peptide transport. Specifically, Dal5p is involved in the uptake of non-N-end rule dipeptides. Moreover, even in the absence of Dal5p and Ptr2p, an additional activity - almost certainly the periplasmic asparaginase II Asp3p - facilitates the utilization of dipeptides with C-terminal asparagine residues by a different strategy. Another, as yet unidentified activity, enables the utilization of dipeptides with C-terminal arginine residues. The identified genes regulating dipeptide utilization were distributed among most of the Gene Ontology functional categories indicating a very wide regulatory network involved in transport and utilization of dipeptides in yeast. It is anticipated that further characterization of how these genes affect peptide utilization should add new insights into the global mechanisms of regulation of transport systems in general and peptide utilization in particular.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I

GENERAL INTRODUCTION

A Review of the Di/tri-peptide Utilization Literature

The utilization of di/tri-peptides is an important cellular activity and has been documented in all organisms from archaea to bacteria, plants, and animals. The introduction of this chapter is a literature survey of what was known about peptide utilization and transport before I undertook the research presented in this dissertation.

Proteins and peptides as a major energy source:

Organisms utilize amino acids and peptides from exogenous proteins for nitrogen/carbon sources and protein synthesis. In the natural environment, the source of proteins and peptides for microorganisms is mainly from decomposition of dead organisms and secreted proteins of living organisms. While microorganisms degrade proteins and peptides in the surrounding environment where they reside, in mammals the digestion of proteins and peptides begins in the stomach and intestine. Both dietary and luminally-secreted proteins and polypeptides undergo limited or complete proteolysis by aminopeptidases, carboxypeptidases, endopeptidases and other pancreatic and intestinal proteolytic enzymes. The brush border peptidases located at the luminal surface of the gut further degrade oligopeptides into di/tri-peptides and amino acids.

Degradation of proteins and peptides:

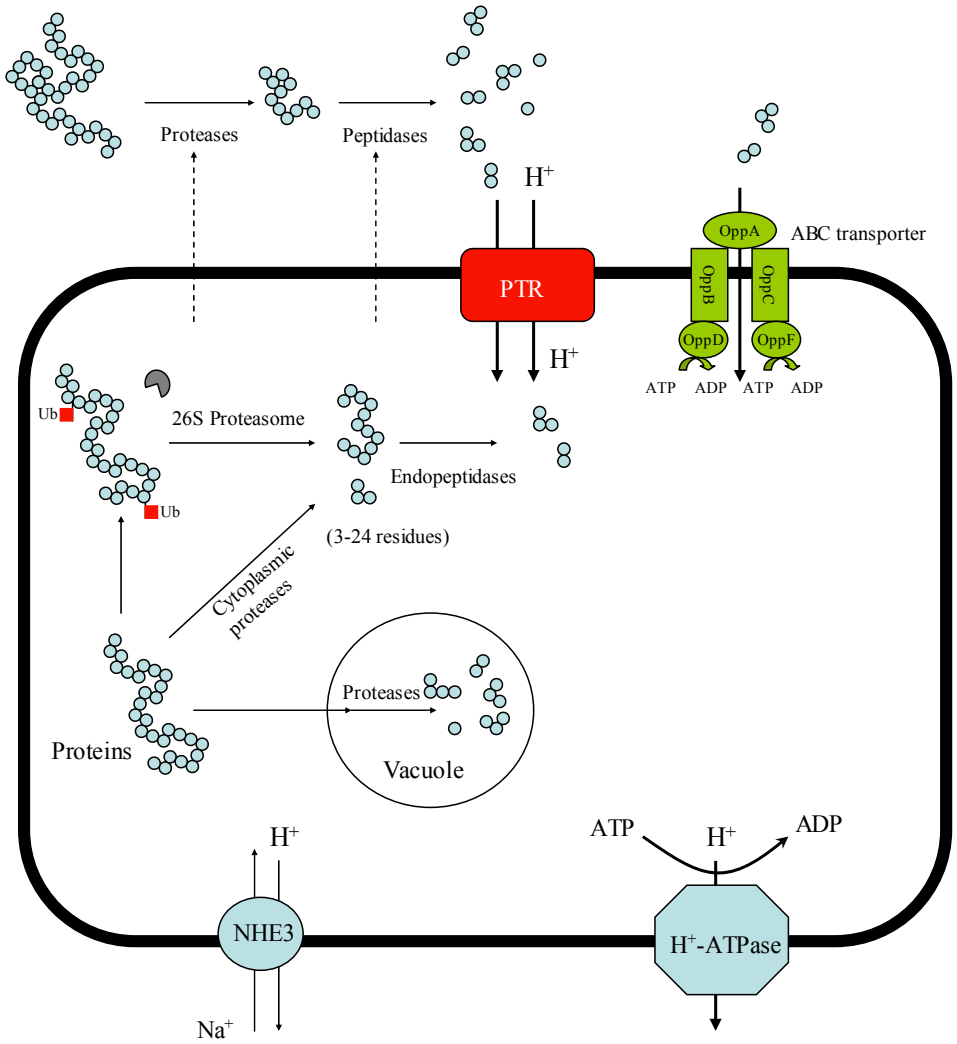
The degradation of proteins is an important process not only for providing a nutritional source for the growth of organisms, but also for the defense against proteinaceous toxins and modulation of the host immune system (DANIEL 2004;

PRITCHARD and COOLBEAR 1993). Degradation of proteins and peptides, mediated by a set of proteases, or peptidases, can take place extracellularly or intracellularly (Figure 1).

Extracellular degradation: Extracellular degradation is mediated by many hydrolytic enzymes, and proteolytic systems can produce oligopeptides, di/tri-peptides and amino acids useful for microbial growth. Proteolytic digestion is particularly important for some pathogenic and parasitic organisms to acquire peptides. For example, the genome of the fungal pathogen *Candida albicans* harbors ten secreted acid proteinase (SAP) genes encoding extracellular aspartic proteinases that appear to allow adaption to variable tissue environments such that these SAP enzymes enable *C. albicans* to grow on medium containing serum albumin as a sole nitrogen source (HUBE and NAGLIK 2001) and can function in environments from pH 2 to 7. SAPs have been shown to be involved during the infection process and are required for pathogenicity (NAGLIK *et al.* 2003). The parasitic organism *Plasmodium falciparum*, the causative agent of intraerythrocytic malaria, can secret proteolytic enzymes plasmepsins I and II (two aspartic proteases), falcipain (a cysteine protease), and endoproteinase to generate a variety of peptides with average length of 8.4 amino acid residues from host hemoglobin. This parasite may solely rely on peptides for growth (KOLAKOVICH *et al.* 1997).

Hydrolytic enzymes can be secreted by the signal peptide-dependent general export pathway, and this secretion process is ubiquitous from bacteria to mammals. Signal peptidase, an endoprotease, removes the N-terminal signal sequence from secretory proteins, and leads to the release of mature polypeptides into the surrounding medium (DEV and RAY 1990). Hydrolytic enzymes can also be exported by ABC

Figure1 Protein degradation and di/tri-peptide transporters in a general model. Exogenous proteins are degraded into oligopeptides extracellularly by secreted proteases, and further degraded into di/tri-peptides and amino acids by peptidases. Proteins can also be degraded into oligopeptides intracellularly by the 26S proteasome via the ubiquitin pathway or by cytoplasmic proteases, and further degraded into di/tri-peptides by endopeptidases. Proteins can be also degraded into peptides and amino acids in the vacuole in eukaryotic organisms. Extracellular di/tri-peptides can be transported into the cell by the PTR system and by ABC transporters. The translocation of di/tri-peptides by the PTR system is coupled with protons, and the H^+ concentration is regulated by several means including the Na+/H+ exchanger (NHE) and the H+-ATPase. Extracellular di/tri-peptides can also be translocated into the cell by an ABC transporter in prokaryotic organisms. This process is coupled with ATP hydrolysis.


Proteases
Peptidases
H+
PTR
H+
OppA
ABC transporter
OppB
OppC
OppD
OppF
ATP
ADP
ATP
ADP
Ub
26S Proteasome
Endopeptidases
Ub
(3-24 residues)
Cytoplasmic proteases
Proteins
Proteases
Vacuole
H+
NHE3
Na+
ATP
H+
ADP
H+-ATPase

transporters in both prokaryotic and eukaryotic organisms. In Gram negative bacteria, proteins lacking an N-terminal signal peptide can be transported by ABC protein-mediated exporters (BINET *et al.* 1997; WANDERSMAN 1998). In addition, a variety of proteinases and peptidases are associated with the cell wall and are involved in extracellular polypeptide degradation (GONZALES and ROBERT-BAUDOUY 1996).

Intracellular degradation: Peptides can also be generated intracellularly due to protein turnover. For example, about 1-2% of intracellular proteins in log phase bacteria cells and 5-12% of proteins in stationary phase cells undergo a degradation process (GONZALES and ROBERT-BAUDOUY 1996). Proteins are hydrolyzed into oligopeptides by the ubiquitin/proteasome system and cytoplasmic proteases/peptidases in prokaryotic and eukaryotic organisms (Figure 1) and by other proteolytic enzymes in vacuoles in eukaryotes. In the ubiquitin system, target proteins are covalently tagged with multi-Ub and processed by an ATP-dependent 26S proteasome containing a 20S core protease and two 19S particles. The 19S particles mediate the binding and unfolding of the target proteins and then transfer them to 20S core for degradation (XIE and VARSHAVSKY 2000). Peptides with three to twenty-four amino acid residues are generated by 26S proteasome with about two-thirds of the generated peptides having less than 8 amino acid residues (SARIC *et al.* 2004). The oligopeptides produced in this process are further degraded into di/tri-peptides or amino acids for nutrient recycling by aminopeptidases and other peptidases. For example, tripeptides can be released from a free N-terminus of larger peptides in eukaryotic cells by tripeptidyl-peptidase II (TPP II), an aminopeptidase associated with the plasma membrane (TOMKINSON and LINDAS 2005). Furthermore, di/tri-peptides derived from proteins or polypeptides can be further degraded into amino

acids by peptidases in both prokaryotic and eukaryotic organisms. For instance, at least four aminopeptidase activities and one single dipeptidase activity were found in yeast and were capable of di/tri-peptide degradation (ROSE *et al.* 1979); and at least five peptidases, pepN, pepC, pepO, pepX, and pepT are involved in di/tri-peptides degradation in *Lactococcus lactis* (KUNJI *et al.* 1996b). Some bacterial strains also possess beta-peptidyl aminopeptidase, which can use unnatural beta-di/tri-peptides for carbon and nitrogen sources (GEUEKE *et al.* 2005). The intracellular or extracellular degradation of proteins provides a variety of peptides and amino acids for the growth and reproduction.

Biological significance of di/tri-peptides

A large number of different size peptides including di/tri-peptides are produced by both microbial proteolytic systems and by gastrointestinal digestion of dietary proteins, such as milk, animal meat, maize, wheat, soybean, or egg (KORHONEN and PIHLANTO 2003; MEISEL and BOCKELMANN 1999; PELLEGRINI 2003; YAMAMOTO *et al.* 2003). These peptides contribute to many cellular functions. For example, casein-derived peptides, produced by the activity of lactic acid bacteria in milk, contain regulatory compounds with hormone–like activity, and other physiologically functional peptides such as opioid peptides, immunostimulating peptides, and angiotension I converting enzyme inhibitors (MEISEL and BOCKELMANN 1999). Some oligopeptides (peptides containing more than three amino acids) are bioactive and are involved in Ca2+, iron, or steroid metabolism, have antiviral/antimicrobial/antifungal or antitumor activity, are neuroactive, inhibit proteases, regulate growth, act as hormones, or are involved in electrolyte turnover, fertility, and coagulation (ADERMANN *et al.* 2004; PELLEGRINI 2003).

However, more and more research has documented that di/tri-peptides have a great physiological significance as well. There are 400 possible different dipeptides and 8000 possible tripeptides composed of the 20 naturally-occurring L-α-amino acids.

Di/tri-peptides serve as a nitrogen and/or carbon source and as a source of amino acids: Since di/tri-peptides can be further degraded by peptidases intracellularly to release amino acids, these peptides can satisfy amino acid auxotrophic requirements in prokaryotic and eukaryotic microorganisms (BECKER *et al.* 1973; MONETON *et al.* 1986) (Table 1).

Di/tri-peptides possess physiological activities whose molecular mechanism has not been elucidated: Di/tri-peptides are not only important nutrient sources, but also some of them possess additional biological activities. A diverse group of biologically active peptides such as antiviral agents, neuroactive peptides, enzyme regulators and inhibitors, hormonal peptides, and immunoactive peptides have been isolated from bacteria, fungi, plants and animals (KUMAR and BHALLA 2005).

Analysis of some naturally-occurring dipeptides reveals a variety of physiological functions (Table 1). Kyotorphin (Tyr-Arg), an analgesic neuropeptide, has morphine-like and naloxone-reversible effects, possibly due to depolarization of the enkephalinergic neurons, which leads to a release of Met-enkephalin from the striatum and spinal cord (SHIOMI *et al.* 1981). In addition, Tyr-Arg also inhibits the isoprenaline-induced increase in twitch tension of rat cardiac muscle, and this inhibition can be reversed by another dipeptide, Leu-Arg (LI *et al.* 2006). Lactokinin (Tyr-Leu) derived from milk proteins by

Table 1 The biological functions of di/tri-peptides and di/tri-peptide derived products

Functions	Di/tri-peptides and Components	Activity	References
Nutrient source	Some Met and Lys contained di/tri-peptides; 284 of 400 possible dipeptides and 11 tripeptides	Auxotrophic requirement; Nitrogen source	(BECKER *et al.* 1973; MONETON *et al.* 1986)
Naturally-occuring dipeptides serve as effective drugs	Kyotorphin (Tyr-Arg)	Analgesic neuropeptide; inhibit isoprenaline- induced increases in twitch tension of rat cardiac muscle	(LI *et al.* 2006; SHIOMI *et al.* 1981)
	Antagonist of kyotorphin (Leu-Arg)	Reverses the inhibitory effect of kyotorphin	(LI *et al.* 2006)
	Lactokinin (Tyr-Leu)	ACE-inhibitory effect	(MEISEL and BOCKELMANN 1999)
Synthetical dipeptides serve as effective drugs	Aspartame (Asp-Phe)	Synthetic nonnutritive sweetener/ Sweet taste	(ERBELDINGER *et al.* 2001)
	Dopa derivative (Dopa-Phe)	Increased absorption of Dopa; effective for Parkinson's disease	(TAMAI *et al.* 1998)
Naturally-occuring tripeptide serve as effective drugs	VPP (Val-Pro-Pro) ; IPP (Ile-Pro-Pro)	Antihypertension	(YAMAMOTO 1997)
	LLY (Leu-Leu-Tyr); YGG (Tyr-Gly-Gly)	Immunopeptide; stimulates the activities of cells of the immune system	(MEISEL and BOCKELMANN 1999)
	YSL or YSV(Tyrosyl-seryl-leucine/valinine)	Inhibit tumor growth	(JIA *et al.* 2005); (YAO *et al.* 2005)
	Thyrotropin releasing hormone (TRH) (L-pyroglutamyl-L-histidyl-L-Prolinamide)	Neuroprotective activity	(FADEN *et al.* 2005)
	Cyclized dipeptides (Cyclo-histidyl-proline diketopiperazine); pyroGlu-Glu-Pro-NH2	TRH-like peptides; Neuroprotective activity	(FADEN *et al.* 2005; PROKAI-TATRAI *et al.* 2003)
	GPE tripeptides (Gly-Pro-Glu)	Stimulates acetylcholine and dopamine release; Neuroprotective activity	(ALONSO DE DIEGO *et al.* 2005)
Di/tri-peptidomimetic products	Substrates similar to tripeptides (Beta-lactam; ACE inhibitors; Bestatin)	Antibiotics; treat hypertension and failing circulatory output; Antitumour	(MEREDITH and BOYD 2000)

the digestion has angiotensin-I-converting enzyme (ACE) inhibitory activity (Meisel and Bockelmann 1999).

Many modified dipeptides have also been chemically synthesized for commercial usage (Table 1). For example, aspartame (Carbobenzoxy-Asp-Phe-OMe) has been widely used as a non-nutritive sweetener (ERBELDINGER *et al.* 2001). The dipeptide derivative, L-Dopa-L-Phe is synthesized to target PepT1 and PepT2, the two major di/tri-peptide transporters in mammals. This compound, significantly increased the absorption of L-dopa (L-3,4-dihydroxyphenylalanine), an effective drug for the treatment of Parkinson's disease, in a tested Caco-2 cell line (TAMAI *et al.* 1998).

Some naturally-occurring tripeptides also showed physiological important activities including antihypertension, stimulation of immune system, inhibition of tumor growth, and neuroprotective activity (Table 1). For example, Val-Pro-Pro and Ile-Pro-Pro, generated from casein by extracellular proteinase followed by peptidase activity in sour milk, were found to act as antihypertensive peptides (YAMAMOTO 1997). In addition, Leu-Leu-Tyr and Tyr-Gly-Gly also derived from the degradation of milk proteins are immunopeptides, which increase the proliferation of human peripheral blood lymphocytes and stimulate activity of the immune system (MEISEL and BOCKELMANN 1999). The tripeptides Tyr-Ser-Leu or Tyr-Ser-Val inhibit the growth of human hepatocarcinoma and increase the survival capability of mice carrying tumor cells (JIA *et al.* 2005; YAO *et al.* 2005). Thyrotropin-releasing hormone (TRH) (L-pyroglutamyl-L-histidyl-L-prolinamide) is a brain hormone and a neuro-tripeptide modulator, which has considerable neuroprotective effects in spinal cord injuries (FADEN *et al.* 2005). The related TRH metabolic product, cyclo-His-Pro (CHP) and pyroGlu-Glu-Pro-NH_2 have a

similar function as that of TRH and CHP and are more resistant to enzymatic degradation (FADEN *et al.* 2005). Tripeptide GPE (Gly-Pro-Glu) binds N-methyl-D-aspartate (NMDA) receptor and promotes the release of acetylcholine and dopamine and shows neuroprotective properties (ALONSO DE DIEGO *et al.* 2005).

Di/tri-peptides regulate cellular processes by mechanisms elucidated at the molecular level: Some di/tri-peptides can regulate gene transcription, protein translation, and potentially modulate enzyme activity and function of regulatory proteins (Table 2) (TROITSKAYA and KODADEK 2004). First, N-end rule dipeptides [peptides with basic (Arg, His, or Lys) or bulky (Ile, Leu, Phe, Trp, or Tyr) residues at the N-terminus] act as ligands to modulate *PTR2* transcription in yeast. Dipeptides with N-terminal residues can bind to the scaffold protein Ptr1p to promote Cup9p degradation via a ubiquitination pathway and up-regulate the transcription of the *PTR2* (TURNER *et al.* 2000). Second, tripeptides such as PAT (Pro-Ala-Thr) and SPF (Ser-Pro-Phe) can recognize the stop codon and serve as functional elements to decipher stop codons in the translation process. These tripeptides can serve as translational release factors to terminate polypeptide synthesis (ITO *et al.* 2000; NAKAMURA and ITO 2002). Third, some di/tri-peptides can regulate enzyme activities. For instance, tripeptidomimetic compound Ac-Asp-Tyr(SO3H)-Nle-NH2 inhibits the function of protein tyrosine phosphatase 1B. As a result, this tripeptide can negatively regulate the insulin signal by dephosphorylation of tyrosine residues on the insulin receptor and promote insulin resistance (LARSEN *et al.* 2002). Furthermore, di/tri-peptides can also serve as, or promote, agonists or antagonists to

Table 2 The mechanisms of biological functions of di/tri-peptides and di/tri-peptide derived products

Functions	Di/tri-peptides and Components	Activity	References
Regulation of transcription	N-end rule dipeptides (dipeptides with bulky or basic N-terminal residues)	Transcriptional regulation on *PTR2*	(TURNER *et al.* 2000)
Regulation of translation	PAT (Pro-Ala-Thr) and SPF (Ser-Pro-Phe) tripeptides	Functional elements to decipher stop codons	(NAKAMURA and ITO 2002)
Regulation of enzyme activity	Ac-Asp-Tyr (SO3H)-Nle-NH2	Inhibitor of protein tyrosine phosphatase 1 B (PTP1B); improve insulin resistance	(LARSEN *et al.* 2002)
Modulation of receptor function	Melanocyte-stimulating hormone release-inhibiting factor (PLG) and analog PAOPA:L-prolyl-L-leucyl-glycinamde	Modulating agonist binding to human dopamine (DA) receptor	(VERMA *et al.* 2005)
	fMLF (N-formyl-L-Methionyl-L-leucyl-L-phenylalanine)	Responsible for neutrophil functions	(DALPIAZ *et al.* 2003)

modulate the function of receptors. For example, hypothalamic tripeptide PLG and its analog 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA) enhance agonist binding to the human dopamine receptor (VERMA *et al.* 2005). The formylpeptide family, derived from bacterial proteins and disrupted cells, as well as the synthetic tripeptide N-formyl-L-methionyl-L-Leucyl-L-phenylalanine can interact with specific receptors in neutrophil plasma membranes and activate a transduction pathway in response to bacterial infection and tissue damage (DALPIAZ *et al.* 2003).

Di/tri-peptides serve as promoieties, soluble agents for covalent attachment of drugs: Di/tri-peptides are also used as the soluble agents for covalent attachment of drugs to increase drug delivery efficiency. The conjugation of di/tri-peptide to drugs usually alters the physical and chemical properties of drugs and di/tri-peptides can serve as a vehicle to deliver drugs via targeting hPepT1/2 transporters in humans (Table 3) (TEMSAMANI and VIDAL 2004). First, di/tri-peptidomimetic prodrugs are designed to increase the solubility and enhance the influx by increasing the affinity of the modified compound. For example, Val-Val-ACV (valyl-valyl-acyclovir), an antiviral nucleoside used to treat herpetic epithelial and stromal keratitis, shows high water-solubility in the physiological pH range in vivo and an increase of absorption of drugs due to high affinity toward the hPEPT1 (ANAND *et al.* 2003a; ANAND *et al.* 2003b). The conjugation of polyoxins and nikkomycins, antifungal agents for inhibiting chitin synthetase, with di/tri-peptides increases the permeability of compounds via the transport system (KRAINER *et al.* 1991). Second, di/tri-peptidomimetic prodrugs are designed to decrease efflux. Val-Val-SQV and Gly-Val-SQV uptake into cells are significantly enhanced by the reduced secretion

Table 3 The significance of di/tri-peptides serving as vehicles to deliver drugs

Significance	Di/tri-peptides and Components	Activity	References
Increase solubility and influx	Val-Val- acyclovir (ACV)/ Gly-Val-ACV	Antiviral nucleoside acyclovir	(ANAND *et al.* 2003a; ANAND *et al.* 2003b)
	Di/tri-peptidyl polyoxin and Nikkomycin	Antifungal drugs	(KRAINER *et al.* 1991)
Decrease efflux	Val-Val-saquinavir (SQV)	Enhanced cellular permeability of saquinavir (SQV) and modulate p-gp mediate efflux	(JAIN *et al.* 2005)
Increase stability	3-(4'-geranyloxy-3'-methoxyphenyl)-2-trans-propenoic acid -Ala-Pro	Activated by intestinal ACE and used in the treatment of different forms of colon cancer	(CURINI *et al.* 2005)

of SQV, an effective HIV protease inhibitor, due to fact that the membrane bound efflux protein P-gp can not recognize the tripeptide prodrug (JAIN *et al.* 2005). Third, di/tri-peptidomimetic prodrugs are designed to stabilize drugs, or protect them from different pH or from enzymatic digestion such as pancreatic and intestine peptidases. Ala-Pro dipeptide is used to conjugate with the anticancer compound 3-(4'-geranyloxy-3'-methoxyphenyl)-2-trans-propenoic acid. This dipeptidyl prodrug is highly stable but can be activated by intestinal angiotension converting enzyme (ACE, dipeptidyl carboxypeptidase) located on the external side of the intestinal brush border membrane of the small and large intestines to release the anticancer compound (CURINI *et al.* 2005). Dipeptides with unnatural amino acids, such as D-Asp-Ala and D-Glu-Ala have also been used to couple with drugs for the protection of the drug from enzyme digestion by enhancing the permeability across the intestine (FRIEDRICHSEN *et al.* 2001). Furthermore, dipeptides also act as effective promoieties to deliver unnatural amino acids. (+)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid is a potent agonist for metabotrophic glutamate receptors, and suppresses enhanced glutamatergic excitations in brain synapses blocking anxiety. A derivative of this compound containing a N-terminal dipeptide addition showed an 8-fold increase in drug absorption and a 300-fold increase in potency compared with its parent chemical (BUENO *et al.* 2005). Other dipeptides, such as Val-Asp, Phe-Gly, and Phe-Asp, were also demonstrated to be promising pro-moieties in drug delivery (VABENO *et al.* 2004a; VABENO *et al.* 2004b). In spite of the fact that many studies have been performed to elucidate the structural requirements for an optimal peptide transport substrate for hPEPT1/2 (DANIEL 2004; MEREDITH and BOYD 2000; NIELSEN and BRODIN 2003), it is not entirely clear how to design the peptide portion of

the prodrug to facilitate drug delivery via PepT1/2 peptide transporters, although the size of drug and modification of N-terminal amino acid side chain should be taken into consideration (FRIEDRICHSEN *et al.* 2001; ERIKSSON *et al.* 2005).

Peptide transporter systems

Di/tri-peptide transport has been documented in a wide variety of organisms including bacteria, fungi, plants, invertebrates, and vertebrates. Influx of di/tri-peptides is mediated by two types of transport systems, ATP-binding cassette (ABC) peptide transporters and proton-driven peptide transporters (Table 4) (STEINER *et al.* 1995).

ABC transporter systems: Three ABC peptide transporters, oligopeptide permease (Opp), dipeptide permease (Dpp), and other single protein peptide permeases have been reported in prokaryotic organisms. The Opp transport system consists of five essential polypeptide proteins, OppA, OppB, OppC, OppD, and OppF (Figure 1). OppA is a substrate binding protein located in the periplasmic space between the cytoplasmic membrane and the outer membrane in gram-negative bacteria, and the binding of a substrate leads to the conformational change of this protein. Similar to eukaryotic cells, which typically have 12 transmembrane domains in their permeases, OppB and OppC are two transmembrane proteins each containing six TMs for a total of 12 membrane-spanning domains. Together, these two proteins create a transmembrane pore for substrate translocation. OppD and OppF located at the cytoplasmic membrane are related

Table 4 ATP-binding cassette (ABC) transport systems involved in di/tri-peptide utilization in prokaryotes

Identified systems	Transmembrane domains	Substrate	Involved proteins	Regulation	Representative organisms
Dpp	2 six-trans-membrane domains	Dipeptides; Limited in tripeptides	Multiple proteins (DppA, DppB, DppC, DppD, DppE)	Constitutively expressed; phosphate limitation down regulates DppA	*E. coli* *S. tryphimurium* *P. Aureuginosa* *S. pyogenes*
Opp	2 six-transmembrane domains	High affinity for tripeptides; limited to dipeptides	Multiple proteins (oppA, oppB, oppC, oppD, oppF)	Induced in anaerobic conditions and media containing Leu Ala; Repressed through Lrp protein in E.coli;	*E. coli* *S. tryphimurium* *P. aureuginosa* *B. burgdorferi* *B. subtilis*
		4-35amino acids residues		Repressed by codY and branched amino acids de-repressed.	*L. lactis*
Opt	Not-reported	larger than tetrapeptides	Multiple proteins [optABCDF two oligopeptide binding proteins (OptS and OptA)]	unknown	*L. lactis*
DtpP	Not-reported	di-/tripeptides (branched-chain Hydrophobic peptides)	Single protein	Induced in the presence of di- and tripeptides containing branched-chain amino acids	*L. lactis*

to ATP binding and hydrolysis, where energy is needed for substrate translocation (PAYNE and SMITH 1994). The Opp transport system has a high affinity for tripeptides and has limited activity for transporting dipeptides in *E.coli* and *S.typhimium*. However, Opp system can also transport 3-5 amino acid residues in *B.subtilis* (SOLOMON *et al.* 2003), and peptides of 4 to 35 amino acids in *L. lactis* (DETMERS *et al.* 1998; DOEVEN *et al.* 2004). OppA is the major determinant of substrate specificity for the Opp transport system, and has been identified in other bacterial organisms with limited differences in OppA (LIN *et al.* 2001). To adapt to the changes in the environment, several versions of OppA substrate binding proteins OppA-1,-2,-3, -4, -5 have also been identified in *B. burgdorferi*. For example, OppA-5 is regulated by temperature stress; while the other functional homolog of OppAs might be responsible for other environmental conditions. Multiple versions of these substrate binding proteins possibly reflect the capabilities of peptide uptake for nutrient requirements and possess an evolutionary advantage in different environments (LIN *et al.* 2001).

Similar to the Opp transport system, the dipeptide permease (Dpp) system also contains five polypeptides (DppA to DppE) and is involved in the uptake of essential amino acids, and dipeptide chemotaxis (Table 4) (PODBIELSKI and LEONARD 1998). The Dpp transporter system transports primarily dipeptides and to a limited extent, tripeptides (PAYNE and SMITH 1994). DppA has structural homology to the substrate-binding OppA protein (ABOUHAMAD *et al.* 1991); DppB and DppC are transmembrane proteins involved in the translocation of substrates; DppD and DppE are membrane associated ATPases that provide energy for translocation of the substrates.

Other ABC transporters such as oligopeptide transporter (Opt system) have also been identified (Table 4). Similarly, the Opt transport system contains multiple proteins OptA, OptB. OptC, OptD, OptF. In *L. lactis*, OptS and OptA, two binding proteins are identified, which might function at different environment conditions. Compared with Opp or Dpp system, Opt has a different genetic organization and mainly transports larger than tetrapeptides as its substrate (LAMARQUE *et al.* 2004). Single protein ABC dipeptide permeases such as DtpP have also been identified in *L. lactis* (KUNJI *et al.* 1996a). DtpP preferentially transports di/tri-peptides composed of hydrophobic residues (branched-chain amino acids). DtpP is driven by ATP or a related energy-rich phophorylated intermediate since the activity is inhibited by o-vanadate, an inhibitor of ATP production in the glycolytic pathway. DtpP is induced by its own substrates such as in the presence of di/tri-peptides with branched-chain amino acids (FOUCAUD *et al.* 1995). However, how ATP is involved in the single protein peptide transport remains unknown.

Regulation of ABC transporter systems: ABC transporter systems are regulated by the cell's environment. Some small molecules in the media can even trigger the expression of the entire transporter activity. For example, the Dpp transporter system is constitutively expressed, but the transport ability is down-regulated when bacteria grow in phosphate-limited medium due to decreased expression of DppA (ABOUHAMAD *et al.* 1991). The Opp transporter system is induced under anaerobic conditions, while the amino acids leucine and alanine up-regulate peptide transport by increasing transcriptional expression of OppA in *E. coli* and *S. tryphimurium*. In addition, some regulatory proteins have been identified that are involved in the expression of the transporter system at the

transcriptional level. For instance, the expression of the Opp operon is repressed by the leucine-responsive regulatory protein (Lrp) in *E. coli*. Mutation of this gene results in constitutive expression of the Opp system (CALVO and MATTHEWS 1994). However, how the Opp system is regulated by Leu and how the Lrp repressor is related to leucine upregulation remains unclear. It might be possible that a sensor system can detect leucine which results in the regulation of Opp transcription. In *L. lactis,* CodY, a transcriptional regulator, interacts with the upstream promoter of the Opp transport system, and branched amino acids directly impact the binding of repressor CodY on the promoter region of the Opp operon (DEN HENGST *et al.* 2005).

Proton-driven di/tri-peptide transport: The proton oligopeptide transporter (POT) or PTR superfamily is another type of peptide transporter involved in the uptake of di/tri-peptides. PTR transporters are symporters in that the influx of di/tri-peptides is coupled with the uptake of protons. As a result, this process leads to a decrease in the pH in the cytosol. The fluctuation of pH in the cytosol is regulated by an antiporter, Na^+/H^+ exchange transporter (NHE) in both prokaryotic and eukaryotic organisms (BOOTH 1985; MADSHUS 1988). NHE exports the proton by uptaking Na^+ across the plasma membrane into the cytosol. Furthermore, Na^+/K^+ pump regulates Na+ intracellular concentration by the exchange of K^+ (Figure 1). In addition, H^+-ATPase is also involved in the proton homeostasis. H^+-ATPase pumps protons out of the cell by hydrolysis of ATP.

PTR family members are present in all cells studied to date (HAUSER *et al.* 2001). At least 40 members are classified into the PTR family in both prokaryotic and eukaryotic organisms (SAIER *et al.* 2006). Among theses transporters, DtpT, Ptr2p, and

PepT1/2 are the most studied peptide transporters in bacteria, yeast, and mammals, respectively (Table 5).

In prokaryotic organisms, the DtpT transporter is predicted to contain 12 transmembrane domains and has high affinity for transporting hydrophilic and charged peptides in *L. lactis* (HAGTING *et al.* 1997). DtpT is also identified in *Listeria monocytogenes*, and is involved in salt stress protection and might also contribute to the virulence of *L. monocytogenes* (WOUTERS *et al.* 2005). In addition, several uncharacterized *E.coli* transporters including YhiP, f493 and Yjd also have the ability to transport di/tri-peptides (SAIER 1999). Another type of peptide transporter, tripeptide permease (Tpp) was identified in *S. typhimurium*, however, it does not carry the signature sequence of PTR transport family nor is it an ABC transporter. The Tpp system has a restricted range of tripeptide substrates containing hydrophobic residues and is not able to transport substrates larger than tetrapeptides (SMITH *et al.* 1999). Expression of Tpp is induced under anaerobic conditions and is positively regulated by the gene products of ompR and anvZ (ABOUHAMAD *et al.* 1991).

In eukaryotic organisms, two distinct peptide transport systems have been reported in a variety of organisms (Hauser et al. 2001): the PTR (Peptide TRansport) system transports di/tri-peptides (STEINER *et al.* 1995) and the OPT (OligoPeptide Transport) system highly favors the transport of peptides of four to five amino acid residues and also glutathione (LUBKOWITZ *et al.* 1997; MIYAKE *et al.* 2002). Both the PTR and OPT transport systems are predicted to contain twelve transmembrane domains, and have specific signature sequences distinguishing them from one another as well as from all other proteins in the database (HAUSER *et al.* 2001). Ptr2p, encoded by the *PTR2*

Table 5 PTR (POT) transporter system in bacteria, yeast and mammalian organisms and the regulatory mechanisms

Transporter Functions	NHE activity	Gene expression or mRNA stability	Protein trafficking	Organism
DtpT	Unknown	1) Induced in anaerobic condition; 2) Up-regulated by Leu and Ala	unknown	Bacteria
PTR2	Unknown	1) Poor nitrogen medium 2) N-end rule dipeptides enhance PTR2 expression 3) Ssy1p-Ptr3p-Ssy5p protein complex induces *PTR2* expression. 4) Stp1/2p is PTR2 transcriptional activation. 5) Cup9p represses *PTR2* transcription 6) Ptr1p, Ubc2p and Ubc4p are involved in the degradation of Cup9p.	1) Protein components of ESCRT I, II, III complex 2) Npr1p, Lst4p	Yeast
PEPT1/2	Intracellular Ca2+ concentration decreases uptake ability	1) High protein diet, fasting, or starvation increases expression. 2) Presence of Phe,Arg, Lys and Gly-Sar, Gly-Phe, Lys-Phe,Asp-Lys increases PEPT1/2 expression via influence the level of transcriptional factor AP-1 3) Tissue specific expression 4) Epidermal growth hormone and thyroid hormone 5) Ligand of σ receptor 6) Organism development, injury and disease states	1) Insulin and leptin increase the amount of transporters in plasma membrane	Mammalian organisms

gene, is the only member of the PTR system transporting di/tri-peptides in *S. cerevisiae.* Highly conserved regions of the PTR family are located in TM2, TM3, and TM5 and the intracellular loop between TM2 and TM3 (MEREDITH and BOYD 2000). Another di/tri-peptide transporter Dal5p has been recently discovered in yeast but does not contain the PTR signature sequence. Dal5p has di/tri-peptide transport activity, and additionally transports allantoate/ureidosuccinate (HOMANN *et al.* 2005).

In mammals, two major di/tri-peptides transporters, PepT1 and PepT2 of the PTR system have been utilized for drug delivery. PepT1 and PepT2 were first identified in the intestine and kidney, respectively (FEI *et al.* 1994; LIU *et al.* 1995). PepT1 has a low affinity but a high capacity for di/tri-peptide transport, while PepT2 has a low capacity but high affinity. Expression and activity of these peptide transporters were also shown in other organs such as lung, placenta, mammary gland, pancreas, pituitary gland, reproductive organs, and the central nervous system (LU and KLAASSEN 2005). Two other peptide transporters, PHT1 and PHT2, have also been identified to transport free histine and certain di/tri-peptides (DANIEL and KOTTRA 2004; MEREDITH and BOYD 2000; NIELSEN and BRODIN 2003). PepT1/2 transporters provide the means for absorption of nutritional peptides and peptidomimetic drugs. Peptidomimetic substances such as beta-lactam antibiotics (Cefadroxil, Cefadrine, cyclacillin, Cefixime, cephalosporins and Ceftibuten), the anticancer compound Bestatin, and angiotension-converting enzyme (ACE) inhibitors (Captopril, Enalapril, and Fosinopril) can also be transported by peptide transporters (RUBIO-ALIAGA and DANIEL 2002), and has been commercialized for treating many diseases. The substrate specificity of these transporters has provided an

mpetus to many pharmaceutical companies to develop peptide transporters as drug delivery systems (HERRERA-RUIZ and KNIPP 2003; LEE 2000).

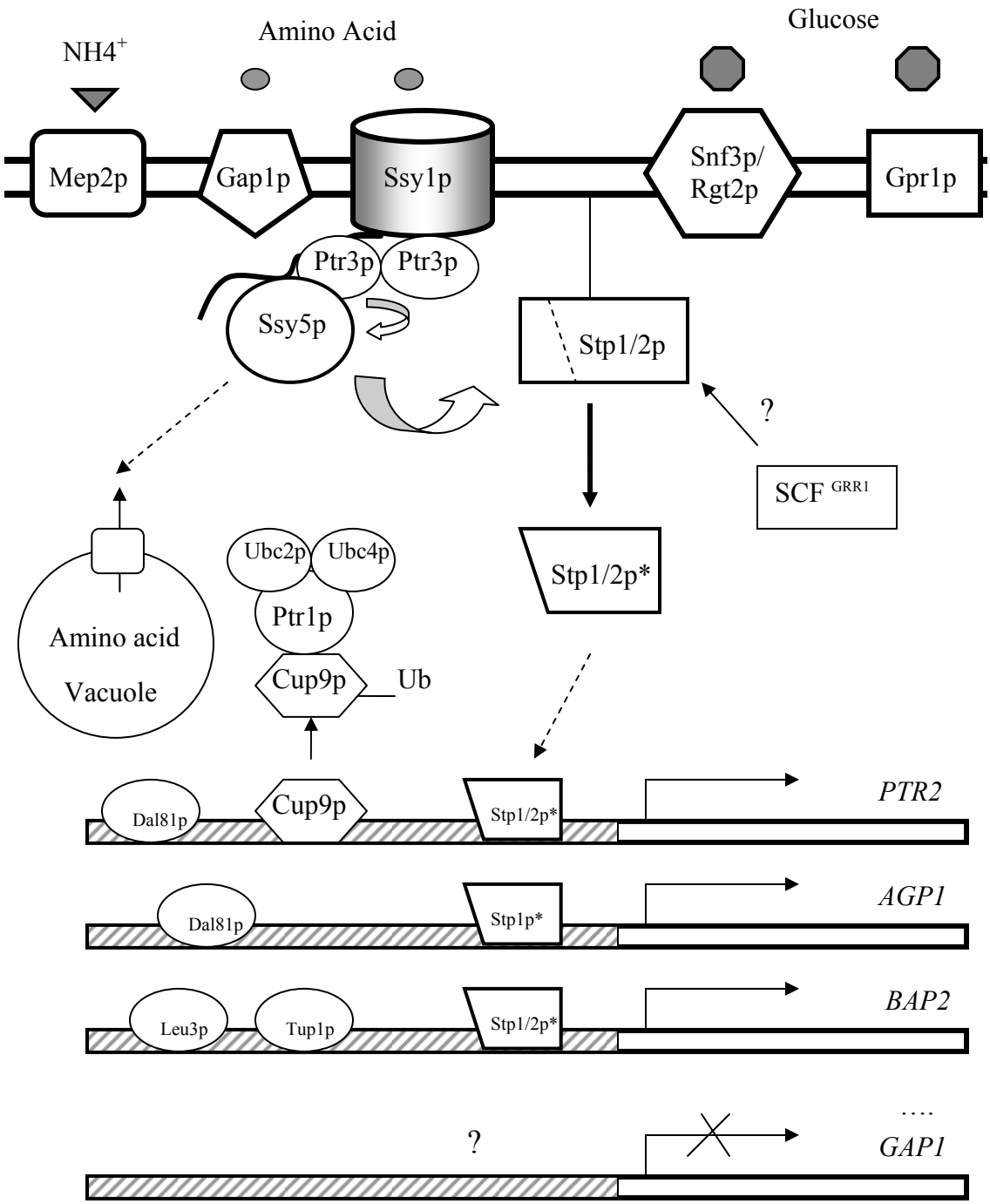
Regulation of PTR transport system: It is not clear whether the members of the PTR transport system are regulated in a similar pattern, however understanding the regulation of one peptide transporter might shed light on the study of another. In prokaryotic organisms, DtpT expression is induced under anaerobic conditions, and growth medium containing Leu or Ala up-regulates peptide transport (PAYNE and SMITH 1994). Further research is needed on the regulation of DtpT expression and function since little information is known for the mechanism of the regulation.

Among the peptide transporters of the PTR system, *PTR2* transcriptional regulation in yeast has been studied in a great detail at the molecular level due to the simplicity of genetic manipulation. This research might provide clues for understanding the genetic regulation of PEPT1/2 for drug delivery in mammalian organisms.

Regulation of di/tri-peptide utilization via transcriptional regulation of* PTR2 *in yeast: Peptide transport is up-regulated by growth media containing poor nitrogen sources, such as allantoin, isoleucine, or proline (ISLAND *et al.* 1991). Under poor nitrogen conditions, *PTR2* expression is highly upregulated and results in more efficient di/tri-peptide transport. In media containing the rich nitrogen source ammonium, *PTR2* expression is down-regulated via nitrogen catabolite repression (MARZLUF 1997).

At least three pathways have been shown to be related to the transcriptional regulation of *PTR2* (Figure 2). First, specific amino acids regulate *PTR2* expression via a

Figure 2 The regulation of SPS protein complex on amino acid permeases and *PTR2*. There are at least three types of sensors in the yeast plasmic membrane. Mep2p sensors NH_4+ concentration; Snf3/Rgt2p and Gpr1p sensor glucose; and Gap1p and Ssy1p sensor amino acids in the extracellular environment. Ssy1p-Ptr3p-Ssy5p form a protein complex and deliver signal to up-regulate the expression level of *PTR2* encoding di/tri-peptide tansporter, and other amino acid permeases such as *AGP1, BAP2*, and down-regulate the expression level of *GAP1*. The regulation of *PTR2* is involved in the modification of Stp2p by trancating the N-terminal of Stp2p. The truncated Stp2p is translocated into nucleus to regulate *PTR2* transcription. SCF ^{GRR1} protein complex is involved in the SPS signal transduction pathway, but the role of this protein complex is not clear. In addition, *PTR2* transcription is also repressed by Cup9p. Cup9p is destabilized by the protein complex of Ptr1p, Ubc2p, and Ubc4p via ubiquitination pathway. SPS protein complex also regulates the release of amino acid from vacuole. The process remains unclear.


NH4+
Amino Acid
Glucose
Mep2p
Gap1p
Ssy1p
Snf3p/
Rgt2p
Gpr1p
Ptr3p
Ptr3p
Ssy5p
Stp1/2p
?
SCF GRR1
Stp1/2p*
Ubc2p
Ubc4p
Ptr1p
Cup9p
Ub
Amino acid
Vacuole
Dal81p
Cup9p
Stp1/2p*
PTR2
Dal81p
Stp1p*
AGP1
Leu3p
Tup1p
Stp1/2p*
BAP2
?
….
GAP1

signal transduction pathway. Peptide utilization was markedly enhanced by addition of micromolar amounts of certain amino acids, most notably leucine and tryptophan, to the growth medium (BAETZ *et al.* 2004; ISLAND *et al.* 1987). The addition of these amino acids has been shown to up-regulate the expression of *PTR2* (PERRY *et al.* 1994). Amino acids regulate *PTR2* expression by the SPS (Ssy1p-Ptr3p-Ssy5p) signal transduction pathway (BARNES *et al.* 1998; FORSBERG *et al.* 2001a; FORSBERG *et al.* 2001b; FORSBERG and LJUNGDAHL 2001b). In the SPS complex, Ssy1p is a transmembrane receptor that senses extracellular amino acids. Similar to the amino acid permeases, Ssy1p contains 12 predicted transmembrane domains and requires Shr3p function for its localization to the cytoplasmic membrane (KLASSON *et al.* 1999). However Ssy1p is not able to transport amino acids, but instead acts as a sensor for the induction of several permease genes and a di/tri-peptide transporter gene *PTR2*. These permeases include branched-chain amino acid permease (*BAP2* and *BAP3*), tyrosine and tryptophan permease (*TAT1*), dicarboxylic amino acid permease (*DIP5*), carnitine transporter (*AGP2*), methionine permease (*MUP1*), broad-specificity amino acid permease (*AGP1*), tyrosine permease (*TAT2*), glutamine permease (*GNP1*), gamma-aminobutyrate (GABA) and Delta-aminolevulinic permease (*UGA4*), arginase gene (*CAR1*), nitrogen catabolite repression (NCR)-sensitive genes (BERMUDEZ MORETTI *et al.* 2005; ECKERT-BOULET *et al.* 2004; FORSBERG *et al.* 2001a; KODAMA *et al.* 2002), reduction of the arginine permease gene (*CAN1*), high-affinity proline permease (*PUT4*), and the general amino acid permease gene (*GAP1*) (FORSBERG *et al.* 2001a; KLASSON *et al.* 1999; KODAMA *et al.* 2002). Compared to other amino acid permease, Ssy1p has a unique extended N-terminal region, which is essential for its function. Plasmid-borne over-expression of this N-terminal region interferes with

Ssy1p function in a dominant negative manner (BERNARD and ANDRE 2001b). The mutation of threonine 382 of Ssy1p to other amino acids results in constitutive amino acid signaling, which further supports that Ssy1p is a sensor responding to extracellular amino acids (BERNARD and ANDRE 2001b; POULSEN *et al.* 2005). In addition to the amino acid sensor Ssy1p, other sensor components, the glucose sensors Snf3/Rgt2p and the ammonium sensor Mep2p have also been studied. Recently, the general amino acid permease, Gap1p, has also been reported to act as an amino acid sensor for the activation of protein kinase A targets, such as the activation of trehalase and repression of *HSP12*, *CTT1* and *SSA3*, which results in the fermentable growth (DONATON *et al.* 2003).

Ptr3p and Ssy5p are hydrophilic proteins, but are associated with the cytoplamic side of the membrane. Yeast two hybrid experiments show protein-protein interactions of Ptr3p-Ssy5p and Ptr3p-Ptr3p (BERNARD and ANDRE 2001a). Ptr3p and Ssy5p interact with the cytoplasmically located N-terminus of Ssy1p, and are required for their function (FORSBERG and LJUNGDAHL 2001a; POULSEN *et al.* 2005). Single and double deletion mutations of these three components showed identical growth phenotypes (FORSBERG and LJUNGDAHL 2001a), and overexpression of Ssy5p suppresses the growth defect caused by the deletion of *SSY5* and *PTR3* (BERNARD and ANDRE 2001a). In addition, the deletion of *SSY1* and *PTR3* caused a change in cell morphology with invasive growth and accumulation of histidine and arginine in the vacuole (KLASSON *et al.* 1999). Two related transcription factors, Stp1p and Stp2p, are downstream of the SPS complex and regulate the expression of *PTR2* and branched-chain amino acid permeases, *BAP2*, *BAP3,* and *AGP1* (DE BOER *et al.* 2000). Stp1p and Stp2p are synthesized in an inactive form and were first identified as necessary for the maturation of pre-tRNAs. The activation of

Stp1p and Stp2p depends on the endoproteolytic-processing of their N-terminal domain mediated by Ptr3p and Ssy5p (ANDREASSON and LJUNGDAHL 2002; ANDREASSON and LJUNGDAHL 2004). In the study of the SPS complex on the regulation of *AGP1* expression, Ssy1p and Ptr3p are involved in signal sensing while Ssy5p is involved in the activation of Stp1p. Ssy5p acts as an endoprotease catalyzing the cleavage of Stp1p in response to extracellular amino acids. Likely Ptr3p self-association brings Ssy5p together, which leads to Ssy5p undergoing Ptr3p-dependent self-processing (ABDEL-SATER *et al.* 2004a). Furthermore, casein kinase I is involved in the phosphorylation of the inactive form of the membrane bound transcription factor Stp1p before undergoing an endoproteolytic process (ABDEL-SATER *et al.* 2004a). The truncated forms of Stp1p and Stp2p are translocated into the nucleus to up-regulate expression of down-stream genes including *PTR2* (ANDREASSON and LJUNGDAHL 2002; ANDREASSON and LJUNGDAHL 2004). The processing of Stp1p/2p is also required for the F-box protein Grr1p, which is a component of the SCF^{Grr1} ubiquitin ligase complex (Skp1p, Hrt1p, Cdc34p, and Cdc53p) that labels proteins for degradation by the proteasome. However, the detailed process of the participants of this protein complex remains unclear in the SPS signal transduction pathway (ABDEL-SATER *et al.* 2004a; ANDREASSON and LJUNGDAHL 2004; BERNARD and ANDRE 2001b).

PTR2 expression is negatively regulated by the repressor Cup9p, which binds the *PTR2* promoter region between nucleotides -488 and -897 upstream of the *PTR2* start codon. In a *cup9* null mutant, *PTR2* is over-expressed and results in a marked increase in dipeptide uptake (BYRD *et al.* 1998). Cup9p is degraded by Ptr1p through the ubiquitinylation pathway. In this pathway, Ptr1p acts as a scaffolding protein or ubiquitin

ligase (E3) for two other proteins, Ubc2p and Ubc4p, which serve as ubiquitin-conjugating (E2) enzymes in the Cup9p degradation process (XIE and VARSHAVSKY 1999). Dipeptides containing N-terminal basic (Arg, Lys, His) or bulky hydrophobic (Phe, Leu, Tyr, Trp and Ile) residues directly bind to two distinct binding sites of Ptr1p, and accelerate the Ptr1p-dependent degradation of Cup9p (TURNER *et al.* 2000). Deletion of *PTR1* results in the stabilization of Cup9p, lack of expression of *PTR2,* and a null peptide-uptake phenotype (DU *et al.* 2002; ISLAND *et al.* 1991; TURNER *et al.* 2000). Other transcription factors, such as Dal81p/Uga35p, have also been reported to be involved in *PTR2* and *AGP1* expression (ABDEL-SATER *et al.* 2004b; IRAQUI *et al.* 1999), however it is not clear how Dal81p responds to the external amino acid sensing pathway (IRAQUI *et al.* 1999). Obviously, the systems regulating *PTR2* expression are complex, and there has not been a systematic study to uncover other proteins involved in the regulation of peptide utilization.

Regulation of di/tri-peptide utilization in mammals: In the mammalian peptide transport system, the mechanisms of the regulation on di/tri-peptide transport are still not clear at the molecular level; however some studies have demonstrated several physiological conditions and possible cellular processes that could impact di/tri-peptide transport activity. These physiological conditions mainly impact di/tri-peptide transport in three aspects (Table 3) (NIELSEN and BRODIN 2003). First, some factors that affect NHE3 activity, defined as Na^+/H^+ exchanger, resulting from the alteration of pH will indirectly alter di/tri-peptide uptake. For example, the uptake of di/tri-peptides is regulated by the intracellular calcium concentration. The calcium concentration might trigger the activation of protein kinase A (PKA) and C (PKC), which lead to alteration of

NHE3 function. As a result, the calcium concentration will eventually influence dipeptide influx via NHE3 activity, and the increase of calcium leads to a decrease of peptide uptake through the PepT1 transporter (MEREDITH and BOYD 2000).

Second, alteration of hPepT1/2 trafficking system will change di/tri-peptide transport. Several hormones such as insulin and leptin show significant alteration in the capability of di/tri-peptides uptake. An increased insulin level immediately elevates the rate of Gly-Gln influx in Caco-2 cells; increases Vmax two fold by increasing the amount of transporters in the plasma membrane without a change in the Km value of the transporter, suggesting peptide transporter activity remains the same (NIELSEN *et al.* 2003; THAMOTHARAN *et al.* 1999).

Third, modification of transcriptional expression of the peptide transporter or its mRNA stability leads to a change in di/tri-peptide transport. A high protein diet, fasting or starvation increases the level of mRNAs of hPepT1 in the intestine (MEREDITH and BOYD 2000; NIELSEN and BRODIN 2003). It is not clear how this diet condition triggers the changes in expression level. In addition, the expression of hPepT1/2 is also up-regulated in the presence of dietary amino acids (Phe, Arg, and Lys) and peptides (Gly-Sar, Gly-Phe, Lys-Phe and Asp-Lys) possibly through the transcription factor AP-1 since the PepT1 gene promoter contains its binding sequence. It is possible that high concentrations of amino acids and peptides up-regulates the expression level of AP-1 (SHIRAGA *et al.* 1999; THAMOTHARAN *et al.* 1998). Expression of the peptide transporter also varies in different tissues. hPEPT1 has higher expression in the small intestine and lower in kidney and liver, and no expression in colon. However, how the expression is changed in different tissues remains unsolved. Epidermal growth factor and thyroid

hormone show a significant alteration in the uptake of di/tri-peptides by increasing the PEPT1 expression (ADIBI 2003; ASHIDA *et al.* 2002). A ligand such as (+)pentazocine for sigma receptor, the plasma membrane receptor binding with nonopiate and nonphencyclidine, can also up-regulate the expression of PEPT1 (FUJITA *et al.* 1999). In addition, the expression of the di/tri-peptide transporter also changes in the development process, injury and disease states such as cancer, as well as diabetes and intestinal inflammation (ADIBI 2003); the mechanisms of these conditions affecting the expression level are largely unknown. Compared with the known gene products involved in the regulation of *PTR2* in yeast, such as SPS sensor, it is not known whether the counterparts in mammals to regulate PEPT1/2 exist and play the similar role.

Rationale for this study:

This study was undertaken to identify possible genes involved in the regulation of peptide utilization in yeast. In my study, I performed a genome-wide screen on a yeast single gene deletion mutant library. In addition, we used high-throughput phenotyping in which we grew yeast cells on 284 different dipeptides or 11 tripeptides as the sole nitrogen source in several genetically diverse strains. Several gene products and signal pathways involved in the regulation of di/tri-peptide utilization have been identified in this study. The identified genes regulating dipeptide utilization represented a very wide regulatory network involved in transport and utilization of dipeptides in yeast. The identified components involved in dipeptide utilization may help understand the regulation of peptide transport in mammals. This is the first such study of a membrane transport system in any organism and initiates a systems biology approach to understanding membrane transport in cells.

REFERENCES

ABDEL-SATER, F., M. EL BAKKOURY, A. URRESTARAZU, S. VISSERS and B. ANDRE, 2004a Amino acid signaling in yeast: casein kinase I and the Ssy5 endoprotease are key determinants of endoproteolytic activation of the membrane-bound Stp1 transcription factor. Mol Cell Biol **24:** 9771-9785.

ABDEL-SATER, F., I. IRAQUI, A. URRESTARAZU and B. ANDRE, 2004b The external amino acid signaling pathway promotes activation of Stp1 and Uga35/Dal81 transcription factors for induction of the AGP1 gene in Saccharomyces cerevisiae. Genetics **166:** 1727-1739.

ABOUHAMAD, W. N., M. MANSON, M. M. GIBSON and C. F. HIGGINS, 1991 Peptide transport and chemotaxis in Escherichia coli and Salmonella typhimurium: characterization of the dipeptide permease (Dpp) and the dipeptide-binding protein. Mol Microbiol **5:** 1035-1047.

ADERMANN, K., H. JOHN, L. STANDKER and W. G. FORSSMANN, 2004 Exploiting natural peptide diversity: novel research tools and drug leads. Curr Opin Biotechnol **15:** 599-606.

ADIBI, S. A., 2003 Regulation of expression of the intestinal oligopeptide transporter (Pept-1) in health and disease. Am J Physiol Gastrointest Liver Physiol **285:** G779-788.

ALONSO DE DIEGO, S. A., P. MUNOZ, R. GONZALEZ-MUNIZ, R. HERRANZ, M. MARTIN-MARTINEZ *et al.*, 2005 Analogues of the neuroprotective tripeptide Gly-Pro-Glu (GPE): synthesis and structure-activity relationships. Bioorg Med Chem Lett **15:** 2279-2283.

ANAND, B. S., J. M. HILL, S. DEY, K. MARUYAMA, P. S. BHATTACHARJEE *et al.*, 2003a In vivo antiviral efficacy of a dipeptide acyclovir prodrug, val-val-acyclovir, against HSV-1 epithelial and stromal keratitis in the rabbit eye model. Invest Ophthalmol Vis Sci **44:** 2529-2534.

ANAND, B. S., J. PATEL and A. K. MITRA, 2003b Interactions of the dipeptide ester prodrugs of acyclovir with the intestinal oligopeptide transporter: competitive inhibition of glycylsarcosine transport in human intestinal cell line-Caco-2. J Pharmacol Exp Ther **304:** 781-791.

ANDREASSON, C., and P. O. LJUNGDAHL, 2002 Receptor-mediated endoproteolytic activation of two transcription factors in yeast. Genes Dev **16:** 3158-3172.

ANDREASSON, C., and P. O. LJUNGDAHL, 2004 The N-terminal regulatory domain of Stp1p is modular and, fused to an artificial transcription factor, confers full Ssy1p-Ptr3p-Ssy5p sensor control. Mol Cell Biol **24:** 7503-7513.

ASHIDA, K., T. KATSURA, H. MOTOHASHI, H. SAITO and K. INUI, 2002 Thyroid hormone regulates the activity and expression of the peptide transporter PEPT1 in Caco-2 cells. Am J Physiol Gastrointest Liver Physiol **282:** G617-623.

BAETZ, K., L. MCHARDY, K. GABLE, T. TARLING, D. REBERIOUX *et al.*, 2004 Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. Proc Natl Acad Sci U S A **101:** 4525-4530.

BARNES, D., W. LAI, M. BRESLAV, F. NAIDER and J. M. BECKER, 1998 PTR3, a novel gene mediating amino acid-inducible regulation of peptide transport in Saccharomyces cerevisiae. Mol Microbiol **29:** 297-310.

BECKER, J. M., F. NAIDER and E. KATCHALSKI, 1973 Peptide utilization in yeast. Studies on methionine and lysine auxotrophs of Saccharomyces cerevisiae. Biochim Biophys Acta **291:** 388-397.

BERMUDEZ MORETTI, M., A. M. PERULLINI, A. BATLLE and S. CORREA GARCIA, 2005 Expression of the UGA4 gene encoding the delta-aminolevulinic and gamma-aminobutyric acids permease in Saccharomyces cerevisiae is controlled by amino acid-sensing systems. Arch Microbiol **184:** 137-140.

BERNARD, F., and B. ANDRE, 2001a Genetic analysis of the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. Mol Microbiol **41:** 489-502.

BERNARD, F., and B. ANDRE, 2001b Ubiquitin and the SCF(Grr1) ubiquitin ligase complex are involved in the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. FEBS Lett **496:** 81-85.

BINET, R., S. LETOFFE, J. M. GHIGO, P. DELEPELAIRE and C. WANDERSMAN, 1997 Protein secretion by Gram-negative bacterial ABC exporters--a review. Gene **192:** 7-11.

BOOTH, I. R., 1985 Regulation of cytoplasmic pH in bacteria. Microbiol Rev **49:** 359-378.

BUENO, A. B., I. COLLADO, A. DE DIOS, C. DOMINGUEZ, J. A. MARTIN *et al.*, 2005 Dipeptides as effective prodrugs of the unnatural amino acid (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740), a selective group II metabotropic glutamate receptor agonist. J Med Chem **48:** 5305-5320.

BYRD, C., G. C. TURNER and A. VARSHAVSKY, 1998 The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. Embo J **17:** 269-277.

CALVO, J. M., and R. G. MATTHEWS, 1994 The leucine-responsive regulatory protein, a global regulator of metabolism in Escherichia coli. Microbiol Rev **58:** 466-490.

CURINI, M., F. EPIFANO and S. GENOVESE, 2005 Synthesis of a novel prodrug of 3-(4'-geranyloxy-3'-methoxyphenyl)-2-trans-propenoic acid for colon delivery. Bioorg Med Chem Lett **15:** 5049-5052.

DALPIAZ, A., S. SPISANI, C. BIONDI, E. FABBRI, M. NALLI *et al.*, 2003 Studies on human neutrophil biological functions by means of formyl-peptide receptor agonists and antagonists. Curr Drug Targets Immune Endocr Metabol Disord **3:** 33-42.

DANIEL, H., 2004 Molecular and integrative physiology of intestinal peptide transport. Annu Rev Physiol **66:** 361-384.

DANIEL, H., and G. KOTTRA, 2004 The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. Pflugers Arch **447:** 610-618.

DE BOER, M., P. S. NIELSEN, J. P. BEBELMAN, H. HEERIKHUIZEN, H. A. ANDERSEN *et al.*, 2000 Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene BAP3 of Saccharomyces cerevisiae. Nucleic Acids Res **28:** 974-981.

DEN HENGST, C. D., P. CURLEY, R. LARSEN, G. BUIST, A. NAUTA *et al.*, 2005 Probing direct interactions between CodY and the oppD promoter of Lactococcus lactis. J Bacteriol **187:** 512-521.

DETMERS, F. J., E. R. KUNJI, F. C. LANFERMEIJER, B. POOLMAN and W. N. KONINGS, 1998 Kinetics and specificity of peptide uptake by the oligopeptide transport system of Lactococcus lactis. Biochemistry **37:** 16671-16679.

DEV, I. K., and P. H. RAY, 1990 Signal peptidases and signal peptide hydrolases. J Bioenerg Biomembr **22:** 271-290.

DOEVEN, M. K., R. ABELE, R. TAMPE and B. POOLMAN, 2004 The binding specificity of OppA determines the selectivity of the oligopeptide ATP-binding cassette transporter. J Biol Chem **279:** 32301-32307.

DONATON, M. C., I. HOLSBEEKS, O. LAGATIE, G. VAN ZEEBROECK, M. CRAUWELS *et al.*, 2003 The Gap1 general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in the yeast Saccharomyces cerevisiae. Mol Microbiol **50:** 911-929.

DU, F., F. NAVARRO-GARCIA, Z. XIA, T. TASAKI and A. VARSHAVSKY, 2002 Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. Proc Natl Acad Sci U S A **99:** 14110-14115.

ECKERT-BOULET, N., P. S. NIELSEN, C. FRIIS, M. M. DOS SANTOS, J. NIELSEN *et al.*, 2004 Transcriptional profiling of extracellular amino acid sensing in Saccharomyces cerevisiae and the role of Stp1p and Stp2p. Yeast **21:** 635-648.

ERBELDINGER, M., X. NI and P. J. HALLING, 2001 Kinetics of enzymatic solid-to-solid peptide synthesis: synthesis of Z-aspartame and control of acid-base conditions by using inorganic salts. Biotechnol Bioeng **72:** 69-76.

ERIKSSON, A. H., P. L. ELM, M. BEGTRUP, R. NIELSEN, B. STEFFANSEN *et al.*, 2005 hPEPT1 affinity and translocation of selected Gln-Sar and Glu-Sar dipeptide derivatives. Mol Pharm **2:** 242-249.

FADEN, A. I., S. M. KNOBLACH, V. A. MOVSESYAN, P. M. T. LEA and I. CERNAK, 2005 Novel neuroprotective tripeptides and dipeptides. Ann N Y Acad Sci **1053:** 472-481.

FEI, Y. J., Y. KANAI, S. NUSSBERGER, V. GANAPATHY, F. H. LEIBACH *et al.*, 1994 Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature **368:** 563-566.

FORSBERG, H., C. F. GILSTRING, A. ZARGARI, P. MARTINEZ and P. O. LJUNGDAHL, 2001a The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Mol Microbiol **42:** 215-228.

FORSBERG, H., M. HAMMAR, C. ANDREASSON, A. MOLINER and P. O. LJUNGDAHL, 2001b Suppressors of ssy1 and ptr3 null mutations define novel amino acid sensor-independent genes in Saccharomyces cerevisiae. Genetics **158:** 973-988.

FORSBERG, H., and P. O. LJUNGDAHL, 2001a Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. Mol Cell Biol **21:** 814-826.

FORSBERG, H., and P. O. LJUNGDAHL, 2001b Sensors of extracellular nutrients in Saccharomyces cerevisiae. Curr Genet **40:** 91-109.

FOUCAUD, C., E. R. KUNJI, A. HAGTING, J. RICHARD, W. N. KONINGS *et al.*, 1995 Specificity of peptide transport systems in Lactococcus lactis: evidence for a third system which transports hydrophobic di- and tripeptides. J Bacteriol **177:** 4652-4657.

FRIEDRICHSEN, G. M., P. JAKOBSEN, M. TAUB and M. BEGTRUP, 2001 Application of enzymatically stable dipeptides for enhancement of intestinal permeability. Synthesis and in vitro evaluation of dipeptide-coupled compounds. Bioorg Med Chem **9:** 2625-2632.

FUJITA, T., Y. MAJIKAWA, S. UMEHISA, N. OKADA, A. YAMAMOTO *et al.*, 1999 sigma Receptor ligand-induced up-regulation of the H(+)/peptide transporter PEPT1 in the human intestinal cell line Caco-2. Biochem Biophys Res Commun **261:** 242-246.

GEUEKE, B., K. NAMOTO, D. SEEBACH and H. P. KOHLER, 2005 A novel beta-peptidyl aminopeptidase (BapA) from strain 3-2W4 cleaves peptide bonds of synthetic beta-tri- and beta-dipeptides. J Bacteriol **187:** 5910-5917.

GONZALES, T., and J. ROBERT-BAUDOUY, 1996 Bacterial aminopeptidases: properties and functions. FEMS Microbiol Rev **18:** 319-344.

HAGTING, A., J. VD VELDE, B. POOLMAN and W. N. KONINGS, 1997 Membrane topology of the di- and tripeptide transport protein of Lactococcus lactis. Biochemistry **36:** 6777-6785.

HAUSER, M., V. NARITA, A. M. DONHARDT, F. NAIDER and J. M. BECKER, 2001 Multiplicity and regulation of genes encoding peptide transporters in Saccharomyces cerevisiae. Mol Membr Biol **18:** 105-112.

HERRERA-RUIZ, D., and G. T. KNIPP, 2003 Current perspectives on established and putative mammalian oligopeptide transporters. J Pharm Sci **92:** 691-714.

HOMANN, O. R., H. CAI, J. M. BECKER and S. L. LINDQUIST, 2005 Harnessing natural diversity to probe metabolic pathways. PLoS Genet **1:** e80.

HUBE, B., and J. NAGLIK, 2001 Candida albicans proteinases: resolving the mystery of a gene family. Microbiology **147:** 1997-2005.

IRAQUI, I., S. VISSERS, F. BERNARD, J. O. DE CRAENE, E. BOLES *et al.*, 1999 Amino acid signaling in Saccharomyces cerevisiae: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. Mol Cell Biol **19:** 989-1001.

ISLAND, M. D., F. NAIDER and J. M. BECKER, 1987 Regulation of dipeptide transport in Saccharomyces cerevisiae by micromolar amino acid concentrations. J Bacteriol **169:** 2132-2136.

ISLAND, M. D., J. R. PERRY, F. NAIDER and J. M. BECKER, 1991 Isolation and characterization of S. cerevisiae mutants deficient in amino acid-inducible peptide transport. Curr Genet **20:** 457-463.

ITO, K., M. UNO and Y. NAKAMURA, 2000 A tripeptide 'anticodon' deciphers stop codons in messenger RNA. Nature **403:** 680-684.

JAIN, R., S. AGARWAL, S. MAJUMDAR, X. ZHU, D. PAL *et al.*, 2005 Evasion of P-gp mediated cellular efflux and permeability enhancement of HIV-protease inhibitor saquinavir by prodrug modification. Int J Pharm **303:** 8-19.

JIA, J., R. LU, S. QIU, H. LI, X. CHE *et al.*, 2005 Preliminary Investigation of the Inhibitory Effects of the Tyroservaltide (YSV) Tripeptide on Human Hepatocarcinoma BEL-7402. Cancer Biol Ther **4:** 993-997.

KLASSON, H., G. R. FINK and P. O. LJUNGDAHL, 1999 Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol Cell Biol **19:** 5405-5416.

KODAMA, Y., F. OMURA, K. TAKAHASHI, K. SHIRAHIGE and T. ASHIKARI, 2002 Genome-wide expression analysis of genes affected by amino acid sensor Ssy1p in Saccharomyces cerevisiae. Curr Genet **41:** 63-72.

KOLAKOVICH, K. A., I. Y. GLUZMAN, K. L. DUFFIN and D. E. GOLDBERG, 1997 Generation of hemoglobin peptides in the acidic digestive vacuole of Plasmodium falciparum implicates peptide transport in amino acid production. Mol Biochem Parasitol **87:** 123-135.

KORHONEN, H., and A. PIHLANTO, 2003 Food-derived bioactive peptides--opportunities for designing future foods. Curr Pharm Des **9:** 1297-1308.

KRAINER, E., J. M. BECKER and F. NAIDER, 1991 Synthesis and biological evaluation of dipeptidyl and tripeptidyl polyoxin and nikkomycin analogues as anticandidal prodrugs. J Med Chem **34:** 174-180.

KUMAR, D., and T. C. BHALLA, 2005 Microbial proteases in peptide synthesis: approaches and applications. Appl Microbiol Biotechnol **68:** 726-736.

KUNJI, E. R., I. MIERAU, A. HAGTING, B. POOLMAN and W. N. KONINGS, 1996a The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek **70:** 187-221.

KUNJI, E. R., I. MIERAU, B. POOLMAN, W. N. KONINGS, G. VENEMA *et al.*, 1996b Fate of peptides in peptidase mutants of Lactococcus lactis. Mol Microbiol **21:** 123-131.

LAMARQUE, M., P. CHARBONNEL, D. AUBEL, J. C. PIARD, D. ATLAN *et al.*, 2004 A multifunction ABC transporter (Opt) contributes to diversity of peptide uptake specificity within the genus Lactococcus. J Bacteriol **186:** 6492-6500.

LARSEN, S. D., T. BARF, C. LILJEBRIS, P. D. MAY, D. OGG *et al.*, 2002 Synthesis and biological activity of a novel class of small molecular weight peptidomimetic competitive inhibitors of protein tyrosine phosphatase 1B. J Med Chem **45:** 598-622.

LEE, V. H., 2000 Membrane transporters. Eur J Pharm Sci **11 Suppl 2:** S41-50.

LI, Y., Y. SAITO, M. SUZUKI, H. UEDA, M. ENDO *et al.*, 2006 Kyotorphin has a novel action on rat cardiac muscle. Biochem Biophys Res Commun **339:** 805-809.

LIN, B., S. A. SHORT, M. ESKILDSEN, M. S. KLEMPNER and L. T. HU, 2001 Functional testing of putative oligopeptide permease (Opp) proteins of Borrelia burgdorferi: a complementation model in opp(-) Escherichia coli. Biochim Biophys Acta **1499:** 222-231.

LIU, W., R. LIANG, S. RAMAMOORTHY, Y. J. FEI, M. E. GANAPATHY *et al.*, 1995 Molecular cloning of PEPT 2, a new member of the H+/peptide cotransporter family, from human kidney. Biochim Biophys Acta **1235:** 461-466.

LU, H., and C. KLAASSEN, 2005 Tissue distribution and thyroid hormone regulation of Pept1 and Pept2 mRNA in rodents. Peptides.

LUBKOWITZ, M. A., L. HAUSER, M. BRESLAV, F. NAIDER and J. M. BECKER, 1997 An oligopeptide transport gene from Candida albicans. Microbiology **143 (Pt 2):** 387-396.

MADSHUS, I. H., 1988 Regulation of intracellular pH in eukaryotic cells. Biochem J **250:** 1-8.

MARZLUF, G. A., 1997 Genetic regulation of nitrogen metabolism in the fungi. Microbiol Mol Biol Rev **61:** 17-32.

MEISEL, H., and W. BOCKELMANN, 1999 Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. Antonie Van Leeuwenhoek **76:** 207-215.

MEREDITH, D., and C. A. BOYD, 2000 Structure and function of eukaryotic peptide transporters. Cell Mol Life Sci **57:** 754-778.

MIYAKE, T., M. KANAYAMA, H. SAMMOTO and B. ONO, 2002 A novel cis-acting cysteine-responsive regulatory element of the gene for the high-affinity glutathione transporter of Saccharomyces cerevisiae. Mol Genet Genomics **266:** 1004-1011.

MONETON, P., P. SARTHOU and F. LE GOFFIC, 1986 Transport and hydrolysis of peptides in Saccharomyces cerevisiae. J Gen Microbiol **132:** 2147-2153.

NAGLIK, J. R., S. J. CHALLACOMBE and B. HUBE, 2003 Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev **67:** 400-428, table of contents.

NAKAMURA, Y., and K. ITO, 2002 A tripeptide discriminator for stop codon recognition. FEBS Lett **514:** 30-33.

NIELSEN, C. U., J. AMSTRUP, R. NIELSEN, B. STEFFANSEN, S. FROKJAER *et al.*, 2003 Epidermal growth factor and insulin short-term increase hPepT1-mediated glycylsarcosine uptake in Caco-2 cells. Acta Physiol Scand **178:** 139-148.

NIELSEN, C. U., and B. BRODIN, 2003 Di/tri-peptide transporters as drug delivery targets: regulation of transport under physiological and patho-physiological conditions. Curr Drug Targets **4:** 373-388.

PAYNE, J. W., and M. W. SMITH, 1994 Peptide transport by micro-organisms. Adv Microb Physiol **36:** 1-80.

PELLEGRINI, A., 2003 Antimicrobial peptides from food proteins. Curr Pharm Des **9:** 1225-1238.

PERRY, J. R., M. A. BASRAI, H. Y. STEINER, F. NAIDER and J. M. BECKER, 1994 Isolation and characterization of a Saccharomyces cerevisiae peptide transport gene. Mol Cell Biol **14:** 104-115.

PODBIELSKI, A., and B. A. LEONARD, 1998 The group A streptococcal dipeptide permease (Dpp) is involved in the uptake of essential amino acids and affects the expression of cysteine protease. Mol Microbiol **28:** 1323-1334.

POULSEN, P., B. WU, R. F. GABER, K. OTTOW, H. A. ANDERSEN *et al.*, 2005 Amino acid sensing by Ssy1. Biochem Soc Trans **33:** 261-264.

PRITCHARD, G. G., and T. COOLBEAR, 1993 The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol Rev **12:** 179-206.

ROSE, B., J. M. BECKER and F. NAIDER, 1979 Peptidase activities in Saccharomyces cerevisiae. J Bacteriol **139:** 220-224.

RUBIO-ALIAGA, I., and H. DANIEL, 2002 Mammalian peptide transporters as targets for drug delivery. Trends Pharmacol Sci **23:** 434-440.

SAIER, M. H., JR., 1999 A functional-phylogenetic system for the classification of transport proteins. J Cell Biochem **Suppl 32-33:** 84-94.

SAIER, M. H., JR., C. V. TRAN and R. D. BARABOTE, 2006 TCDB: the Transporter Classification Database for membrane transport protein analyses and information. Nucleic Acids Res **34:** D181-186.

SARIC, T., C. I. GRAEF and A. L. GOLDBERG, 2004 Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases. J Biol Chem **279:** 46723-46732.

SHIOMI, H., Y. KURAISHI, H. UEDA, Y. HARADA, H. AMANO *et al.*, 1981 Mechanism of kyotorphin-induced release of Met-enkephalin from guinea pig striatum and spinal cord. Brain Res **221:** 161-169.

SHIRAGA, T., K. MIYAMOTO, H. TANAKA, H. YAMAMOTO, Y. TAKETANI *et al.*, 1999 Cellular and molecular mechanisms of dietary regulation on rat intestinal H+/Peptide transporter PepT1. Gastroenterology **116:** 354-362.

SMITH, M. W., D. R. TYREMAN, G. M. PAYNE, N. J. MARSHALL and J. W. PAYNE, 1999 Substrate specificity of the periplasmic dipeptide-binding protein from Escherichia coli: experimental basis for the design of peptide prodrugs. Microbiology **145 (Pt 10):** 2891-2901.

SOLOMON, J., L. SU, S. SHYN and A. D. GROSSMAN, 2003 Isolation and characterization of mutants of the Bacillus subtilis oligopeptide permease with altered specificity of oligopeptide transport. J Bacteriol **185:** 6425-6433.

STEINER, H. Y., F. NAIDER and J. M. BECKER, 1995 The PTR family: a new group of peptide transporters. Mol Microbiol **16:** 825-834.

TAMAI, I., T. NAKANISHI, H. NAKAHARA, Y. SAI, V. GANAPATHY *et al.*, 1998 Improvement of L-dopa absorption by dipeptidyl derivation, utilizing peptide transporter PepT1. J Pharm Sci **87:** 1542-1546.

TEMSAMANI, J., and P. VIDAL, 2004 The use of cell-penetrating peptides for drug delivery. Drug Discov Today **9:** 1012-1019.

THAMOTHARAN, M., S. Z. BAWANI, X. ZHOU and S. A. ADIBI, 1998 Mechanism of dipeptide stimulation of its own transport in a human intestinal cell line. Proc Assoc Am Physicians **110:** 361-368.

THAMOTHARAN, M., S. Z. BAWANI, X. ZHOU and S. A. ADIBI, 1999 Hormonal regulation of oligopeptide transporter pept-1 in a human intestinal cell line. Am J Physiol **276:** C821-826.

TOMKINSON, B., and A. C. LINDAS, 2005 Tripeptidyl-peptidase II: a multi-purpose peptidase. Int J Biochem Cell Biol **37:** 1933-1937.

TROITSKAYA, L. A., and T. KODADEK, 2004 Peptides as modulators of enzymes and regulatory proteins. Methods **32:** 406-415.

TURNER, G. C., F. DU and A. VARSHAVSKY, 2000 Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. Nature **405:** 579-583.

VABENO, J., T. LEJON, C. U. NIELSEN, B. STEFFANSEN, W. CHEN *et al.*, 2004a Phe-Gly dipeptidomimetics designed for the di-/tripeptide transporters PEPT1 and PEPT2: synthesis and biological investigations. J Med Chem **47:** 1060-1069.

VABENO, J., C. U. NIELSEN, T. INGEBRIGTSEN, T. LEJON, B. STEFFANSEN *et al.*, 2004b Dipeptidomimetic ketomethylene isosteres as pro-moieties for drug transport via the human intestinal di-/tripeptide transporter hPEPT1: design, synthesis, stability, and biological investigations. J Med Chem **47:** 4755-4765.

VERMA, V., A. MANN, W. COSTAIN, G. PONTORIERO, J. M. CASTELLANO *et al.*, 2005 Modulation of agonist binding to human dopamine receptor subtypes by L-prolyl-L-leucyl-glycinamide and a peptidomimetic analog. J Pharmacol Exp Ther **315:** 1228-1236.

WANDERSMAN, C., 1998 Protein and peptide secretion by ABC exporters. Res Microbiol **149:** 163-170.

WOUTERS, J. A., T. HAIN, A. DARJI, E. HUFNER, H. WEMEKAMP-KAMPHUIS *et al.*, 2005 Identification and characterization of Di- and tripeptide transporter DtpT of Listeria monocytogenes EGD-e. Appl Environ Microbiol **71:** 5771-5778.

XIE, Y., and A. VARSHAVSKY, 1999 The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. Embo J **18:** 6832-6844.

XIE, Y., and A. VARSHAVSKY, 2000 Physical association of ubiquitin ligases and the 26S proteasome. Proc Natl Acad Sci U S A **97:** 2497-2502.

YAMAMOTO, N., 1997 Antihypertensive peptides derived from food proteins. Biopolymers **43:** 129-134.

YAMAMOTO, N., M. EJIRI and S. MIZUNO, 2003 Biogenic peptides and their potential use. Curr Pharm Des **9:** 1345-1355.

YAO, Z., R. LU, J. JIA, P. ZHAO, J. YANG *et al.*, 2005 The effect of tripeptide tyroserleutide (YSL) on animal models of hepatocarcinoma. Peptides.

PART II

GENOME-WIDE SCREEN REVEALS A WIDE REGULATORY NETWORK FOR DI/TRI-PEPTIDE UTILIZATION IN *Saccharomyces cerevisiae*

Part II was published in its entirety as Houjian Cai, Sarah Kauffman, Fred Naider, and Jeffrey M. Becker. 2006. Genome-wide screen reveals a wide regulatory network for di/tri-peptide utilization in *Saccharomyces cerevisiae.* Genetics*,* Mar.;172(3):1459-76. Houjian Cai performed all the studies in the part with the exception that Sarah Kauffman transforming a plasmid containing Ptr2p-GFP to the 103 deletion mutants. Toxic dipeptide (Ala-Eth) was provided from Dr. Naider.

ABSTRACT

Small peptides of two to six residues serve as important sources of amino acids and nitrogen required for growth by a variety of organisms. In the yeast *Saccharomyces cerevisiae,* the membrane transport protein Ptr2p, encoded by *PTR2,* mediates the uptake of di/tri-peptides. To identify genes involved in regulation of dipeptide utilization, we performed a systematic, functional examination of this process in a haploid, non-essential, single-gene deletion mutant library. We have identified 103 candidate genes: 57 genes whose deletion decreased dipeptide utilization and 46 genes whose deletion enhanced dipeptide utilization. Based on Ptr2p-GFP expression studies, together with *PTR2* expression analysis and dipeptide uptake assays, 42 genes were ascribed to the regulation of *PTR2* expression, 37 genes were involved in Ptr2p localization, and 24 genes did not apparently affect Ptr2p-GFP expression or localization. The 103 genes regulating dipeptide utilization were distributed among most of the Gene Ontology functional categories indicating a very wide regulatory network involved in transport and utilization of dipeptides in yeast. It is anticipated that further characterization of how these genes affect peptide utilization should add new insights into the global mechanisms of regulation of transport systems in general and peptide utilization in particular.

INTRODUCTION

Peptides can serve as a major nutritional source of amino acids and nitrogen for microbial cell growth and for human nutrition as well (DANIEL 2004; PRITCHARD and COOLBEAR 1993). Peptides are generated by the action of proteases and peptidases in environments where microbes reside (DANIEL 2004; GONZALES and ROBERT-BAUDOUY 1996). All cellular organisms from bacteria to fungi, plants and mammals are capable of taking up small peptides (two to six amino acids) through a system mediated by cytoplasmic membrane transporters specific for peptides (HAUSER *et al.* 2001). In mammalian systems, two major peptide transporters, PepT1 and PepT2, have been found in the intestine and kidney, respectively (FEI *et al.* 1994; LIU *et al.* 1995). The PepT1 transporter provides the means for absorption of nutritional peptides and peptidomimetic drugs, such as beta-lactam antibiotics, angiotensin converting enzyme inhibitors, renin inhibitors, and antivirals. The substrate specificity of these transporters has provided an impetus to many pharmaceutical companies to develop peptide transporters as drug delivery systems (HERRERA-RUIZ and KNIPP 2003; LEE 2000).

Two peptide transport systems have been characterized in the model eukaryote *Saccharomyces cerevisiae*: the PTR (Peptide TRansport) system transports di/tri-peptides (STEINER *et al.* 1995) and the OPT (OligoPeptide Transport) system highly favors transport of peptides of four to five amino acid residues and glutathione (LUBKOWITZ *et al.* 1997; MIYAKE *et al.* 2002). While PTR family members are present in all cells studied to date and are in the same family as the mammalian transporters PepT1 and PepT2, OPT family members have been found only in plants and fungi (HAUSER *et al.* 2001). Both The PTR and OPT transport systems are dependent on the proton-motive force, are

predicted to have twelve transmembrane domains, and have specific signature sequences distinguishing them from one another and from all other proteins in the database (HAUSER *et al.* 2001). Yet the amino acid sequence of the PTR and OPT members places them in different, unrelated families of transport proteins with presumably totally separate evolutionary origins. The only *S. cerevisiae* member of the PTR system is Ptr2p, encoded by the *PTR2* gene. Di/tri-peptide utilization is regulated by a number of means including *PTR2* transcriptional regulation.

In early studies pre-dating the cloning of *PTR2*, peptide utilization was shown to be up-regulated by growing cells on organic nitrogen sources such as allantoin, isoleucine, or proline defined as poor nitrogen sources in comparison to the rich nitrogen source ammonium sulfate (ISLAND *et al.* 1991). Peptide utilization was also stimulated markedly by addition of micromolar amounts of certain amino acids, most notably leucine and tryptophan, to the growth medium (BAETZ *et al.* 2004; ISLAND *et al.* 1987). These environmental conditions were later shown to up-regulate the expression of *PTR2* (PERRY *et al.* 1994). Although nitrogen source apparently regulates *PTR2* expression via nitrogen catabolite repression (MARZLUF 1997), amino acids regulate *PTR2* expression via the SPS (Ssy1p-Ptr3p-Ssy5p) signal transduction pathway (BARNES *et al.* 1998; FORSBERG *et al.* 2001a; FORSBERG *et al.* 2001b; FORSBERG and LJUNGDAHL 2001b).

In the SPS complex, Ssy1p is a transmembrane receptor that senses extracellular amino acids. Ptr3p and Ssy5p interact with the cytoplasmically located N-terminus of Ssy1p (FORSBERG and LJUNGDAHL 2001a; POULSEN *et al.* 2005). Two related transcription factors, Stp1p and Stp2p, are down-stream of the SPS complex and regulate the expression of *PTR2* and branched-chain amino acid permeases (DE BOER *et al.* 2000).

Stp1p and Stp2p are synthesized in an inactive form, and the activation of Stp1p and Stp2p depends on the endoproteolytic-processing of their N-terminus domain mediated by Ptr3p and Ssy5p. The truncated forms of Stp1p and Stp2p are translocated into the nucleus to up-regulate expression of down-stream genes including *PTR2* (ANDREASSON and LJUNGDAHL 2002; ANDREASSON and LJUNGDAHL 2004).

In addition to the SPS complex regulation, *PTR2* expression is positively regulated by the import of di/tri-peptides with basic (Arg, His, or Lys) and bulky (Ile, Leu, Phe, Trp, or Tyr) N-terminal residues that bind to Ubr1p to allosterically activate Ubr1p-mediated degradation of the *PTR2* repressor Cup9p (BYRD *et al.* 1998; DU *et al.* 2002; TURNER *et al.* 2000). Relief of Cup9p repression of *PTR2* results in enhanced *PTR2* expression. In a *cup9* null mutant strain, *PTR2* is over-expressed, resulting in a marked increase in dipeptide uptake (BYRD *et al.* 1998). Cup9p is degraded by Ptr1p through the ubiquitinylation pathway. In this pathway, Ptr1p acts as a scaffolding protein or ubiquitin ligase (E3) for two other proteins, Ubc2p and Ubc4p, which serve as ubiquitin-conjugating (E2) enzymes in the Cup9p degradation process (XIE and VARSHAVSKY 1999). Deletion of *PTR1* results in the stabilization of Cup9p, lack of expression of *PTR2,* and a null peptide-uptake phenotype (DU *et al.* 2002; ISLAND *et al.* 1991; TURNER *et al.* 2000). Other transcription factors, such as Dal81p/Uga35p, have also been reported to be involved in *PTR2* expression, in ways apparently different from their involvement in the SPS or Cup9p pathways (IRAQUI *et al.* 1999). Obviously, the systems regulating *PTR2* expression are numerous and complex, and there has not been a systematic study to uncover other proteins involved in the regulation of peptide utilization.

Systematic screening of yeast deletion mutant collections has been successfully applied in identifying genes related to drug resistance (AOUIDA *et al.* 2004; BAETZ *et al.* 2004; PAGE *et al.* 2003), human disease (STEINMETZ *et al.* 2002), telomere function (ASKREE *et al.* 2004), centromeric cohesion (MARSTON *et al.* 2004), vacuolar protein sorting (BONANGELINO *et al.* 2002), and other biological processes (SCHERENS and GOFFEAU 2004). In order to identify other gene products involved in the regulation of peptide utilization in yeast, we conducted a genome-wide screen using the haploid, non-essential, single-gene deletion strain library. We have identified a total of 103 Open Reading Frames (ORFs), accounting for about 2 percent of the non-essential deletion mutants as being involved in causing increased or decreased utilization of peptides for growth. We monitored Ptr2p-GFP expression in the identified 103 deletion mutant strains, and genes involved in multiple cellular functions including transcriptional regulation and membrane trafficking were revealed as being involved in dipeptide utilization. The data generated provide a global view of molecular components regulating dipeptide utilization by *S. cerevisiae.*

MATERIAL AND METHODS

Strains, media, and mutant library growth assay screen: A yeast haploid, non-essential, single-gene deletion mutant strain library was purchased from Open Biosystems (Huntsville, AL) containing 4827 deletion strains, 4750 in the BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) background, and 77 strains in the BY4739 (*MATα leu2Δ0 lys2Δ0 ura3Δ0*) strain background. The list of strains contained in the library is available at the company's website (http://openbiosystems.com/yeast_knock_outs.php). The deletion mutants were arrayed in 96-well microtiter plates kept at -80˚C in YPD broth containing G418 and glycerol (1% yeast extract, 2% peptone, 2% dextrose, 200 μg/mL G418, and 15% glycerol). After thawing, the deletion mutants were inoculated into minimal medium supplemented with amino acids to satisfy the auxotrophic requirements using a 96-well plate replicator. The minimal medium used in this study is referred to as MM+HLKU and contained (per liter) 20g dextrose, 1.7g Yeast Nitrogen Base (Difco) without $(NH_4)_2SO_4$ and amino acids, 1g allantoin as nitrogen source, supplemented with 20μg/mL His (H), 30μg/mL Leu (L), 30μg/mL Lys (K), and 20μg/mL Ura (U). The plates were incubated at 30˚C for 2 days, then the liquid medium growth assay was performed as follows. Five microliters from each well were inoculated into 200μL of three different liquid media: (1) MM+HLKU medium as described above, (2) MM+(H-L)HKU medium, or (3) MM+(H-L)HKU+Trp medium. The MM+(H-L)HKU and MM+(H-L)HKU+Trp media contained 80μM His-Leu dipeptide (H-L) in place of leucine with all other components identical to the MM+HLKU medium, except that tryptophan (30μg/mL) was added to the MM+(H-L)HKU+Trp medium. The purpose of the tryptophan addition was to induce *PTR2* expression as shown in previous studies

(ISLAND *et al.* 1987). The efficiency of the utilization of His-Leu was reflected in cell growth. The mini-cultures in the microtiter plates were grown at 30˚C with shaking at 200 rpm. The OD_{620} was measured at incubation times of 0, 12, 24, 48, and 72 hours with a 96-well plate reader (Labsystems Multiskan MCC/340, Finland). In some circumstances, growth at incubation times of 36, 60, 84, and 96 hours were also recorded. The *ptr2* deletion mutant was not able to grow in the His-Leu supplemented medium, although its growth in the medium MM+HKLU was excellent. Strains either defective in peptide utilization (*ptr1* deletion) or over-expressing *PTR2* (*cup9* deletion) were inoculated into wells of each plate as controls showing defective or enhanced dipeptide utilization, respectively. In this manner, deletion strains were identified that grew more, or less, efficiently than wild-type in MM+(H-L)HKU or MM+(H-L)HKU+Trp medium.

Solid medium growth assay: Using the same principle as that employed in the liquid medium growth assay, a solid medium growth assay was used to determine whether the candidate gene deletion strains obtained from the liquid medium growth screen could utilize dipeptide on solid medium as their substrate to satisfy auxotrophic requirements. The deletion mutant candidates from the screen, wild type BY4742, *cup9*, and *ptr1* deletion mutants were grown overnight in MM+HLKU liquid medium at 30˚C while shaking at 200rpm. Cells were harvested by centrifugation and washed three times with sterile, distilled water. Cells were then counted by hemacytometer and adjusted to the cell number of $5x10^6$/mL. Five microliters of a suspension containing $2.5x10^4$ and $2.5x10^3$ yeast cells were spotted onto agar plates containing one of three different media: (1) MM+HLKU, (2) MM+(H-L)HKU, or (3) MM+(H-L)HKU+Trp. The plates were

incubated at 30˚C, and growth was recorded after 2 days. A score was given to the growth of each strain compared to the growth of wild-type, *cup9*, and *ptr1* deletion mutant strains.

Toxic halo assays and osmotic sensitivity assay: For the toxic dipeptide halo assay, the sensitivity of deletion mutants to the toxic dipeptide Ala-Eth was measured as previously described (ISLAND *et al.* 1987). Ethionine (Eth) is an analog of methionine, and utilization of Eth causes cell death. This assay is more sensitive than the previously described dipeptide growth assays. Yeast cells having a functional dipeptide transport system will take up the toxic dipeptide and die (indicated by a clear halo of growth inhibition on plates spotted with ethioninyl-containing peptides). Cells with a defective dipeptide uptake system will not take up the dipeptide efficiently and will survive (as indicated by a small halo or no halo). Cells were grown overnight in MM+HLKU medium, then harvested and washed three times with sterile, distilled water. Yeast cells were counted and adjusted to the cell number of $5x10^6$/mL. One milliliter of the cell suspension was added to 0.8% noble agar (3mL) and plated onto solid MM+HLKU medium. Two 6mm sterile paper disks containing either 0.2 or 0.1µmoles of Ala-Eth were placed on the lawn of cells. The halo size was measured after two days of incubation at 30˚C. The halo size formed by each deletion mutant was compared to the halo size of the wild-type strain by dividing the halo size of the mutant by the halo size of the wild-type strain and multiplied by 100 to get a percentage. A strain that gave a higher than 100% value was considered to be deleted for a gene that would was involved in

down-regulation of peptide utilization, whereas a strain deleted for a gene involved in up-regulation would give a lower than 100% value in this test.

For the canavanine toxicity assay, the procedure was the same as the toxic dipeptide halo assay. Two 6 mm sterile paper disks containing 1µg of canavanine were placed on the lawn of cells. The size of halo was measured and the image was taken after two days incubation at 30˚C.

For the osmotic sensitivity assay, the wild type and deletion mutant strains were grown overnight in MM+HLKU medium, then harvested and washed three times with sterile, distilled water. Yeast cells were counted and adjusted to the cell number of $5x10^6$/mL, then cells were further diluted to $5x10^5$/mL, $5x10^4$/mL, and $5x10^3$/mL.Ten microliters of each cell suspension was spotted into MM+HLKU media containing 0M, 0.4M, and 1.0M NaCl.

Uptake assays: The strains were grown overnight in MM+HLKU, then sub-cultured into a fresh medium. Cells were harvested in log phase, washed with 2% glucose and adjusted to a final concentration to $2x10^8$ cells/mL. The uptake assay was initiated by combining equal volumes of pre-warmed (30˚C) cells and uptake assay mixture [2% glucose, 20mM sodium citrate/potassium phosphate, pH 5.5, 320µM Leu-Leu (Sigma), and 2µCi/ml [^{3}H]Leu-Leu (ISLAND *et al.* 1987)]. After 10 minutes, portions (100µL) were removed onto a membrane filter and washed four times by vacuum filtration with 1mL ice cold water. The radioactivity retained on the filter was determined by liquid scintillation spectrometry, and results were reported as nmol Leu-Leu uptake/$1x10^9$ cells/10 min. The accumulation of [^{3}H]Leu-Leu in *ptr2* strain was subtracted from that of

the tested strains, and the percentage of the accumulation of [^{3}H]Leu-Leu in each deletion mutant vs the wild-type was calculated.

Reporter gene assay: The centromeric plasmid pRD1, which contained a selectable *URA3* marker and the *lacZ* gene under the control of the *PTR2* promoter was transformed into the tested deletion mutant strains. The reporter gene assay was performed using a protocol adapted from Hoffman (HOFFMAN *et al.* 2002). The strains were grown overnight in MM+HLK, and sub-cultured into a fresh medium. Cells were harvested in log phase, washed with sterile water and adjusted to a final concentration to $1x10^8$ cells/mL. Cell suspension (100µL) was added to a well of a 96-well plate, then 20µl FDG (Fluorescein di-β-D-galactopyranoside) solution was added. The FDG solution was prepared by mixing solution 1 (0.1mM FDG diluted in 25mM PIPES, pH7.2) and solution 2 (5% Triton X-100 diluted in 250mM PIPES, pH7.2) in equal amounts just prior to use. The plate was incubated at 37˚C for 1.5 hours, then read with a fluorescence multi-well plate reader (Wallac Victor2, 1420 multilabel counter, PerkinElmer Life and Analytical Sciences, Inc., MA) using 485nm and 530nm as excitation and emission wavelengths, respectively.

Classification of the obtained genes: The classification of the 103 genes identified through this screen was based on the Gene Ontology (GO) annotation found in the Saccharomyces Genome Database (SGD) (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper) (CHRISTIE et al. 2004), where 34 functional categories of biological process were given. Among the 103 genes, some genes fell into several

categories due to their multiple known functions. The detailed gene function could also be referenced from Yeast Proteome Database (YPD) database (https://proteome.incyte.com/control /tools/ proteome) (HODGES et al. 1999).

Northern analysis of *PTR2* mRNA in deletion mutant strains: Approximately $3x10^8$ cells of select candidate deletion mutant strains were harvested after overnight growth in MM+HLKU medium. Yeast total RNA was isolated using an extraction kit (RiboPure^{TM}-yeast, Ambion, Austin, TX). Total RNA was quantified by monitoring absorbance at 260nm, and 20µg was loaded per lane into a formaldehyde-reducing gel. Gels were run in buffer containing MOPS (pH 7.0) for 5 hours at 75V and then transferred by capillary action onto a nylon hybridization membrane in 20x SSC. Total RNA was UV cross-linked to the membrane with a Stratalinker UV source. The blot was prehybridized at 65°C in Church buffer (7% SDS, 1% BSA, 1mM EDTA, and 250 mM Na_2HPO4, pH 7.2) for 2 hours and hybridized overnight in the same buffer containing radioactive probes at 65°C. Radioactive probes were prepared as follows. A 1.7kb fragment of *PTR2* was obtained by PCR amplification using the primers PTR2-F (Northern) and PTR2-R (Northern), and a 0.92 kb fragment of *ACT1* was obtained by PCR amplification using the primers ACT1-F (Northern) and ACT1-R (Northern) (primer sequences are represented in Supplemental Table S1). DNA fragments were labeled with (α-^{32}P)-dATP by random-primed probe synthesis. Blots were rinsed with post-hybridization buffer (0.1X SSC, 0.1% SDS) at 65°C, and the RNA images were developed with a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA). The expression of *PTR2* in each mutant was analyzed at least twice in separate experiments, with similar expression found

in each experiment. The quantification of *PTR2* and *ACT1* mRNA was performed using the software ImageQuant5.0 (GE Healthcare, Technologies, Waukesha, Wisconsin).

Real-Time RT PCR: Approximately 3 x 10^8 cells of the tested deletion mutant strains were harvested after overnight growth in MM+HLKU medium. Yeast total RNA was isolated using RiboPure^{TM}-Yeast extraction kit (Ambion, Austin, TX), and then treated with TURBO DNA-free^{TM} Kit to eliminate the genomic DNA contamination (Ambion, Austin, TX). The amount of total RNA was quantified by monitoring absorbance at 260nm. To check that the genomic DNA had been eliminated, the obtained total RNA was used for the template to PCR amplify the target genes, and no PCR product was obtained. cDNA was synthesized using M-MLV RT (Invitrogen; 400 U reverse transcriptase) in a reaction mixture containing 1μg Oligo(dT), 1 mM deooxynucleoside triphosphates, 14 U anti-RNase and 1μg of total RNA at 42°C for 45min, and the reaction was stopped after 5 minutes by incubation at 72°C.

Real-Time PCR analysis was performed in the DNA Engine Opticon^{TM} (MJ Research, Boston, MA). QuantiTect^{TM} SYBR Green PCR Kit (Qiagen, Valencia, CA) and the primers (the sequences are presented in Supplemental Table S1) resulting in about 100bp amplicon were applied in the PCR reaction. The reaction contained 12.5 μl of 2x qPCR reaction mix, 15pmol of Primers, and run with a cycle of 50ºC for 2min, 95ºC for 15 min, followed by 40 cycles of 95ºC for 15s, 54ºC for 30s, and 72ºC for 15s. A standard curve for each primer set was performed with 1, 1:10, 1:100, 1:1000, 1:10000, 1:100000 dilutions of the wild type cDNA. The C_T value, the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence, was determined

using the software program. The copy number of *PTR2, PMA1*, and *ACT1* genes in each deletion mutant strain was calculated based on the standard curve. The ratio of the fold change of target genes (*PTR2* and *PMA1*) *vs* the fold change of the internal control gene (*ACT1*) in the tested mutant strains was calculated to show up-regulation or down-regulation. The ratio of the fold change in the wild type was standardized as 1.0.

Ptr2p localization in deletion mutant strains: In order to trace the localization of Ptr2p, a Ptr2p-GFP construct was created as follows. The primers PTR2-FLAG-GFP-F and PTR2-Flag-GFP-R (see Supplemental Table S1 for the sequence) were used to PCR amplify two tandem copies of the GFP gene using pKW430 [a gift of Dr. Mary Miller, Rhodes College, Memphis, TN (STADE *et al.* 1997)] as the template. The amplified PCR product (1.5kb) had two copies of GFP sequence with 40bp of flanking sequence homologous to pMS2, which contained *PTR2* with its endogenous promoter and terminator sequences and both FLAG and His tags located at the C terminus. The plasmid was linearized with *Age*I at a unique restriction site located between the FLAG and His tags. The centromeric plasmid pMS4, containing Ptr2p-FLAG-GFP_2-His6 and the selectable marker *URA3*, was created by homologous recombination.

To test the localization of Ptr2p-GFP in different yeast strains, the pMS4 construct was transformed into each deletion mutant of interest and transformants selected by growth in the absence of uracil. Yeast strains carrying pMS4 were pre-grown in MM+HLK overnight, then inoculated into fresh MM+HLK at an initial cell concentration of $2x10^6$ cells/mL. Cells were concentrated by centrifugation and observed at 10 hours after inoculation by fluorescence microscopy using a 470-490nm excitation

wavelength and 515nm emission filter fitted to an Olympus microscope. Images were taken with a MicroFire^{TM} camera (Model S99809, Olympus). All mutants were in the log phase at the 10 hour time point as ascertained by the high proportion of cells with buds and the low density of cells under the experimental conditions used. To visualize the amount of GFP expression, each image was captured with the same exposure time. Significantly higher and lower GFP signal could be distinguished from the captured images. For those images with higher GFP signal, the images were taken with a decreased exposure time to be visualized clearly.

RESULTS

Primary Screening of Deletion Mutants to Identify Strains Demonstrating Increased or Decreased Dipeptide Utilization

In order to identify genes involved in dipeptide utilization, we screened a library of 4826 haploid, single-gene deletion strains for mutants with an altered dipeptide utilization profile. The leucine auxotrophic marker in the BY4742 strain background *(MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0)* was used to monitor how well each strain was able to utilize the dipeptide His-Leu. Under the conditions of the screen, cells could grow only if they were able to take up the dipeptide His-Leu from the medium and release leucine from the peptide intracellularly. Numerous previous studies have shown that there is no extracellular peptidase or protease in *S. cerevisiae* able to catalyze the hydrolysis of dipeptides under the growth conditions used. In this screening, any mutant strain demonstrating either an enhanced or decreased growth was considered to have a deletion in a gene involved in dipeptide utilization. Since Ptr2p is the only dipeptide transporter expressed in this yeast under the conditions used for the screen, we expected that genes related to the regulation of *PTR2* expression or Ptr2p function would comprise the list of candidates. The yeast deletion mutant library was generated by the Saccharomyces Genome Deletion Project, which involved several laboratories (WINZELER *et al.* 1999). Only non-essential genes are represented in this deletion mutant collection.

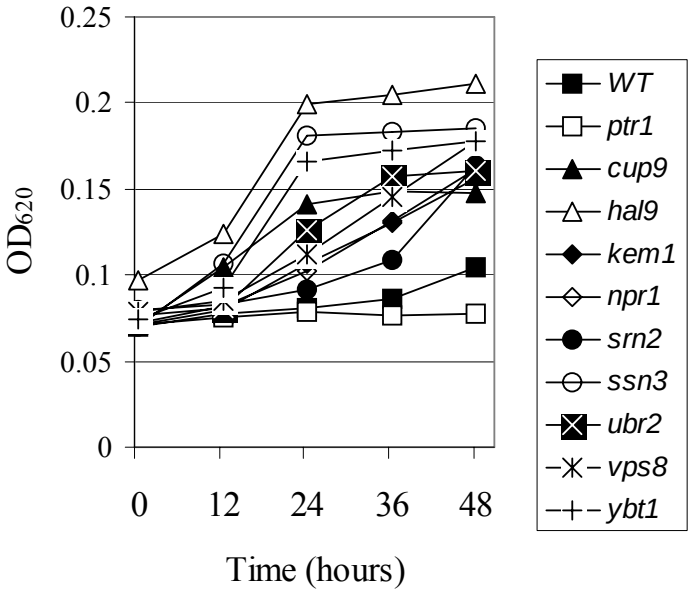
In the primary screening, yeast deletion mutants were grown in a poor nitrogen medium with allantoin as the nitrogen source. These conditions are known to partially up-regulate *PTR2* expression (ISLAND *et al.* 1991). All deletion mutant strains not capable of growing in allantoin as the sole nitrogen source were necessarily excluded from this

study. One hundred and nine strains of the haploid collection were identified in this category. For a full list of the 109 strains see supplemental data Table S2. The rest of the deletion series, including strains known to be deficient (*ptr1*) and hyper-active (*cup9*) for dipeptide utilization, grew similarly in the MM+HLKU medium.

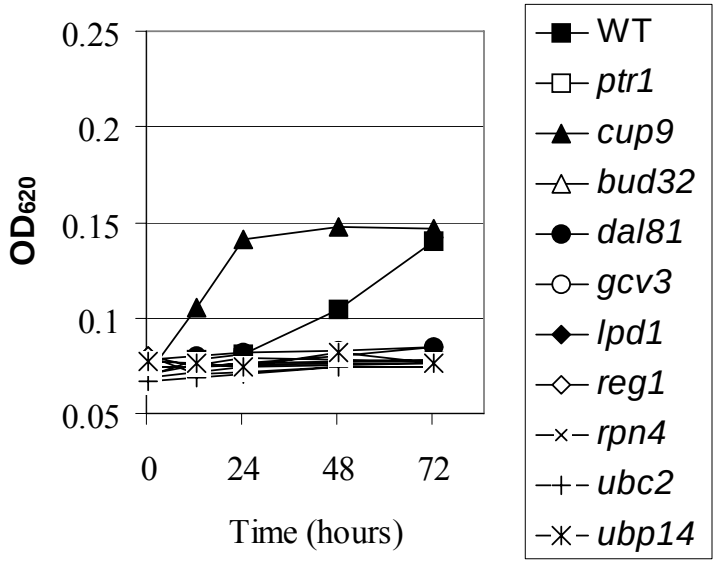
A screen was performed to the mutant collection in medium [MM+(H-L)HKU+Trp] containing His-Leu as the only leucine source. Tryptophan up-regulates *PTR2* expression via the SPS system over the enhancement induced by growth on MM alone thus providing the maximal dipeptide utilization phenotype (FORSBERG and LJUNGDAHL 2001b; ISLAND *et al.* 1987). The screen was also performed without tryptophan addition with identical results observed (data not shown). Two hundred seventy-eight deletion mutants were obtained from the primary screening that showed either enhanced dipeptide utilization (the deleted genes are "negative regulator genes") or decreased dipeptide utilization (the deleted genes are "positive regulator genes"). As controls for comparison of strains that show increased or decreased dipeptide utilization, *cup9* and *ptr1* mutants were used. Cup9p is a repressor of *PTR2* transcriptional expression; the *cup9* deletion mutant strain has a higher expression level of *PTR2* in comparison to that of the wild-type (BYRD *et al.* 1998), therefore the hyper-active strain (*cup9*) grew better than wild-type on dipeptide (Figure 1 A). Ptr1p is required for degradation of Cup9p; in the *ptr1* deletion mutant strain Cup9p is stabilized (DU *et al.* 2002; ISLAND *et al.* 1991), and there is an undetectable level of *PTR2* expression preventing this strain from growing on dipeptide (Figure 1 A). For clarity of presentation and to present data representative of the phenotypes among the mutants identified, we highlight in the Results section of this paper eighteen deletion mutant strains (*bud32,*

Figure 1 Growth patterns of various selected strains that demonstrate increased or decreased dipeptide utilization. A) The growth pattern of wild-type (BY4742), *ptr1*, *cup9*, and eight other deletion mutant strains in MM+H(H-L)KU+Trp medium. B) The growth pattern of wild-type (BY4742), *ptr1*, *cup9*, and nine other deletion mutant strains in MM+H(H-L)KU+Trp medium.

A)


0.25
0.2
0.15
0.1
0.05
0
OD620
0
12
24
36
48
Time (hours)
WT
ptr1
cup9
hal9
kem1
npr1
srn2
ssn3
ubr2
vps8
ybt1

B)


0.25
0.2
0.15
0.1
0.05
OD620
0
24
48
72
Time (hours)
WT
ptr1
cup9
bud32
dal81
gcv3
lpd1
reg1
rpn4
ubc2
ubp14

cup9, dal81, gcv3, hal9, kem1, lpd1, npr1, ptr1, reg1, rpn4, ssn3, srn2, ubc2, ubp14, ubr2, vps8, and ybt1).

Growth patterns of deletion mutant strains *hal9, kem1, npr1, srn2, ssn3*, *ubr2, vps8,* and *ybt1* were similar to that of the *cup9* deletion mutant in that they had a shorter lag phase and a higher growth rate compared to the wild type (Figure 1A). Therefore, these deletion strains were considered to carry a deletion in a gene involved in decreasing dipeptide utilization. Growth patterns of deletion mutants *bud32, dal81, gcv3, lpd1, reg1, rpn4, ubc2,* and *ubp14,* had a similar growth pattern to the *ptr1* deletion strain which did not grow in MM+(H-L)HKU+Trp medium (Figure 1 B). These deletion mutants were considered to carry a deletion in a gene involved in increasing dipeptide utilization.

Verification of Candidate Genes: Solid Media Growth Assay and Toxic Dipeptide Halo Assay

The candidate mutants were further tested in a solid medium growth assay and a toxic dipeptide halo assay. The deletion mutant strains were grown on solid medium plates containing MM+HLKU, MM+(H-L)HKU, or MM+(H-L)HKU+Trp. Eighteen representative deletion mutant strains with enhanced or decreased dipeptide utilization are shown in Figure 2. All the tested deletion mutant strains grew to a similar extent as compared to that of the wild type in MM+HLKU. The deletion mutants *hal9, kem1, npr1, srn2, ssn3, ubr2, vps8,* and *ybt1* had a similar growth pattern to the *cup9* deletion mutant strain, which grew better than wild-type in both MM+(H-L)HKU+Trp (Figure 2) and MM+(H-L)HKU (data not shown). In contrast, the deletion mutants *bud32, dal81, gcv3, lpd1, reg1, rpn4, ubc2,* and *ubp14* had a similar growth pattern to the *ptr1* deletion

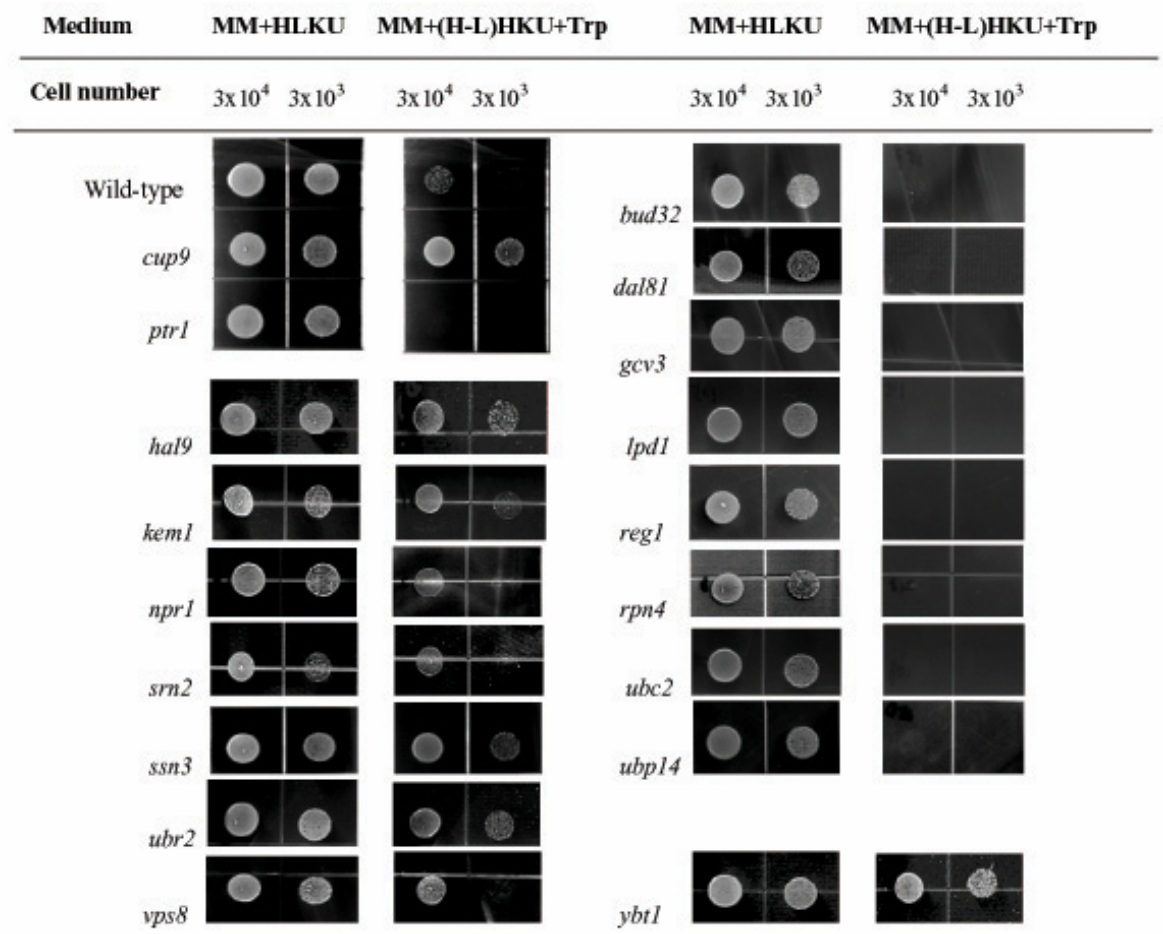


Figure 2 Growth of selected strains on solid medium. Two cell dilutions, $3x10^4$ cells /mL, and $3x10^3$ cells/mL were tested on MM+HLKU and MM+(H-L)HKU+Trp media.

mutant strain, which could not grow in MM+(H-L)HKU+Trp medium (Figure 2) or in MM+(H-L)HKU (data not shown). The growth phenotype found in the liquid growth screening of these chosen candidate mutants with respect to the regulation of dipeptide utilization was therefore confirmed in this solid medium test.

The mutants identified in the initial screen were also subjected to a toxic dipeptide assay for further verification of the peptide transport phenotype. In this assay, cells were grown on MM+HLKU and tested for their sensitivity to Ala-Eth. The halo size of each deletion mutant strain was measured and compared to that of wild-type. A larger halo indicated that the deletion mutant strain was more sensitive to Ala-Eth toxic dipeptide than the wild-type, while a smaller halo indicated less sensitivity. As expected, *cup9* developed a larger halo than that of wild-type and *ptr1* was not sensitive to the toxic dipeptide. Similar to that of *cup9*, deletion strains *hal9, kem1, npr1, srn2, ssn3, ubr2, vps8,* and *ybt1* developed a larger halo than that of wild-type (Table 1), indicating the deleted genes negatively regulated dipeptide utilization. Conversely, similar to that of *ptr1,* deletion strains *bud32, dal81, gcv3, lpd1, reg1, rpn4, ubc2,* and *ubp14* were not sensitive to toxic dipeptide (Table 2), indicating that the deleted genes positively regulated dipeptide utilization.

Of the 278 strains identified in the initial liquid growth screen, 103 strains showed increased or decreased peptide utilization in all three assays: liquid and solid media growth and the toxic dipeptide halo assay (Table S3 and S4 listed in order of biological process according to Gene Ontology annotation). These 103 genes were considered to be the set of genes involved as either negative or positive regulators of peptide utilization as determined by the three experimental tests. As expected, five genes (*PTR1*, *CUP9*,

Table 1 The response of deletion mutants with increased dipeptide utilization to Ala-Eth, NaCl, and canavanine.[a]

Gene name	Description of gene product	Toxicity of Ala-Eth (% of wild type)[b]	NaCl Sensitivity[c]	Canavanine toxicity (% of the wild type)[d]
Wild-type		100		
CUP9	Specific RNA polymerase II transcription factor activity	120	-	-
HAL9	Specific RNA polymerase II transcription factor activity	131	-	-
KEM1	5'-3' exoribonuclease activity	135	More sensitive	Less sensitive
NPR1	Serine/threonine protein kinase	129	-	Less sensitive
SRN2	Class E vacuolar sorting protein	109	More sensitive	More sensitive
SSN3	Cyclin-dependent protein kinase activity	114	-	-
UBR2	Ubiquitin-protein ligase activity	129	-	-
VPS8	Vacuolar sorting	121	More sensitive	More sensitive
YBT1	Bile acid transporter activity	156	-	-

[a]These data are also presented in supplementary Table S3, Figure S4, and Figure S5.
[b] The halo size formed by each deletion mutant was compared to the halo size of the wild-type strain by dividing the halo size of the mutant by the halo size of the wild-type strain and multiplied by 100 to get a percentage.
[c] "-" indicates that the deletion strain had no difference in its sensitivity to NaCl compared to that of the wild-type. A strain that was more sensitive exhibited greater growth inhibition to 1.0 M NaCl in comparison to that shown by the wild-type.
[d] "-" indicates that the deletion strain had no difference in sensitivity to canavanine compared to that of the wild-type.

Table 2 The response of deletion mutants with decreased dipeptide utilization to Ala-Eth, NaCl, and canavanine.[a]

Gene name	Description of gene product	Toxicity of Ala-Eth (% of wild type)[b]	NaCl Sensitivity[c]	Canavanine toxicity (% of the wild type)[d]
Wild-type		100		
PTR1	Ubiquitin-protein ligase activity	0	-	-
BUD32	Protein serine/threonine kinase activity	0 (Fuzzy halo)	More sensitive	Less sensitive
DAL81	Specific RNA polymerase II transcription factor activity	0	-	More sensitive
GCV3	Glycine dehydrogenase (decarboxylating) activity	0	-	More sensitive
LPD1	Dihydrolipoyl dehydrogenase activity	0	-	More sensitive
REG1	Protein phosphatase type 1 activity	0 (Fuzzy halo)	-	More sensitive
RPN4	Transcriptional activator activity	0	-	-
UBC2	Ubiquitin conjugating enzyme activity	0	-	-
UBP14	Ubiquitin-specific protease activity	73	-	-

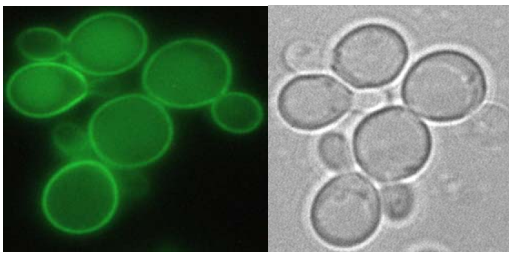
[a]These data are also presented in supplementary Table S3, Figure S4, and Figure S5.
[b] The halo size formed by each deletion mutant was compared to the halo size of the wild-type strain by dividing the halo size of the mutant by the halo size of the wild-type strain and multiplied by 100 to get a percentage.
[c] "-" indicates that the deletion strain had no difference in its sensitivity to NaCl compared to that of the wild-type. A strain that was more sensitive exhibited greater growth inhibition to 1.0 M NaCl in comparison to that shown by the wild-type.
[d] "-" indicates that the deletion strain had no difference in sensitivity to canavanine compared to that of the wild-type.

UBC2, *DAL81/UGA35*, and *STP2)* previously known to be involved in dipeptide utilization were identified among the 103 genes, which validated the utility of the screening method. Three other genes (*SSY1*, *PTR3*, and *SSY5*), known to affect dipeptide utilization via the regulation of *PTR2* mRNA expression, were not found in the screen since they are not included in the deletion mutant library. The number of deletion mutant strains found by our methods to modulate dipeptide utilization accounted for about 2% of the entire collection. Of the 46 genes whose deletion mutant strains enhanced dipeptide utilization (Table S3), 20 encoded proteins with more than a 30% protein sequence identity to a human protein (Table S3, footnote a). Additionally, of the 57 genes whose deletion mutant strain decreased dipeptide utilization (Table S4), 33 encoded proteins with more than a 30% sequence identity to a human protein (Table S4, footnote a). According to Gene Ontology (GO) annotation in SGD, the 103 genes covered 29 out of the 34 given categories in the Biological Processes GO categories. The categories of each gene are indicated in Table S3 and S4.

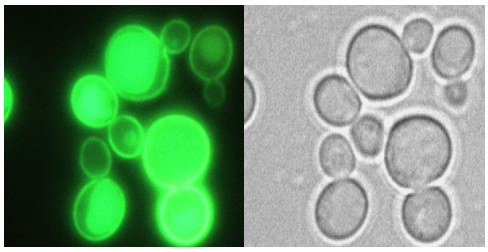
Ptr2p-GFP Expression and Localization in the Identified Deletion Strains

In order to further explore the impact of the identified 103 genes on dipeptide utilization, we measured Ptr2p expression and localization using a Ptr2p-GFP chimera. This construct (pMS4), which encoded Ptr2p-GFP under the control of its native promoter, was transformed into the 103 deletion strains, and Ptr2p-GFP in log phase cells was observed by fluorescence microscopy. The addition of the GFP tag did not affect the function of Ptr2p as demonstrated by halo and uptake assays (data not shown). Ptr2p-GFP was primarily localized to the plasma membrane in wild-type log-phase cells (Figure 3).

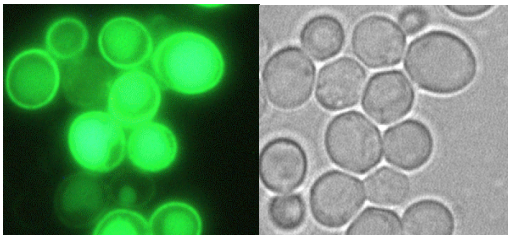
Figure 3 Ptr2p-GFP expression and localization in the wild-type (WT) and 9 deletion strains that demonstrated increased dipeptide utilization. The right-hand panel of each strain shows phase microscopy of the same field as the left-hand panel which shows the fluorescently-labeled Ptr2p-GFP. Deletion strain *ssn3* showed a very high amount of GFP signal under the same exposure time as that of the wild-type.



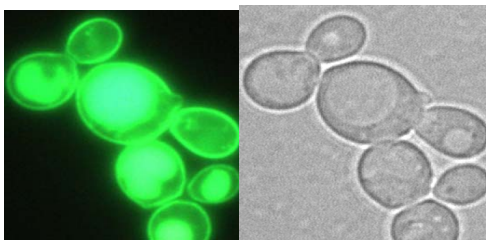
WT



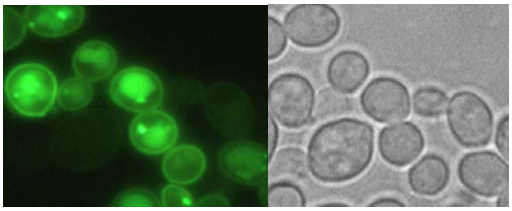
cup9



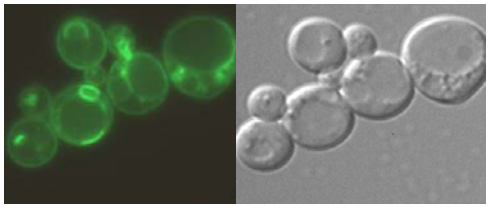
hal9



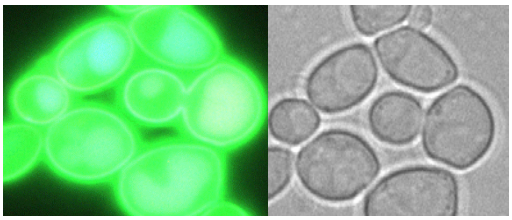
kem1



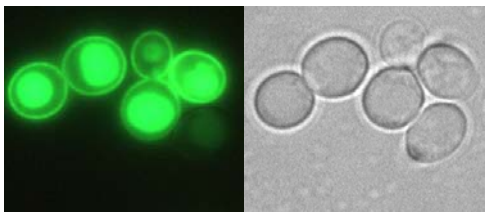
npr1



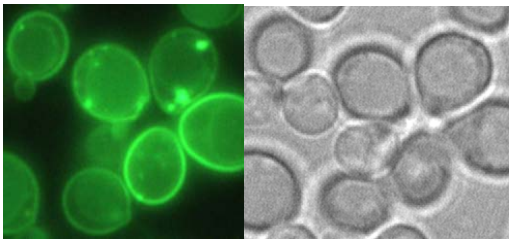
srn2



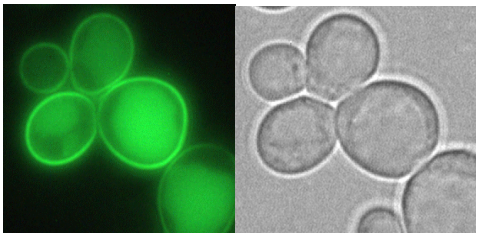
ssn3



ubr2



vps8



ybt1

The amount of the Ptr2p-GFP expression signal and its localization in the 103 deletion mutant strains revealed seven phenotypic patterns (Table 3).

Ptr2p-GFP expression and localization in deletion strains with increased dipeptide utilization: The Ptr2p-GFP expression signal and localization in 46 deletion strains with increased dipeptide utilization are shown in Figure S1 (9 deletion strains and wild type are shown in Figure 3 as representative of the full set).

Strains with enhanced Ptr2p-GFP expression signal: A higher level of Ptr2p-GFP expression signal in the plasma membrane was observed in the *cup9* deletion strain as compared to the wild-type (Figure 3 and Table 3). The enhanced Ptr2p-GFP expression level corresponded with the phenotype of increased dipeptide utilization as reflected in growth on dipeptide in liquid and solid medium and toxicity to Ala-Eth (Figure 1, Figure 2, and Table 1). In addition, Ptr2p-GFP was also observed in the vacuole, possibly due to the over-expressed *PTR2* in the *cup9* strain. Similar to the *cup9* strain, the deletion of *HAL9, KEM1*, *SSN3,* and *UBR2* showed an increased Ptr2p-GFP expression signal in both the plasma membrane and the vacuole. Enhanced Ptr2p-GFP expression signal was also observed in 18 other deletion strains including four unknown gene deletion mutants, *yfr044c, ylr114c, ynl123w,* and *yor322c* (Table 3 and Figure S1). Consistently, the increased Ptr2p-GFP expression agreed with increased sensitivity to toxic dipeptide and better growth on dipeptide substrate (Table S3).

Strains with altered Ptr2p-GFP localization: Fourteen deletion strains with increased dipeptide utilization showed altered localization of Ptr2p-GFP (Table 3 and Figure S1). An example of three deletion mutants, *npr1, srn2*, and *vps8* is shown in Figure 3. These

Table 3 Ptr2p-GFP expression and localization in 103 identified strains with decreased or increased dipeptide utilization.[a]

Phenotypic Category	Increased dipeptide utilization (46 strains)	Decreased dipeptide utilization (57 strains)
Strains with enhanced Ptr2p-GFP expression	*arp5, arp8,* ***cup9****, csm1, dbr1, eaf3, hal9, ies6, kem1, mrps9, pat1, sfp1, srb8, ssd1, ssn2, ssn3, ssn8, tom72, ubr2, yor322c, ynl123w, yfr044c, ylr114c*	*asm4, ydr015c, ydr290w*
Strains with altered Ptr2p-GFP localization	*bni1, def1, eaf7, lst4, npr1, sac6, snf7, srn2, tpm1, vam10, vps8, vps36, ypl073c, ynl295w*	*bem4, bud32 , csf1, etr1, gcv2, gcv3, hfm1, ilm1, isa1, isa2, mck1, mre11, npl3, shp1, reg1, rmd12, rps9b, tsa1, vbm1, ydr433w, yjl046w, ydr157w, yjl175w*
Strains with no apparent effect on Ptr2p-GFP localization or expression	*cik1, kti12, mlh1, mrp17, pho2, rim101, tif3, ybt1, ydr417c*	*bud28, bye1, elf1, hfa1, ira2, lat1, lpd1, mct1, oar1, pdb1, pdx1, prd1, rad23, spo21, ypl098c*
Strains with decreased Ptr2p-GFP expression	No mutant strains were found in this category.	***dal81****, hof1, ipk1, nfu1, pho85,* ***ptr1,*** *rpl21a, rpn4, shm2,* ***stp2,*** *taf14, thp1, uba3, ubp14,* ***ubc2,*** *ypr174c*

[a]Ptr2p-GFP expression and localization in the underlined strains are shown in Figures 3 and 4. The underlined strains were the representatives chosen to highlight in the Results section of this paper. Ptr2p-GFP expression and localization of all the listed strains are shown in supplemental data (Figures S1 and S3). The strains in bold (***cup9, dal81, ptr1, stp2,*** and ***ubc2***) contain the deleted genes previously identified as being involved in regulation of *PTR2* transcription.

gene products had GO annotations of transport, vesicle-mediated transport, cytokinesis, or related to protein modification. Ptr2p-GFP signal in the *srn2* and *vps8* deletion mutants was mainly localized to the plasma membrane and the endosome. In addition, Ptr2p-GFP remained in the endosome in stationary phase cells and targeting of Ptr2p-GFP to the vacuole was defective (data not shown). Similar to *srn2* and *vps8* strains, the deletion strains *eaf7, sac6, snf7, vps36,* and *ypl073c* exhibited Ptr2p-GFP localization to the plasma membrane and to the endosome (Figure S1), suggesting that these gene products are involved in a similar cellular process regulating dipeptide utilization. Snf7p, Srn2p, and Vps36p are three key proteins involved in ESCRT protein complexes required for endocytotic degradation of membrane proteins (BOWERS *et al.* 2004).

To further characterize the ESCRT protein complexes involved in Ptr2p endocytotic degradation, deletion mutant strains of other components of the ESCRT protein complexes (*vps2, vps20, vps22, vps23, vps24, vps25,* and *vps28*) were also transformed with the pMS4 construct. The localization of Ptr2p-GFP in these strains was similar to that in *snf7, srn2,* and *vps36* strains (Figure S2). Additionally, *vps2, vps20, vps22, vps23, vps24, vps25,* and *vps28* strains were more sensitive to toxic dipeptide as compared to the wild type (Table S5), confirming the role of the ESCRT protein complex in dipeptide utilization.

The deletion strain *npr1* showed an accumulation of Ptr2p-GFP signal in both endosomal vesicles and the vacuole in addition to the plasma membrane localization (Figure 3). The Ptr2p sorting process is likely regulated by Npr1p, a protein kinase involved in the regulation of vesicle transport systems (Table S3). Similar to Ptr2p-GFP localization in the *npr1* strain, deletion strains *bni1*, *def1*, *lst4,* and *tpm1 s*howed Ptr2p-

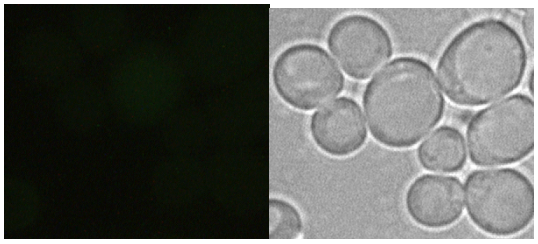
GFP localization to the plasma membrane and the vacuole (Figure S1), implicating them as well as being involved in the protein trafficking of Ptr2p.

Strains with no apparent effect on Ptr2p-GFP expression or localization: The deletion strain *ybt1* showed no apparent difference in the expression level of Ptr2p-GFP signal in the cytoplasmic membrane (Figure 3), although there was an increase in the Ptr2p-GFP signal in the vacuole. In this strain dipeptide utilization was increased (Figure 1, Figure 2, and Table 1). In addition, seven other deletion strains, *cik1, kti12, mlh1, mrp17*, *pho2*, *rim101, tif3,* and *ydr417c* demonstrated increased dipeptide utilization with no apparent effect on Ptr2p-GFP expression in the cytoplasmic membrane (Table 3 and Figure S1). These gene products might impact dipeptide utilization independently of Ptr2p function. For example, Ybt1p, known as an ABC transporter and localized on the vacuolar membrane, may be involved in the uptake of dipeptides into the vacuole. The deletion of this gene resulted in increased growth response and dipeptide toxicity.

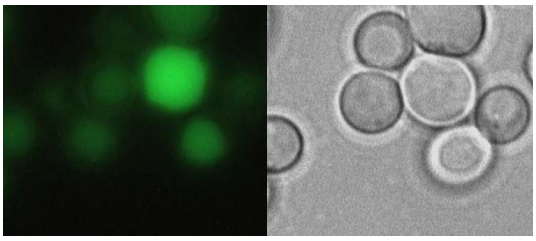
Ptr2p-GFP expression and localization in deletion strains with decreased dipeptide utilization: The expression level of Ptr2p-GFP signal and localization of nine representative strains are shown in Figure 4, and all 57 deletion strains listed in Table 3 with decreased dipeptide utilization are shown in Figure S3.

Gene deletion enhanced the expression level of Ptr2p-GFP signal: The deletion strains *asm4, ydr015c* and *ydr290w* showed an enhanced expression level of Ptr2p-GFP at the cytoplasmic membrane and the vacuole (Figure S4). These strains showed a decreased sensitivity to toxic dipeptide, however, with no growth change on dipeptide (Table S4). In the *asm4* strain, a fuzzy halo was observed in the toxic dipeptide halo assay indicating

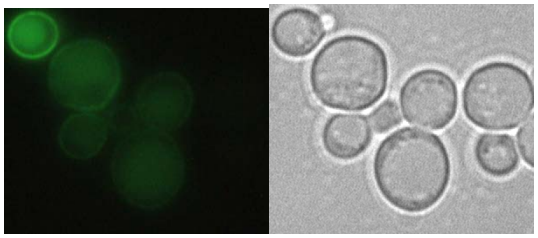
Figure 4 Ptr2p-GFP expression and localization in 9 selected deletion strains that demonstrated decreased dipeptide utilization. The right-hand panel of each strain shows phase microscopy of the same field as the left-hand panel which shows the fluorescently-labeled Ptr2p-GFP.



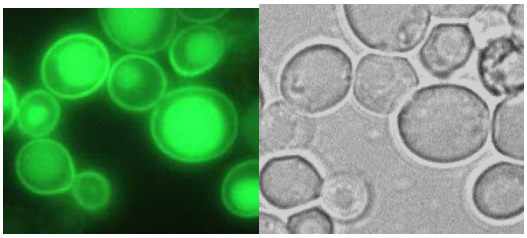
ptr1



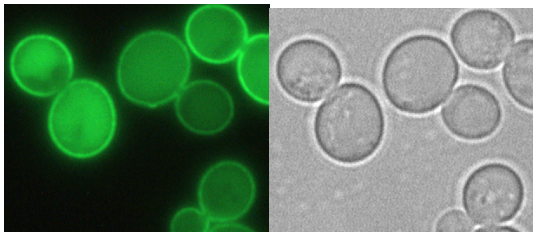
bud32



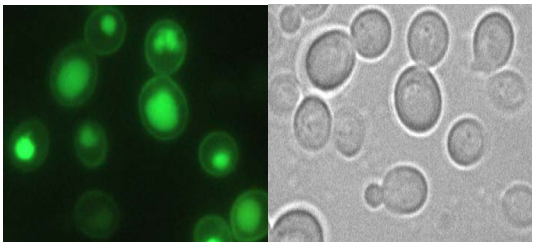
dal81



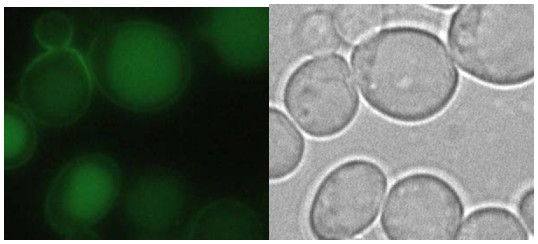
gcv3



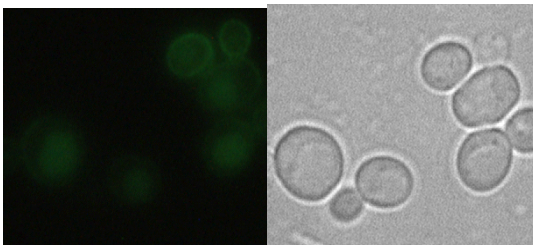
lpd1



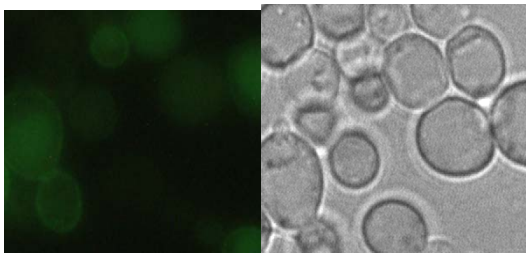
reg1



rpn4



ubc2



ubp14

a transient growth inhibition. *ASM4* encodes a component of the karyopherin docking complex of the nuclear pore (MARELLI *et al.* 1998) indicating that some cellular protein involved in ethionine toxicity requires Asm4p for its full expression.

Strains with altered Ptr2p-GFP localization: Compared to the wild-type, deletion strains *bud32* and *reg1* showed higher expression level of Ptr2p-GFP signal at the vacuole and less expression in the plasma membrane (*reg1*) or no visible plasma membrane expression (*bud32*) (Figure 4). In addition, deletion strain *gcv3* showed Ptr2p-GFP localization both at the vacuole and plasma membrane (Figure 4). Similar to the Ptr2p-GFP localization at the vacuole in *bud32, reg1*, or *gcv3* strains, the deletion strains *bem4, csf1, etr1, gcv2, hfm1, ilm1, isa1, isa2, mck1*, *mre11, npl3, rps9b, rmd12, shp1, tsa1, vbm1,* and four unknown gene deletion strains (*ydr157w, ydr433w, yjl046w,* and *yjl175w*) resulted in Ptr2p-GFP localization both at the vacuole and plasma membrane (Table S4 and Figure S3).

Strains with no apparent effect on the expression or localization of Ptr2p-GFP: No apparent change in the expression or localization of Ptr2p-GFP was observed in the *lpd1* deletion strain (Figure 4). Similarly, Ptr2p-GFP localization and expression level was not different in the deletion strains *bye1, bud28, elf1, hfa1, ira2, lat1, mct1, oar1, pdb1, pdx1, rad23,* and *ypl098c* as compared to the wild-type (Table S4 and Figure S3).

Strains with decreased expression of Ptr2p-GFP: The expression level of Ptr2p-GFP signal was lower in the *ptr1, dal81, rpn4, ubc2,* and *ubp14* deletion strain as compared to the wild-type (Figure 4). A decreased Ptr2p-GFP expression signal was also observed in the deletion strains *hof1, ipk1, nfu1, pho85, rpl21a, shm2, stp2, taf14, thp1, uba3,* and *ypr174c* (Table S4 and Figure S3). The decreased Ptr2p-GFP expression signal in these

strains was consistent with a decrease of dipeptide utilization which was previously documented (Table S4) and is consistent with the observation that Ptr1p, Stp2p, Ubc2p, and Dal81p positively regulate *PTR2* transcription (ANDREASSON and LJUNGDAHL 2002; BERNARD and ANDRE 2001; DU *et al.* 2002).

Transcriptional Regulation of *PTR2*

In order to explore whether strains with enhanced or reduced expression level of Ptr2p-GFP showed a change in the transcriptional regulation of *PTR2*, the fold change of *PTR2* mRNA compared to that of the wild type was measured by Real-Time reverse transcription PCR in the eighteen representative deletion strains. In control experiments, *PTR2* mRNA expression level in the *cup9* strain was up-regulated more than nine times that of the wild-type (Table 4). This result also agreed with the increased *PTR2* mRNA level in Northern analysis and *lacZ* activity as compared to the wild-type (data not shown). The increase in *PTR2* mRNA expression in the *cup9* strain is consistent with the increased expression level of Ptr2p-GFP signal (Figure 3). Similar to that of the *cup9* strain, *PTR2* mRNA expression level was highly up-regulated in *ssn3* and *kem1*. *PTR2* mRNA expression was up-regulated in *ubr2*, *hal9*, *ybt1*, *vpc8*, and *gcv3* less than two-fold. In contrast to the *cup9, hal9, kem1, ssn3, ubr2, vps8,* and *ybt1* strains, which showed increased dipeptide utilization, the *gcv3* strain exhibited a decrease in peptide utilization (Table S4). The apparent discrepancy between decrease in utilization and robust expression in *gcv3* strain could result from a post-translational alteration of Ptr2p function or the alteration of the metabolic pathway(s) of dipeptide utilization.

Table 4 The expression of *PTR2* and *PMA1* in various strains.

Strains	*PTR2* expression[a]	*PMA1* Expression[a]
ubc2	0.06 (0.02)	0.94 (0.50)
ptr1	0.07 (0.03)	1.08 (0.40)
ubp14	0.08 (0.04)	1.09 (0.48)
rpn4	0.21 (0.02)	1.23 (0.25)
npr1	0.48 (0.1)	1.33 (0.08)
dal81	0.79 (0.07)	1.70 (0.31)
reg1	0.88 (0.06)	1.03 (0.31)
lpd1	0.82 (0.19)	2.30 (0.03)
srn2	0.92 (0.05)	0.87 (0.08)
bud32	0.94 (0.10)	1.44 (0.44)
ubr2	1.23 (0.62)	2.95 (0.35)
hal9	1.37 (0.21)	1.93 (0.35)
ybt1	1.51 (0.14)	2.37 (0.25)
vps8	1.66 (0.22)	2.09 (0.44)
gcv3	1.93 (0.56)	1.22 (0.33)
ssn3	5.00 (1.17)	2.41 (0.47)
cup9	9.36 (2.60)	0.98 (0.21)
kem1	12.12 (1.47)	3.06 (0.24)

[a]Expression level measured by Real-Time RT-PCR analysis is shown as a ratio calculated by the fold change of the target gene (*PTR2* or *PMA1*) in the mutant compared to that of the wild-type divided by the fold change of *ACT1* in the mutant compared to that of the wild-type. The datum in the bracket is the standard deviation.

PTR2 mRNA expression level in the *ptr1* strain was only 7% that of the wild type (Table 4) in accordance with the known requirement of *Ptr1p* for *PTR2* transcription (BYRD et al. 1998; TURNER et al. 2000). This result also agreed with the decreased *PTR2* mRNA level in Northern analysis and lacZ activity as compared to the wild-type (data not shown). The decrease in *PTR2* mRNA expression in the *ptr1* strain is consistent with the decreased expression level of Ptr2p-GFP signal (Figure 4). Similar to the *ptr1* strain, *PTR2* mRNA expression was highly down-regulated in *ubc2, ubp14,* and *rpn4* strains. The expression of *PTR2* mRNA in *npr1*, *dal81, reg1, lpd1, srn2,* and *bud32* strains was not more than two-fold less than that of the wild-type.

Dipeptide Uptake Capability

In order to explore whether altered dipeptide utilization was correlated directly to flux of dipeptide, the accumulation of [^{3}H]Leu-Leu dipeptide was measured in the eighteen representative strains (Figure 5). In a control experiment, the accumulation of [^{3}H]Leu-Leu increased remarkably in the *cup9* strain as compared to the wild-type (Figure 5). Similar to the *cup9* strain, the accumulation of [^{3}H]Leu-Leu was higher in the *hal9, kem1, npr1, srn2, ssn3, ubr2, vps8*, and *ybt1* strains as compared to that of the wild-type. The increased uptake in these strains was reflected in their increased ability to utilize dipeptide. In contrast to the increased accumulation of dipeptide in *hal9, kem1, npr1, srn2, ssn3, ubr2, vps8,* and *ybt1* strains, the accumulation of [^{3}H]Leu-Leu in *gcv3* was not significantly different as compared to the wild-type, suggesting that deletion of this gene did not impact the import of dipeptide even though dipeptide utilization was decreased.

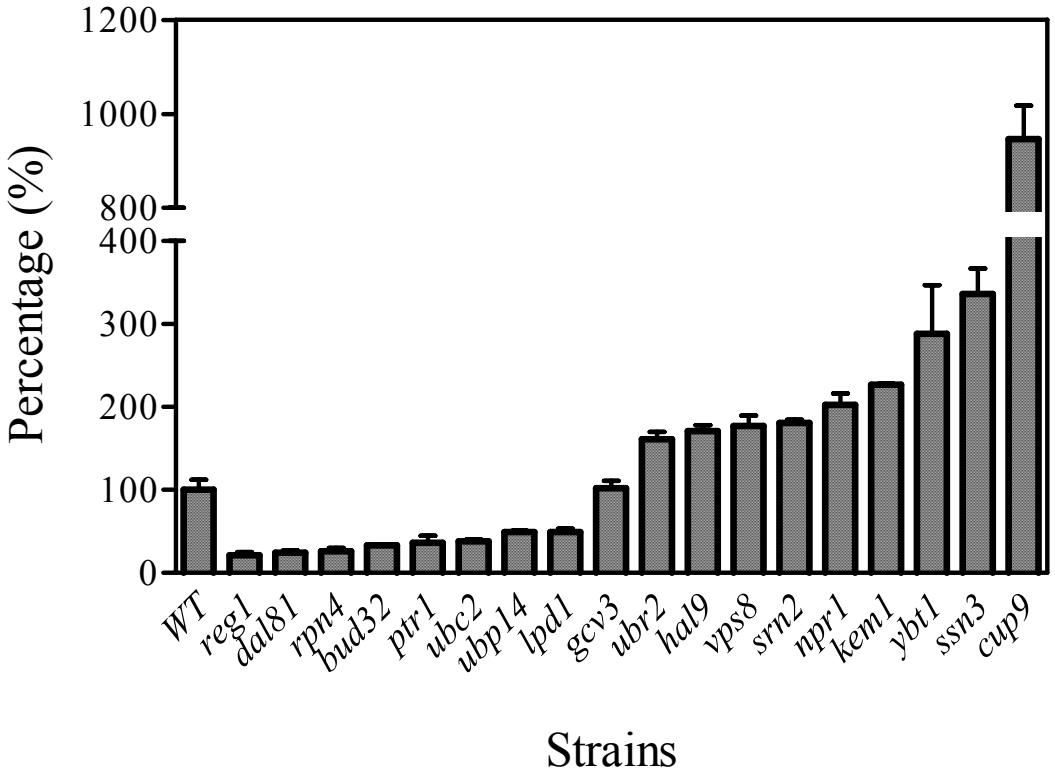


Figure 5 The uptake of [^{3}H]Leu-Leu dipeptide in log phase cells of the wild-type, *cup9*, *ptr1*, and the 16 deletion strains *bud32*, *dal81*, *gcv3*, *hal9*, *kem1*, *lpd1*, *npr1*, *reg1*, *rpn4*, *srn2*, *ssn3*, *ubc2*, *ubr2*, *ubp14*, *vps8*, and *ybt1*. The percentage of the accumulation of [^{3}H]Leu-Leu for each deletion mutant was calculated as [(the accumulation of [^{3}H]Leu-Leu in each mutant strain)/(the accumulation of [^{3}H]Leu-Leu in the wild-type) X 100].

The accumulation of [^{3}H]Leu-Leu in the *ptr1* strain was lower than that of the wild-type. The reduced accumulation of dipeptide was consistent with the decreased expression level of *PTR2* mRNA and Ptr2p-GFP in the *ptr1* strain. Similar to the *ptr1* strain, the accumulation of [^{3}H]Leu-Leu was lower in the *bud32, dal81, lpd1, reg1, rpn4, ubc2*, and *ubp14* strains as compared to that of the wild-type (Figure 5). The decreased uptake in these strains was reflected in their decreased expression of *PTR2* in *dal81, rpn4, ubc2,* and *ubp14* strains (Table 4), decreased amount of Ptr2p-GFP in the cytoplasmic membrane (Figure 4) and the alteration localization of Ptr2p in *bud32* and *reg1* strains (Figure 4). In contrast, although the transport of dipeptide was reduced in the *lpd1* strain, the expression level of Ptr2p-GFP remained similar to that in the wild-type (Figure 4).

Effects of Gene Deletions on Osmotic and Canavanine Sensitivity and *PMA1* Expression

In order to determine whether gene deletions also affected general membrane properties, additional assays were performed. Osmotic sensitivity, toxicity of canavanine, and the expression level of *PMA1* encoding a proton transporter localized in the cytoplasmic membrane were examined. NaCl at 1.0 M inhibits the growth of wild type *S. cerevisiae* by causing osmotic de-stabilization of the cell membrane (HOHMANN 2002). NaCl at 1.0 M significantly inhibited the growth of all strains on MM+HLKU plates. Fourteen deletion mutant strains, *cup9, dal81, gcv3, hal9, lpd1, npr1, ptr1, reg1, rpn4, ssn3, ubc2, ubp14, ubr2,* and *ybt1,* showed similar effects to NaCl as that of the wild type (Table 1 and 2, and Figure S4) suggesting that the deletion of those genes did not change

the osmotic sensitivity of the deletion mutant strains. In contrast, four deletion mutant strains, *bud32, kem1, srn2,* and *vps8,* showed greater sensitivity to 1.0 M NaCl than that of the wild-type (Tables 1 and 2 and Figure S4).

Canavanine is transported by the arginine permease, and changes in canavanine toxicity have been correlated directly to arginine permease function (HAMPSEY 1997). In the canavanine toxicity assay, nine deletion mutant strains*, cup9, hal9, ptr1, rpn4, ssn3, ubc2, ubp14, ubr2,* and *ybt1,* did not show significant alteration in the canavanine sensitivity as compared with that of the wild-type. In contrast, six deletion mutants, *dal81, gcv3, lpd1, reg1, srn2,* and *vps8* were more sensitive to canavanine, and three deletion mutant strains, *bud32, kem1,* and *npr1* were less sensitive than the wild type (Table 1 and 2 and Figure S5).

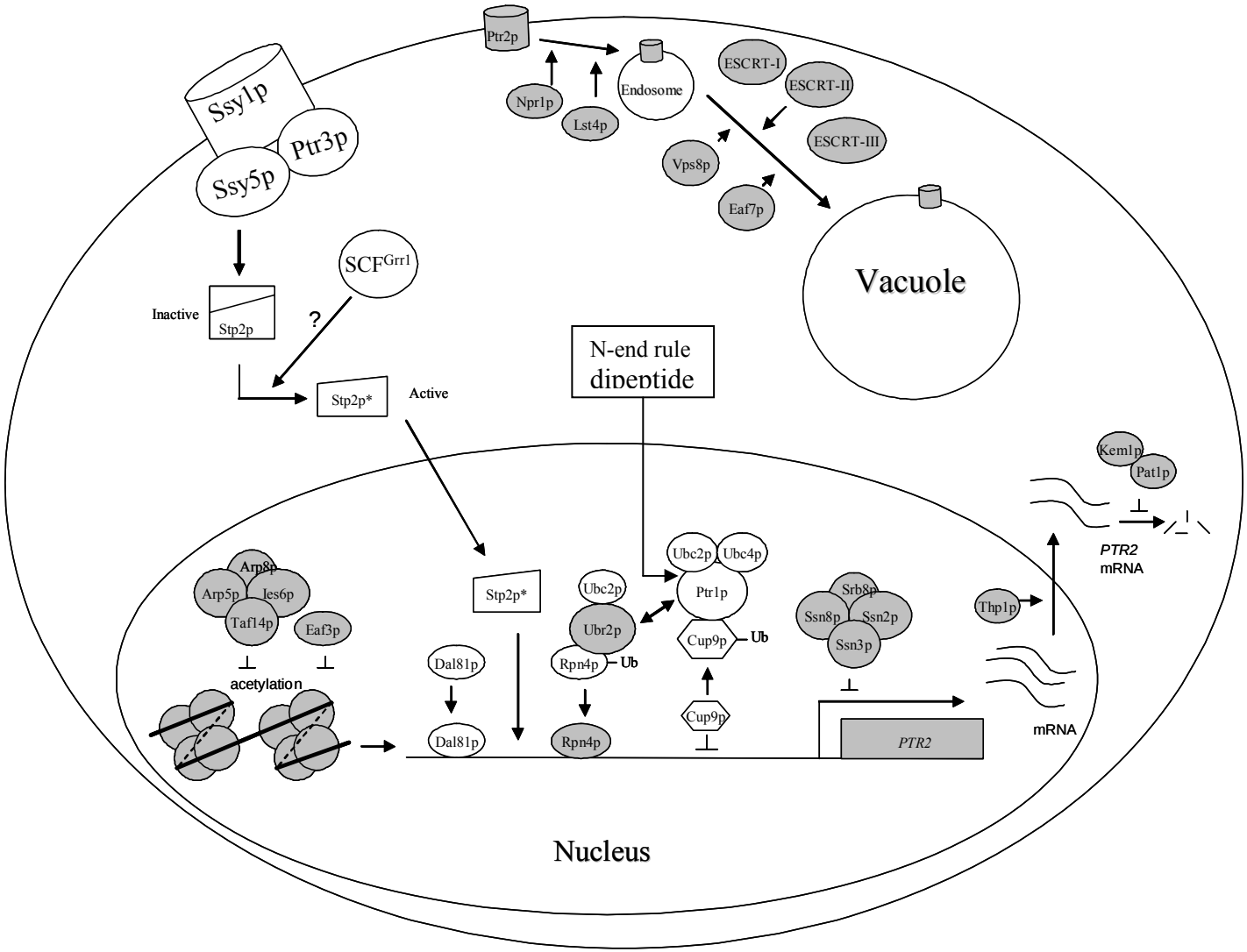
To examine whether the gene representative deletion mutants were also affected in the expression of other transport proteins, the expression level of *PMA1* encoding a proton transporter was measured. *PMA1* mRNA expression level in *cup9* and *ptr1* strains was similar to that of the wild type (Table 4). In contrast, *PMA1* mRNA level was up-regulated more than two-fold in *kem1, lpd1, ssn3, ubr2, vps8,* and *ybt1* strains, and less than two-fold in *bud32, dal81, gcv3, hal9, npr1, reg1, rpn4, srn2, ubc2,* and *ubp14* strains (Table 4). Overall these changes in expression levels of *PMA1* in the various mutant strains did not correlate with the changes in expression in *PTR2* except for up-regulation of both genes in the *ssn3* and *kem1* strains.

DISCUSSION

We have systematically screened a haploid, single-gene, deletion mutant library and identified 103 genes involved in dipeptide utilization. To our knowledge, this is the first such screen for a membrane transport system. *PTR2* expression is known to be regulated by amino acids via the Ssy1p-Ptr3p-Ssy5p (SPS) protein complex and by dipeptides themselves (Figure 6). Import of di/tri-peptides containing basic or bulky hydrophobic N-terminal residues (N-end Rule peptides) induces *PTR2* by reducing cellular levels of the *PTR2* repressor Cup9p (BYRD *et al.* 1998; DU *et al.* 2002; TURNER *et al.* 2000). In the SPS complex regulatory pathway a few down-stream proteins, such as *STP2* and components of SCF^{Grr1} ubiquitin ligase complex (Skp1p, Hrt1p, Cdc34p, and Cdc53p), are involved in the Ssy1p-induced signal transduction pathway via proteolytic processing of Stp2p (ANDREASSON and LJUNGDAHL 2002; BERNARD and ANDRE 2001). Due to the complex nature of signal transduction pathways, such as the well-studied pheromone-mediated mitogen-activated protein (MAP) kinase pathway in yeast (GUSTIN *et al.* 1998), we expected that other regulatory proteins would be identified in this screen.

The 103 genes identified in the screen cover a number of different Biological Processes based on the Gene Ontology annotation. It is clear from the broad range of mutants identified that genes involved in both direct and indirect regulation of peptide utilization were discovered. Such a global array of genes supports the inter-connectivity of networks involved in regulating biological processes (CHEN *et al.* 2003; LEE *et al.* 2002), but such a heterogeneous collection of interacting genes has not previously identified as specifically regulating membrane transport systems.

Figure 6 A hypothetical model for the regulation of dipeptide utilization in *S. cerevisiae*. The proteins with clear background represent those that have been reported previously to be involved in *PTR2* regulation. Grey background represents those proteins that have been determined in this study. The SPS protein complex (Ssy1p-Ptr3p-Ssy5p) is an amino acid sensor, which leads to a cleavage of the N-terminal of Stp2p. The activated form of Stp2p is localized to the nucleus and regulates *PTR2* expression. In addition, Dal81p synergistically regulates *PTR2* expression together with Stp2p. Other proteins, Rpn4p, a positive regulator, and Cup9p, a negative regulator, bind at the *PTR2* promoter region and are also involved in *PTR2* transcription. The stability of Rpn4p is modulated by Ubr2p and Ubc2p, and the stability of Cup9p by Ptr1p, Ubc2p, and Ubc4p via the ubiquitination pathway. Binding of N-end Rule dipeptides to Ptr1p/Ubr1p results in ubiquitin-mediated degradation of Cup9p. Ubr2p competes with Ptr1p for the degradation of Cup9p. Arp5p, Arp8p, Ies6p, and Taf14p (components of the INO80 complex) together with Eaf3p possibly negatively regulates *PTR2* transcription via repressing the acetylation of histones in the *PTR2* coding region. The transcription of *PTR2* is negatively regulated by a RNA polymerase mediator protein complex, which includes Ssn2p, Ssn3p, Ssn8p, and Srb8p. Thp1p might be involved in the translocation of *PTR2* mRNA from the nucleus to the cytosol. The interaction between Kem1p and Pat1p is potentially involved in the degradation of *PTR2* mRNA. Dipeptide utilization is also modulated by the Ptr2p trafficking system; ESCRT-I, -II, III, together with Vps8p and Eaf7p, appear to regulate Ptr2p internalization from the endosome to the vacuole. In addition, Npr1 and Lst4p are also involved in Ptr2p trafficking and affect the retention of Ptr2p in the plasma membrane.


Ptr2p
Ssy1p
Ptr3p
Ssy5p
Npr1p
Lst4p
Endosome
ESCRT-I
ESCRT-II
ESCRT-III
Vps8p
Eaf7p
Vacuole
SCF^Grr1
Inactive
Stp2p
?
Stp2p*
Active
N-end rule
dipeptide
Kem1p
Pat1p
PTR2
mRNA
Arp8p
Arp5p
Ies6p
Taf14p
Eaf3p
acetylation
Stp2p*
Ubc2p
Ubc4p
Ptr1p
Ubr2p
Rpn4p
Ub
Cup9p
Srb8p
Ssn8p
Ssn2p
Ssn3p
Thp1p
Dal81p
PTR2
mRNA
Nucleus

Genes that regulate *PTR2* transcription: In this study we have considerably expanded the identification of genes involved in *PTR2* transcriptional regulation. The deletion of these genes results in the alteration of dipeptide utilization.

First, we have shown that dipeptide utilization can be affected by gene products involved in the modification of the nucleosome, namely Arp5p, Arp8p, Ies6p and Taf14p. These proteins belong to the components of the chromatin remodeling INO80 protein complex that carries ATPase activity, DNA binding, and nucleosome mobilization (SANDERS *et al.* 2002), and that preferentially interacts with histones H3 and H4 (SHEN *et al.* 2003). Another histone- modification-related gene product, Eaf3p, was also identified in our screen. Eaf3p is a component of the NuA4 histone acetyltransferase complex which maintains the acetylation of histone H3 and H4 (REID *et al.* 2004). It is possible that these chromatin remodeling proteins negatively regulate *PTR2* transcription via the modification of histones. A model representing the potential involvement of these proteins and others is shown (Figure 6).

Second, four gene products with transcription factor activity, Cup9p, Dal81p, Stp2p, and Rpn4p were shown to regulate *PTR2* expression and affected dipeptide utilization. Cup9p binds the region between -488 and -897 upstream of the *PTR2* start codon (BYRD *et al.* 1998), while Rpn4p may bind between -610 and -603 due to the existence of the Rpn4p binding consensus sequence (5'-GGTGGAAA-3') present at this location (MANNHAUPT *et al.* 1999). Cup9p is a repressor of *PTR2*, whereas Rpn4p is a "positive-regulator" as reflected in the observation that the *cup9* deletion strain showed an increase in peptide utilization and the *rpn4* strain has decreased peptide utilization. Subsequent to amino acid induction via the SPS system and its conversion into the active

form after proteolytically processing and translocation into the nucleus (ANDREASSON and LJUNGDAHL 2002; ANDREASSON and LJUNGDAHL 2004), the active form of Stp2p (truncated at the N-terminus) binds to the upstream activation sequence (UAS) of the promoter region of *BAP2* and *BAP3* (DE BOER *et al.* 2000; NIELSEN *et al.* 2001). Because *BAP2, BAP3* and *PTR2* are simultaneously regulated in response to amino acid induction via the SPS-Stp2p system, the active form of Stp2p likely also binds to the *PTR2* promoter region and regulates its transcription. Dal81p is a transcriptional activator essential for *PTR2* expression under the induction by the SPS signal pathway as well (BERNARD and ANDRE 2001). Dal81p acts synergistically with Stp1p to regulate the transcription of *AGP1* in response to the amino acid induction, and the sequence 5'-CGGC-3' of a UAS element is important for *AGP1* transcriptional regulation (ABDEL-SATER *et al.* 2004). Since the *PTR2* promoter region has this consensus sequence and because Dal81p was identified as important for *PTR2* transcription and dipeptide utilization in our screening, we speculate that Dal81p and Stp2p act synergistically in *PTR2* transcription in a similar manner.

Third, other gene products identified in this screen such as Ubr2p, Ptr1p, and Ubc2p regulate *PTR2* expression by modulating the stability of Rpn4p and Cup9p (Figure 6). Cup9p stability is regulated by Ptr1p, Ubc2p, and Ubc4p (DU *et al.* 2002). Ubr2p is associated with Rpn4p to regulate proteasome gene expression through the ubiquitination pathway (WANG *et al.* 2004). In addition, Ubr2p has high amino acid sequence similarity to Ptr1p presumably allowing a competition with Ptr1p for the stabilization of Cup9p (BARTEL *et al.* 1990). Therefore, Ubr2p probably regulates *PTR2* transcription by impacting the stability of both Rpn4p and Cup9p (Figure 6).

Fourth, we identified one other gene product (*SSN3*) involved in *PTR2* transcription that is known to be general transcription factor, which also affected *PMA1* expression. Ssn3p/Srb10p, Ssn8p/Srb11p, Ssn2p/Srb9p, and Srb8p have been identified as a cyclin-dependent serine/threonine protein kinase complex that functions as a mediator of RNA polymerase (KORNBERG 2005). This complex is also involved in glucose signaling pathway (BALCIUNAS and RONNE 1995; KUCHIN *et al.* 1995). Deletion of these genes leads to de-repression of a wide variety of down-stream genes, including *GAL* and *SUC*, which are important for galactose and raffinose metabolism, respectively (KUCHIN *et al.* 2000; MYER and YOUNG 1998). The deletion of *SSN3* resulted in a change of *PMA1* and *PTR2* expression level.

Finally, we identified gene products involved in *PTR2* transcription by means that are not clear. For example, *UBP14* was important for *PTR2* expression since deletion of these genes significantly decreased *PTR2* mRNA level (Table 4), and the strain carrying deletion of this gene exhibited decreased peptide utilization. Ubp14p has ubiquitin-specific protease activity (Table S4). It is probably modulating *PTR2* transcription via the ubiquitination pathway. In addition, the deletion of *UBP14* had no effect on the expression of *PMA1* or on sensitivity to NaCl and canavanine. Further experiments are needed to provide some insight into whether the gene product acts directly or indirectly to control *PTR2* expression.

Genes involved in *PTR2* mRNA maturation: Gene products (Kem1p, Pat1p, and Thp1p) involved in post-transcriptional regulation impacting the stability and transport of *PTR2* mRNA also affected dipeptide utilization (Figure 6). Both Kem1p and Pat1p

belong to an mRNA decay protein complex (BONNEROT et al. 2000; BOUVERET et al. 2000). *PTR2* mRNA was highly up-regulated in the kem1 strain (Table 4). It is likely that *PTR2* mRNA was more stable in *kem1* and *pat1* strains, which resulted in more Ptr2p being synthesized leading to enhanced Ptr2p-GFP expression (Figure 3) and increased dipeptide utilization (Table 1 and Figure 1 and 2). The deletion of *KEM1* resulted as also an increased expression level of *PMA1* and changes sensitivity to NaCl and canavanine (Table 1). It is not surprising that the deletion of this gene affected several phenotypes related to membrane function, since a deletion in this gene would impact gene expression of many different genes. Another gene shown to impacting dipeptide utilization was Thp1p, which is involved in mRNA export from the nucleus to the cytoplasm (FISCHER et al. 2002). The *thp1* strain probably has a defect in transporting *PTR2* mRNA into the cytoplasm for protein synthesis. Therefore this mutant demonstrated a decrease in peptide utilization.

Genes that regulate Ptr2p trafficking: This screen revealed that dipeptide utilization is regulated by the trafficking system. Deletion of components of ESCRT-I, ESCRT-II, and ESCRT-III protein complexes showed a defect in Ptr2p-GFP trafficking from the endosome to the vacuole (Figure 6 and Figure S2). These protein complexes are essential for sorting ubiquitinylated membrane proteins from the plasma membrane to the multivesicular body and to the vacuole for further degradation (RAIBORG et al. 2003). Ptr2p has been shown to be ubiquitinylated (HITCHCOCK et al. 2003). Strains *srn2*, *vps36* and *snf7* demonstrated increased dipeptide utilization perhaps due to an increased residence time of Ptr2p at the cytoplasmic membrane. In addition, the *eaf7* and *vps8*

strains showed Ptr2p localization similar to that of the ESCRT protein mutants. Surprisingly, Eaf7p is annotated as a component of the NuA4 HAT complex, which is an essential histone acetyltransferase complex that acetylates the N-terminal tails of histones H4 and H2A (KROGAN et al. 2004). It is possible that Eaf7p has dual functions or indirectly affects Ptr2p localization through other proteins. Both *srn2* and *vps8* strains showed an altered sensitivity to NaCl and canavanine. It has been reported that the osmotic sensitivity changes if vacuolar development is affected (HAMPSEY 1997). Thus a disruption of the protein sorting process that affects the vacuole could lead to alteration of osmotic sensitivity.

Deletion mutants (*npr1*, *lst4*) with increased dipeptide utilization and mutants (*bud32*, *reg1*) with decreased utilization showed an accumulation of Ptr2p-GFP in vesicles. *NPR1* encodes a Ser/Thr protein kinase, involved in post-translational control of Gap1p. Npr1p is required for Gap1p to be targeted to the plasma membrane, and an *npr1* deletion mutant loses Gap1p function by sorting Gap1p from the Golgi into the vacuole by-passing the plasma membrane (DE CRAENE *et al.* 2001). Similarly, mutations in *LST4* reduced the function of Galp1p and other amino acid permeases by the interruption of sorting of these transporters to the cell surface (ROBERG *et al.* 1997). Our observations indicated that *NPR1* and *LST4* regulate Ptr2p differently from the way they regulate Gap1p. It is not clear how Lst4p and Npr1p regulate Ptr2p in a manner opposite to that of their regulation of Gap1p. Bud32p is a Ser/Thr protein kinase involving in polar bud site selection and changing the vacuolar morphology (BONANGELINO et al. 2002). Reg1p is a regulatory subunit for protein phosphatase Glc7p involved in the repression of many

glucose-regulated genes and vesicular trafficking (CUI *et al.* 2004). The involvement of Bud32p and Reg1p in dipeptide utilization reflects their roles in Ptr2p trafficking.

Genes that regulate dipeptide utilization independent of Ptr2p: The alteration of dipeptide utilization also results from Ptr2p-independent metabolic processes as indicated by the observation of no apparent phenotypic change in expression or localization of Ptr2p-GFP in a number of mutants (Table 3). Mutation in genes encoding a variety of cellular metabolic processes (Figure S6) (NAGARAJAN and STORMS 1997; SCHNEIDER et al. 1997; STOOPS et al. 1997), such as the conversion of pyruvate into acetyl-CoA by pyruvate dehydrogenase complex (Pdx1p, Lpd1, Pdb1p, and Lat1p), the conversion of acetyl-CoA to malonyl-CoA by Hfa1p, fatty acid synthesis by fatty acid synthase complex (Oar1p, Mct1p, and Etr1p), glycine degradation (Gcv2p, Gcv3p, and Lpd1p), and a peptidase activity (Prd1p) resulted in a decrease of dipeptide utilization (Table 3). In these mutants the impairment of the dipeptide utilization appeared to be independent of Ptr2p transport activity since Ptr2p-GFP could be observed at the plasma membrane (Figure S3). The assay of [^{3}H]Leu-Leu uptake in *gcv3* strains suggested that the import of dipeptide was still maintained at wild-type level which showed that Ptr2p was functional (Figure 5), indicating that the decrease of dipeptide utilization resulted from the inability of the mutant to release amino acid from accumulated dipeptide or from a metabolic block in using the released amino acid for protein biosynthesis. Prd1p is a metalloendoproteinase (BUCHLER *et al.* 1994). The deletion mutant might be impaired in the degradation of intracellularly transported dipeptide which would result in a decrease of dipeptide utilization.

Deletion strains *cik1, kti12, mlh1, mrp17, pho2, rim101, tif3, ybt1* and *ydr417c* showed no apparent effect on Ptr2p-GFP localization or expression but demonstrated increased dipeptide utilization. The utilization of dipeptide in these strains appears to involve Ptr2p-independent cellular processes as well. For example, Ybt1p belongs to the ABC transporter family exhibiting ATP-dependent bile acid transport (ORTIZ et al. 1997; PAULSEN et al. 1998). As expected for an ABC transporter, the *ybt1* mutant was not only more sensitive to the toxic dipeptide Ala-Eth, but also this strain was more sensitive to ethionine and Lys-Ala-Eth (data not shown). We speculate that this protein might be involved in the transport of dipeptide into the vacuole. In the deletion mutant the defect in the uptake of dipeptide into the vacuole would lead to an increase of dipeptide availability in the cytoplasm. It is also possible that the deletion of a gene might result in a de-repression or change in function of other transport systems that may mediate dipeptide utilization in the mutant without alteration of *PTR2* expression or Ptr2p function. We are currently working on unraveling the involvement of these metabolic genes in dipeptide utilization.

Unknown genes that regulate dipeptide utilization: Fifteen unknown gene deletion mutant strains were identified in this screen, including seven strains exhibiting increased and eight strains showing decreased dipeptide utilization (Table 3). Deletion mutants *yor322c, ynl123w, yfr044c,* and *ylr114c* showed enhanced expression level of Ptr2p-GFP signal compared to wild-type, and *ypr174c* had reduced expression of Ptr2p-GFP; these results were consistent with dipeptide utilization in these strains, suggesting these unknown genes products are involved in the regulation of *PTR2* expression. Deletion mutants *ynl295w, ypl073c, yjl175w, ydr157w,* and *ydr433w* showed accumulation of

Ptr2p-GFP signal in the vesicles or the vacuole, suggestion these unknown gene products are involved in Ptr2p trafficking and localization. Similar to the expression level of Ptr2p-GFP in the *asm4* strain, enhanced Ptr2p-GFP expression was observed in the deletion mutant strains of unknown function *ydr015c* and *ydr290w.* These two mutants, however, showed a decreased sensitivity to toxic dipeptide and no growth change on dipeptide; it is not clear how these genes regulate dipeptide utilization.

Overall, this investigation has provided a list of genes involved directly and indirectly in the utilization of dipeptides in yeast. In our screen, 53 genes encoding proteins with more than 30% identity to human genes have been identified (See Tables S3 and S4, footnote a). To date no genes have been reported in the regulation of the human *PTR2* homologs PEPT1 and PEPT2 (DANIEL 2004). Further investigation of these human genes may promote understanding of the regulation of dipeptide utilization in mammalian systems. This study represents the first such global analysis of a membrane transport process and as such provides a rich starting ground for future investigations.

Acknowledgements

We thank Dr. Melinda Hauser, Dr. Amy Wiles and Dr. Tom Masi for helpful discussions and critical review of the manuscript.

REFERENCES

ABDEL-SATER, F., I. IRAQUI, A. URRESTARAZU and B. ANDRE, 2004 The external amino acid signaling pathway promotes activation of Stp1 and Uga35/Dal81 transcription factors for induction of the AGP1 gene in Saccharomyces cerevisiae. Genetics **166:** 1727-1739.

ANDREASSON, C., and P. O. LJUNGDAHL, 2002 Receptor-mediated endoproteolytic activation of two transcription factors in yeast. Genes Dev **16:** 3158-3172.

ANDREASSON, C., and P. O. LJUNGDAHL, 2004 The N-terminal regulatory domain of Stp1p is modular and, fused to an artificial transcription factor, confers full Ssy1p-Ptr3p-Ssy5p sensor control. Mol Cell Biol **24:** 7503-7513.

AOUIDA, M., N. PAGE, A. LEDUC, M. PETER and D. RAMOTAR, 2004 A genome-wide screen in Saccharomyces cerevisiae reveals altered transport as a mechanism of resistance to the anticancer drug bleomycin. Cancer Res **64:** 1102-1109.

ASKREE, S. H., T. YEHUDA, S. SMOLIKOV, R. GUREVICH, J. HAWK *et al.*, 2004 A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length. Proc Natl Acad Sci U S A **101:** 8658-8663.

BAETZ, K., L. MCHARDY, K. GABLE, T. TARLING, D. REBERIOUX *et al.*, 2004 Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. Proc Natl Acad Sci U S A **101:** 4525-4530.

BALCIUNAS, D., and H. RONNE, 1995 Three subunits of the RNA polymerase II mediator complex are involved in glucose repression. Nucleic Acids Res **23:** 4421-4425.

BARNES, D., W. LAI, M. BRESLAV, F. NAIDER and J. M. BECKER, 1998 PTR3, a novel gene mediating amino acid-inducible regulation of peptide transport in Saccharomyces cerevisiae. Mol Microbiol **29:** 297-310.

BARTEL, B., I. WUNNING and A. VARSHAVSKY, 1990 The recognition component of the N-end rule pathway. Embo J **9:** 3179-3189.

BERNARD, F., and B. ANDRE, 2001 Ubiquitin and the SCF(Grr1) ubiquitin ligase complex are involved in the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. FEBS Lett **496:** 81-85.

BONANGELINO, C. J., E. M. CHAVEZ and J. S. BONIFACINO, 2002 Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae. Mol Biol Cell **13:** 2486-2501.

BONNEROT, C., R. BOECK and B. LAPEYRE, 2000 The two proteins Pat1p (Mrt1p) and Spb8p interact in vivo, are required for mRNA decay, and are functionally linked to Pab1p. Mol Cell Biol **20:** 5939-5946.

BOUVERET, E., G. RIGAUT, A. SHEVCHENKO, M. WILM and B. SERAPHIN, 2000 A Sm-like protein complex that participates in mRNA degradation. Embo J **19:** 1661-1671.

BOWERS, K., J. LOTTRIDGE, S. B. HELLIWELL, L. M. GOLDTHWAITE, J. P. LUZIO *et al.*, 2004 Protein-protein interactions of ESCRT complexes in the yeast Saccharomyces cerevisiae. Traffic **5:** 194-210.

BUCHLER, M., U. TISLJAR and D. H. WOLF, 1994 Proteinase yscD (oligopeptidase yscD). Structure, function and relationship of the yeast enzyme with mammalian thimet oligopeptidase (metalloendopeptidase, EP 24.15). Eur J Biochem **219:** 627-639.

BYRD, C., G. C. TURNER and A. VARSHAVSKY, 1998 The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. Embo J **17:** 269-277.

CHEN, Y., Y. LIU, G. K. M., J. M. BECKER, Y. XU *et al.*, 2003 A computational study on the signal transduction pathway for amino acid and peptide transport in yeast: bridging the gap between high-throughput data and traditional biology. Applied Genomics and Proteomics **2:** 43-50.

CHRISTIE, K. R., S. WENG, R. BALAKRISHNAN, M. C. COSTANZO, K. DOLINSKI *et al.*, 2004 Saccharomyces Genome Database (SGD) provides tools to identify and analyze sequences from Saccharomyces cerevisiae and related sequences from other organisms. Nucleic Acids Res **32 Database issue:** D311-314.

CUI, D. Y., C. R. BROWN and H. L. CHIANG, 2004 The type 1 phosphatase Reg1p-Glc7p is required for the glucose-induced degradation of fructose-1,6-bisphosphatase in the vacuole. J Biol Chem **279:** 9713-9724.

DANIEL, H., 2004 Molecular and integrative physiology of intestinal peptide transport. Annu Rev Physiol **66:** 361-384.

DE BOER, M., P. S. NIELSEN, J. P. BEBELMAN, H. HEERIKHUIZEN, H. A. ANDERSEN *et al.*, 2000 Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene BAP3 of Saccharomyces cerevisiae. Nucleic Acids Res **28:** 974-981.

DE CRAENE, J. O., O. SOETENS and B. ANDRE, 2001 The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. J Biol Chem **276:** 43939-43948.

DU, F., F. NAVARRO-GARCIA, Z. XIA, T. TASAKI and A. VARSHAVSKY, 2002 Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. Proc Natl Acad Sci U S A **99:** 14110-14115.

FEI, Y. J., Y. KANAI, S. NUSSBERGER, V. GANAPATHY, F. H. LEIBACH *et al.*, 1994 Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature **368:** 563-566.

FISCHER, T., K. STRASSER, A. RACZ, S. RODRIGUEZ-NAVARRO, M. OPPIZZI *et al.*, 2002 The mRNA export machinery requires the novel Sac3p-Thp1p complex to dock at the nucleoplasmic entrance of the nuclear pores. Embo J **21:** 5843-5852.

FORSBERG, H., C. F. GILSTRING, A. ZARGARI, P. MARTINEZ and P. O. LJUNGDAHL, 2001a The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Mol Microbiol **42:** 215-228.

FORSBERG, H., M. HAMMAR, C. ANDREASSON, A. MOLINER and P. O. LJUNGDAHL, 2001b Suppressors of ssy1 and ptr3 null mutations define novel amino acid sensor-independent genes in Saccharomyces cerevisiae. Genetics **158:** 973-988.

FORSBERG, H., and P. O. LJUNGDAHL, 2001a Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. Mol Cell Biol **21:** 814-826.

FORSBERG, H., and P. O. LJUNGDAHL, 2001b Sensors of extracellular nutrients in Saccharomyces cerevisiae. Curr Genet **40:** 91-109.

GONZALES, T., and J. ROBERT-BAUDOUY, 1996 Bacterial aminopeptidases: properties and functions. FEMS Microbiol Rev **18:** 319-344.

GUSTIN, M. C., J. ALBERTYN, M. ALEXANDER and K. DAVENPORT, 1998 MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev **62:** 1264-1300.

HAMPSEY, M., 1997 A review of phenotypes in Saccharomyces cerevisiae. Yeast **13:** 1099-1133.

HAUSER, M., V. NARITA, A. M. DONHARDT, F. NAIDER and J. M. BECKER, 2001 Multiplicity and regulation of genes encoding peptide transporters in Saccharomyces cerevisiae. Mol Membr Biol **18:** 105-112.

HERRERA-RUIZ, D., and G. T. KNIPP, 2003 Current perspectives on established and putative mammalian oligopeptide transporters. J Pharm Sci **92:** 691-714.

HITCHCOCK, A. L., K. AULD, S. P. GYGI and P. A. SILVER, 2003 A subset of membrane-associated proteins is ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery. Proc Natl Acad Sci U S A **100:** 12735-12740.

HODGES, P. E., A. H. MCKEE, B. P. DAVIS, W. E. PAYNE and J. I. GARRELS, 1999 The Yeast Proteome Database (YPD): a model for the organization and presentation of genome-wide functional data. Nucleic Acids Res **27:** 69-73.

HOFFMAN, G. A., T. R. GARRISON and H. G. DOHLMAN, 2002 Analysis of RGS proteins in Saccharomyces cerevisiae. Methods Enzymol **344:** 617-631.

HOHMANN, S., 2002 Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev **66:** 300-372.

IRAQUI, I., S. VISSERS, F. BERNARD, J. O. DE CRAENE, E. BOLES *et al.*, 1999 Amino acid signaling in Saccharomyces cerevisiae: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. Mol Cell Biol **19:** 989-1001.

ISLAND, M. D., F. NAIDER and J. M. BECKER, 1987 Regulation of dipeptide transport in Saccharomyces cerevisiae by micromolar amino acid concentrations. J Bacteriol **169:** 2132-2136.

ISLAND, M. D., J. R. PERRY, F. NAIDER and J. M. BECKER, 1991 Isolation and characterization of S. cerevisiae mutants deficient in amino acid-inducible peptide transport. Curr Genet **20:** 457-463.

KORNBERG, R. D., 2005 Mediator and the mechanism of transcriptional activation. Trends Biochem Sci **30:** 235-239.

KROGAN, N. J., K. BAETZ, M. C. KEOGH, N. DATTA, C. SAWA *et al.*, 2004 Regulation of chromosome stability by the histone H2A variant Htz1, the Swr1 chromatin remodeling complex, and the histone acetyltransferase NuA4. Proc Natl Acad Sci U S A **101:** 13513-13518.

KUCHIN, S., I. TREICH and M. CARLSON, 2000 A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. Proc Natl Acad Sci U S A **97:** 7916-7920.

KUCHIN, S., P. YEGHIAYAN and M. CARLSON, 1995 Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast. Proc Natl Acad Sci U S A **92:** 4006-4010.

LEE, T. I., N. J. RINALDI, F. ROBERT, D. T. ODOM, Z. BAR-JOSEPH *et al.*, 2002 Transcriptional regulatory networks in Saccharomyces cerevisiae. Science **298:** 799-804.

LEE, V. H., 2000 Membrane transporters. Eur J Pharm Sci **11 Suppl 2:** S41-50.

LIU, W., R. LIANG, S. RAMAMOORTHY, Y. J. FEI, M. E. GANAPATHY *et al.*, 1995 Molecular cloning of PEPT 2, a new member of the H+/peptide cotransporter family, from human kidney. Biochim Biophys Acta **1235:** 461-466.

LUBKOWITZ, M. A., L. HAUSER, M. BRESLAV, F. NAIDER and J. M. BECKER, 1997 An oligopeptide transport gene from Candida albicans. Microbiology **143 (Pt 2):** 387-396.

MANNHAUPT, G., R. SCHNALL, V. KARPOV, I. VETTER and H. FELDMANN, 1999 Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett **450:** 27-34.

MARELLI, M., J. D. AITCHISON and R. W. WOZNIAK, 1998 Specific binding of the karyopherin Kap121p to a subunit of the nuclear pore complex containing Nup53p, Nup59p, and Nup170p. J Cell Biol **143:** 1813-1830.

MARSTON, A. L., W. H. THAM, H. SHAH and A. AMON, 2004 A genome-wide screen identifies genes required for centromeric cohesion. Science **303:** 1367-1370.

MARZLUF, G. A., 1997 Genetic regulation of nitrogen metabolism in the fungi. Microbiol Mol Biol Rev **61:** 17-32.

MIYAKE, T., M. KANAYAMA, H. SAMMOTO and B. ONO, 2002 A novel cis-acting cysteine-responsive regulatory element of the gene for the high-affinity glutathione transporter of Saccharomyces cerevisiae. Mol Genet Genomics **266:** 1004-1011.

MYER, V. E., and R. A. YOUNG, 1998 RNA polymerase II holoenzymes and subcomplexes. J Biol Chem **273:** 27757-27760.

NAGARAJAN, L., and R. K. STORMS, 1997 Molecular characterization of GCV3, the Saccharomyces cerevisiae gene coding for the glycine cleavage system hydrogen carrier protein. J Biol Chem **272:** 4444-4450.

NIELSEN, P. S., B. VAN DEN HAZEL, T. DIDION, M. DE BOER, M. JORGENSEN *et al.*, 2001 Transcriptional regulation of the Saccharomyces cerevisiae amino acid permease gene BAP2. Mol Gen Genet **264:** 613-622.

ORTIZ, D. F., M. V. ST PIERRE, A. ABDULMESSIH and I. M. ARIAS, 1997 A yeast ATP-binding cassette-type protein mediating ATP-dependent bile acid transport. J Biol Chem **272:** 15358-15365.

PAGE, N., M. GERARD-VINCENT, P. MENARD, M. BEAULIEU, M. AZUMA *et al.*, 2003 A Saccharomyces cerevisiae genome-wide mutant screen for altered sensitivity to K1 killer toxin. Genetics **163:** 875-894.

PAULSEN, I. T., M. K. SLIWINSKI, B. NELISSEN, A. GOFFEAU and M. H. SAIER, JR., 1998 Unified inventory of established and putative transporters encoded within the complete genome of Saccharomyces cerevisiae. FEBS Lett **430:** 116-125.

PERRY, J. R., M. A. BASRAI, H. Y. STEINER, F. NAIDER and J. M. BECKER, 1994 Isolation and characterization of a Saccharomyces cerevisiae peptide transport gene. Mol Cell Biol **14:** 104-115.

POULSEN, P., B. WU, R. F. GABER, K. OTTOW, H. A. ANDERSEN *et al.*, 2005 Amino acid sensing by Ssy1. Biochem Soc Trans **33:** 261-264.

PRITCHARD, G. G., and T. COOLBEAR, 1993 The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol Rev **12:** 179-206.

RAIBORG, C., T. E. RUSTEN and H. STENMARK, 2003 Protein sorting into multivesicular endosomes. Curr Opin Cell Biol **15:** 446-455.

REID, J. L., Z. MOQTADERI and K. STRUHL, 2004 Eaf3 regulates the global pattern of histone acetylation in Saccharomyces cerevisiae. Mol Cell Biol **24:** 757-764.

ROBERG, K. J., S. BICKEL, N. ROWLEY and C. A. KAISER, 1997 Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae by SEC13, LST4, LST7 and LST8. Genetics **147:** 1569-1584.

SANDERS, S. L., J. JENNINGS, A. CANUTESCU, A. J. LINK and P. A. WEIL, 2002 Proteomics of the eukaryotic transcription machinery: identification of proteins associated with components of yeast TFIID by multidimensional mass spectrometry. Mol Cell Biol **22:** 4723-4738.

SCHERENS, B., and A. GOFFEAU, 2004 The uses of genome-wide yeast mutant collections. Genome Biol **5:** 229.

SCHNEIDER, R., B. BRORS, F. BURGER, S. CAMRATH and H. WEISS, 1997 Two genes of the putative mitochondrial fatty acid synthase in the genome of Saccharomyces cerevisiae. Curr Genet **32:** 384-388.

SHEN, X., R. RANALLO, E. CHOI and C. WU, 2003 Involvement of actin-related proteins in ATP-dependent chromatin remodeling. Mol Cell **12:** 147-155.

STADE, K., C. S. FORD, C. GUTHRIE and K. WEIS, 1997 Exportin 1 (Crm1p) is an essential nuclear export factor. Cell **90:** 1041-1050.

STEINER, H. Y., F. NAIDER and J. M. BECKER, 1995 The PTR family: a new group of peptide transporters. Mol Microbiol **16:** 825-834.

STEINMETZ, L. M., C. SCHARFE, A. M. DEUTSCHBAUER, D. MOKRANJAC, Z. S. HERMAN *et al.*, 2002 Systematic screen for human disease genes in yeast. Nat Genet **31:** 400-404.

STOOPS, J. K., R. H. CHENG, M. A. YAZDI, C. Y. MAENG, J. P. SCHROETER *et al.*, 1997 On the unique structural organization of the Saccharomyces cerevisiae pyruvate dehydrogenase complex. J Biol Chem **272:** 5757-5764.

TURNER, G. C., F. DU and A. VARSHAVSKY, 2000 Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. Nature **405:** 579-583.

WANG, L., X. MAO, D. JU and Y. XIE, 2004 Rpn4 is a physiological substrate of the Ubr2 ubiquitin ligase. J Biol Chem **279:** 55218-55223.

WINZELER, E. A., D. D. SHOEMAKER, A. ASTROMOFF, H. LIANG, K. ANDERSON *et al.*, 1999 Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science **285:** 901-906.

XIE, Y., and A. VARSHAVSKY, 1999 The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. Embo J **18:** 6832-6844.

APPENDIX

Supplement Table S1: The sequence of the primers used in the construction of PTR2-GFP, Northern analysis, and Real-Time PCR

Primers	Sequence
PTR2-FLAG-GFP-F	(5'- gatccaccaccaaatatGACTACAAGGACGACGATGACAAGcttgatatcaacaagacaggg-3')
PTR2-Flag-GFP-R	(5'-GATGATGATGACTGCTGCCGCTGCCGCGCGGCACACCGGTcctcgagccgcttttcttgtac-3')
PTR2-F (Northern)	(5'-TTCCGCACCATTCCAAAACTACAT-3')
PTR2-R (Northern)	(5'-GCAGCACAGAAAACTCCCGTAAAC-3')
ACT1-F (Northern)	(5'-GCCGGTTTTGCCGGTGACGAC-3')
ACT1-R (Northern)	(5'-GGAAGATGGAGCCAAAGCGTG-3').
PTR2 (F)	(5'-cagtgaccgttgatcctaaat-3')
PTR2 (R)	(5'-ctgaagcacaaccagaacaaa-3')
ACT1 (F)	(5'-ccaccatgttcccaggtatt-3')
ACT1 (R)	(5'-ccaatccagacggagtactt-3')
PMA1 (F)	(5'-ctggtccattctggtcttcta-3')
PMA1 (R)	(5'-ccaccaaccgaataaggtaaa-3')

Supplement Table S2 One hundred and nine strains of the haploid, deletion mutant collection were not capable of growing in MM+HLKU with allantoin as the sole nitrogen source. These deletion mutant strains were excluded from this study. The strains are listed alphabetically by gene name.

ORF name	Gene Name	Gene description in SGD
YLR304C	*ACO1*	Mitochondrial aconitase, required for the tricarboxylic acid (TCA) cycle; mutation leads to glutamate auxotrophy
YAR015W	*ADE1*	N-succinyl-5-aminoimidazole-4-carboxamide ribotide (SAICAR) synthetase, required for 'de novo' purine nucleotide biosynthesis; red pigment accumulates in mutant cells deprived of adenine
YOR128C	*ADE2*	Phosphoribosylaminoimidazole carboxylase, catalyzes a step in the 'de novo' purine nucleotide biosynthetic pathway; red pigment accumulates in mutant cells deprived of adenine
YGR204W	*ADE3*	Cytoplasmic trifunctional enzyme C1-tetrahydrofolate synthase, involved in single carbon metabolism and required for biosynthesis of purines, thymidylate, methionine, and histidine
YMR300C	*ADE4*	Phosphoribosylpyrophosphate amidotransferase (PRPPAT; amidophosphoribosyltransferase), catalyzes first step of the 'de novo' purine nucleotide biosynthetic pathway
YGL234W	*ADE5,7*	Bifunctional enzyme of the 'de novo' purine nucleotide biosynthetic pathway, contains aminoimidazole ribotide synthetase and glycinamide ribotide synthetase activities
YGR061C	*ADE6*	Formylglycinamidine-ribonucleotide (FGAM)-synthetase, catalyzes a step in the 'de novo' purine nucleotide biosynthetic pathway
YDR408C	*ADE8*	Phosphoribosyl-glycinamide transformylase, catalyzes a step in the 'de novo' purine nucleotide biosynthetic pathway
YNL220W	*ADE12*	Adenylosuccinate synthase, catalyzes the first committed step in the 'de novo' biosynthesis of adenosine
YOL058W	*ARG1*	Arginosuccinate synthetase, catalyzes the formation of L-argininosuccinate from citrulline and L-aspartate in the arginine biosynthesis pathway; potential Cdc28p substrate
YJL071W	*ARG2*	Acetylglutamate synthase (glutamate N-acetyltransferase), mitochondrial enzyme that catalyzes the first step in the biosynthesis of the arginine precursor ornithine; forms a complex with Arg5,6p
YJL088W	*ARG3*	Ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase), catalyzes the sixth step in the biosynthesis of the arginine precursor ornithine
YHR018C	*ARG4*	Argininosuccinate lyase, catalyzes the final step in the arginine biosynthesis pathway
YER069W	*ARG5,6*	Bifunctional enzyme with N-acetyl-gamma-glutamyl-phosphate reductase and acetylglutamate kinase activities, catalyzes the second and third steps in the biosynthesis of the arginine precursor ornithine; forms a complex with Arg2p
YDR127W	*ARO1*	Pentafunctional arom protein, catalyzes steps 2 through 6 in the biosynthesis of chorismate, which is a precursor to aromatic amino acids
YGL148W	*ARO2*	Bifunctional chorismate synthase and flavin reductase, catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to form chorismate, which is a precursor to aromatic amino acids

Supplement Table S2 Continued

ORF name	Gene Name	Gene description in SGD
YEL029C	*BUD16*	Protein involved in bud-site selection; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern; has similarity to pyridoxal kinases
YER014C-A	*BUD25*	Protein involved in bud-site selection; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern
YLR226W	*BUR2*	Bypass UAS Requirement; involved in transcriptional regulation
YJR122W	*CAF17*	Mitochondrial protein that interacts with Ccr4p in the two-hybrid system; 3'-untranslated region contains a putative mRNA localization element common to genes encoding mitochondrial proteins
YJR060W	*CBF1*	Helix-loop-helix protein that binds the motif CACRTG (R=A or G), which is present at several sites including MET gene promoters and centromere DNA element I (CDEI); required for nucleosome positioning at this motif; targets Isw1p to DNA
YDR364C	*CDC40*	Pre-mRNA splicing factor, important for catalytic step II of pre-mRNA splicing and plays a role in cell cycle progression; required for DNA synthesis during mitosis and meiosis; has WD repeats
YGL029W	*CGR1*	Coiled-coil protein that may contribute to compartmentalization of nucleolar constituents; expression is growth-regulated
YGL206C	*CHC1*	Vesicle coat protein; presumed vesicle coat protein
YOR303W	*CPA1*	Small subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline, an arginine precursor; translationally regulated by an attenuator peptide encoded by YOR302W within the CPA1 mRNA 5'-leader
YJR109C	*CPA2*	Large subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline, an arginine precursor
YML112W	*CTK3*	Gamma subunit of C-terminal domain kinase I (CTDK-I), which phosphorylates the C-terminal repeated domain of the RNA polymerase II large subunit (Rpo21p) to affect both transcription and pre-mRNA 3' end processing
YGR155W	*CYS4*	Cystathionine beta-synthase, catalyzes the first committed step of transsulfuration in cysteine biosynthesis
YDR294C	*DPL1*	Dihydrosphingosine phosphate lyase, regulates intracellular levels of sphingolipid long-chain base phosphates (LCBPs), degrades phosphorylated long chain bases, prefers C16 dihydrosphingosine-l-phosphate as a substrate
YJR137C	*ECM17*	Sulfite reductase beta subunit, involved in amino acid biosynthesis, transcription repressed by methionine
YJL201W	*ECM25*	Non-essential protein of unknown function; promoter contains a consensus binding sequence for factor Abf1p
YHL030W	*ECM29*	Major component of the proteasome; tethers the proteasome core particle to the regulatory particle, and enhances the stability of the proteasome
YFR019W	*FAB1*	1-phosphatidylinositol-3-phosphate 5-kinase; vacuolar membrane kinase that generates phosphatidylinositol (3,5)P2, which is involved in vacuolar sorting and homeostasis
YPR062W	*FCY1*	Cytosine deaminase, involved in salvage pathways of pyrimidine metabolism
YER060W-A	*FCY22*	Putative purine-cytosine permease, very similar to Fcy2p but cannot substitute for its function

Supplement Table S2 Continued

ORF name	Gene Name	Gene description in SGD
YOR382W	*FIT2*	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall
YOR381W	*FRE3*	Ferric reductase, reduces siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels
YDL198C	*GGC1*	Mitochondrial GTP/GDP transporter, essential for mitochondrial genome maintenance; has a role in mitochondrial iron transport; member of the mitochondrial carrier family; (putative) mitochondrial carrier protein
YHR183W	*GND1*	6-phosphogluconate dehydrogenase, decarboxylating; converts 6-phosphogluconate + NADP to ribulose-5-phosphate + NADPH + CO2; Phosphogluconate Dehydrogenase (Decarboxylating)
YJL184W	*GON7*	Protein of unknown function, proposed to be involved in the transfer of mannosylphosphate groups onto N-linked oligosaccharides; also proposed to be involved in responding to osmotic stress
YER057C	*HMF1*	Member of the p14.5 protein family with similarity to Mmf1p, functionally complements Mmf1p function when targeted to mitochondria; heat shock inducible; high-dosage growth inhibitor; forms a homotrimer in vitro
YDR158W	*HOM2*	Aspartic beta semi-aldehyde dehydrogenase, catalyzes the second step in the common pathway for methionine and threonine biosynthesis; expression regulated by Gcn4p and the general control of amino acid synthesis
YJR139C	*HOM6*	Homoserine dehydrogenase (L-homoserine:NADP oxidoreductase), dimeric enzyme that catalyzes the third step in the common pathway for methionine and threonine biosynthesis; enzyme has nucleotide-binding, dimerization and catalytic regions
YER086W	*ILV1*	Threonine deaminase, catalyzes the first step in isoleucine biosynthesis; expression is under general amino acid control; ILV1 locus exhibits highly positioned nucleosomes whose organization is independent of known ILV1 regulation
YJR050W	*ISY1*	Component of the spliceosome complex involved in pre-mRNA splicing, auxiliary splicing factor that may modulate Syf1p activity and help optimize splicing; isy1 syf2 double mutation activates the spindle checkpoint, causing cell cycle arrest
YDR017C	*KCS1*	Inositol hexaphosphate kinase, phosphorylates inositol hexakisphosphate (InsP6) to diphosphoinositol polyphosphates, required for proper vacuole morphology and involved in salt stress response, contains two leucine heptad repeats
YFR001W	*LOC1*	Nuclear protein involved in asymmetric localization of ASH1 mRNA; binds double-stranded RNA in vitro
YKR069W	*MET1*	S-adenosyl-L-methionine uroporphyrinogen III transmethylase, involved in sulfate assimilation, methionine metabolism, and siroheme biosynthesis
YJR010W	*MET3*	ATP sulfurylase, catalyzes the primary step of intracellular sulfate activation, essential for assimilatory reduction of sulfate to sulfide, involved in methionine metabolism
YER091C	*MET6*	Cobalamin-independent methionine synthase, involved in amino acid biosynthesis; also called N5-methyltetrahydrofolate homocysteine methyltransferase or 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase
YOR241W	*MET7*	Folylpolyglutamate synthetase, catalyzes extension of the glutamate chains of the folate coenzymes, required for methionine synthesis and for maintenance of mitochondrial DNA, present in both the cytoplasm and mitochondria

Supplement Table S2 Continued

ORF name	Gene Name	Gene description in SGD
YBR213W	*MET8*	Bifunctional dehydrogenase and ferrochelatase, involved in the biosynthesis of siroheme; also involved in the expression of PAPS reductase and sulfite reductase
YFR030W	*MET10*	Subunit alpha of assimilatory sulfite reductase, which is responsible for the conversion of sulfite into sulfide
YKL001C	*MET14*	Adenylylsulfate kinase, required for sulfate assimilation and involved in methionine metabolism
YPR167C	*MET16*	3'-phosphoadenylsulfate reductase, reduces 3'-phosphoadenylyl sulfate to adenosine-3',5'-bisphosphate and free sulfite using reduced thioredoxin as cosubstrate, involved in sulfate assimilation and methionine metabolism
YLR303W	*MET17*	O-acetyl homoserine-O-acetyl serine sulfhydrylase, required for sulfur amino acid synthesis
YIL128W	*MET18*	DNA repair and TFIIH regulator, required for both nucleotide excision repair (NER) and RNA polymerase II (RNAP II) transcription; possible role in assembly of a multiprotein complex(es) required for NER and RNAP II transcription
YIR017C	*MET28*	Transcriptional activator in the Cbf1p-Met4p-Met28p complex, participates in the regulation of sulfur metabolism
YML128C	*MSC1*	Protein of unknown function, green fluorescent protein (GFP)-fusion protein localizes to the endoplasmic reticulum; msc1 mutants are defective in directing meiotic recombination events to homologous chromatids
YMR091C	*NPL6*	Nuclear protein that may have a role in nuclear protein import
YJR073C	*OPI3*	Phospholipid methyltransferase (methylene-fatty-acyl-phospholipid synthase), catalyzes the last two steps in phosphatidylcholine biosynthesis
YOR130C	*ORT1*	Ornithine transporter of the mitochondrial inner membrane, exports ornithine from mitochondria as part of arginine biosynthesis; human ortholog is associated with hyperammonaemia-hyperornithinaemia-homocitrullinuria (HHH) syndrome
YDR323C	*PEP7*	Multivalent adaptor protein that facilitates vesicle-mediated vacuolar protein sorting by ensuring high-fidelity vesicle docking and fusion, which are essential for targeting of vesicles to the endosome; required for vacuole inheritance
YNL316C	*PHA2*	Prephenate dehydratase
YPL188W	*POS5*	Mitochondrial NADH kinase, phosphorylates NADH; also phosphorylates NAD(+) with lower specificity; required for the response to oxidative stress
YGR006W	*PRP18*	Splicing factor involved in the positioning of the 3' splice site during the second catalytic step of splicing, recruited to the spliceosome by Slu7p
YHR001W-A	*QCR10*	8.5 kDa subunit of the ubiqunol-cytochrome c oxidoreductase complex
YOL143C	*RIB4*	Lumazine synthase (6,7-dimethyl-8-ribityllumazine synthase, also known as DMRL synthase); catalyzes synthesis of immediate precursor to riboflavin
YER070W	*RNR1*	Ribonucleotide-diphosphate reductase (RNR), large subunit; the RNR complex catalyzes the rate-limiting step in dNTP synthesis and is regulated by DNA replication and DNA damage checkpoint pathways via localization of the small subunits
YBL093C	*ROX3*	RNA polymerase II holoenzyme component

Supplement Table S2 Continued

ORF name	Gene Name	Gene description in SGD
YIL069C	*RPS24B*	Protein component of the small (40S) ribosomal subunit; identical to Rps24Ap and has similarity to rat S24 ribosomal protein
YOR184W	*SER1*	Phosphoserine transaminase
YGR208W	*SER2*	Phosphoserine phosphatase
YJR104C	*SOD1*	Cu, Zn superoxide dismutase
YHR178W	*STB5*	Binds Sin3p in two-hybrid assay; Zinc finger
YDL185W	*TFP1*	Vacuolar ATPase V1 domain subunit A; protein precursor is spliced to yield the extein Tfp1p and the intein Vde (PI-SceI), which is a site-specific endonuclease
YHR025W	*THR1*	Homoserine kinase
YCR053W	*THR4*	Threonine synthase
YJL138C	*TIF2*	Translation initiation factor eIF4A
YPR074C	*TKL1*	Transketolase 1
YGR260W	*TNA1*	High affinity nicotinic acid plasma membrane permease
YJL129C	*TRK1*	180 kDa high affinity potassium transporter
YDR120C	*TRM1*	tRNA methyltransferase, localizes to both the nucleus and mitochondrion to produce the modified base N2,N2-dimethylguanosine in tRNAs in both compartments
YER090W	*TRP2*	Anthranilate synthase Component I
YKL211C	*TRP3*	Anthranilate synthase Component II and indole-3-phosphate (multifunctional enzyme)
YDR354W	*TRP4*	Anthranilate phosphoribosyl transferase
YGL026C	*TRP5*	Tryptophan synthetase
YBR166C	*TYR1*	Step of tyrosine biosynthesis pathway; Prephenate dehydrogenase (NADP+)
YDL077C	*VAM6*	Protein involved in vacuolar morphogenesis
YPR036W	*VMA13*	Vacuolar ATPase V1 domain subunit H (54 kDa)
YBR127C	*VMA2*	Vacuolar H+ ATPase regulatory subunit (subunit B) of the catalytic (V1) sector
YPL065W	*VPS28*	Soluble, hydrophilic protein involved in transport of precursors for soluble vauolar hydrolases from the late endosome to the vacuole; involved in vacuolar protein targeting
YDR495C	*VPS3*	Vacuolar sorting protein
YLR240W	*VPS34*	Phosphatidylinositol 3-kinase

Supplement Table S2 Continued

ORF name	Gene Name	Gene description in SGD
YGL095C	*VPS45*	Protein of the Sec1p family, essential for vacuolar protein sorting; required for the function of both Pep12p and the early endosome/late Golgi SNARE Tlg2p
YLR322W	*VPS65*	Dubious open reading frame, unlikely to encode a protein; not conserved in closely related Saccharomyces species; 75% of ORF overlaps the verified gene SFH1; deletion causes a vacuolar protein sorting defect
YML097C	*VPS9*	Protein required for Golgi to vacuole trafficking, has similarity with mammalian ras inhibitors
YEL004W	*YEA4*	Shows sequence similarity to GOG5, a gene involved in vanadate resistance; similar to Gog5, which is involved in vanadate resistance
YNL241C	*ZWF1*	Glucose-6-phosphate dehydrogenase
YOR302W		CPA1 uORF , Arginine attenuator peptide, regulates translation of the CPA1 mRNA
YOR302W		CPA1 uORF , Arginine attenuator peptide, regulates translation of the CPA1 mRNA
YHR039C-B		Slow growth
YER068C-A		Questionable ORF from MIPS
YER091C-A		Hypothetical ORF
YIL039W		Hypothetical ORF
YDR008C		Hypothetical ORF
YOL125W		Hypothetical ORF
YDR442W		Hypothetical ORF
YER137C		Hypothetical ORF

Supplement Table S3 List of 46 genes whose deletion mutant strain enhanced dipeptide utilization. In the solid medium plate, deletion strains were grown in MM+(H-L)HKU with or without Trp. In the toxicity halo assay, deletion strains were grown in MM+HLKU medium supplied with 0.1μmol Ala-Eth in the disk. According to the Gene Ontology (GO) in SGD (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper), these genes cover 23 out of 34 categories of "Biological Processes". A given gene is only listed once, although based on its functions some genes are classified into multiple categories. The gene in bold (*CUP9*) was previously identified as being involved in the regulation of *PTR2* transcription.

Gene name	Description of gene product	Growth in solid medium plate with His-Leu		Toxicity of Ala-Eth (% of the wild type)	Functional categories of Biological Processes[b]
		No inducer (Without Trp)	Inducer (With Trp)		
Wild-type		-	+	100	
RIM101[a]	Specific transcriptional repressor activity	++	++	109	1 2 8 20
SSN3[a]	Cyclin-dependent protein kinase activity	++	++	114	1 2 6 8
SSN8[a]	Cyclin-dependent protein kinase regulator activity	++	++	106	1 2 8
BNI1	Cytoskeletal regulatory protein binding	++	++	109	1 4 5 9 10 17 21
CIK1	Microtubule motor activity	++	++	124	1 4 8 9
CSM1	Required for monopolar attachment of homologous chromosomes to the spindle	+	++	109	1 7 8
MLH1[a]	DNA binding; ATPase activity	+	++	106	1 7 8
PAT1	Required for faithful chromosome transmission and normal translation initiation	+	++	121	1 13
SRB8	RNA polymerase II transcription mediator activity	++	++	109	2
SSN2	General RNA polymerase II transcription factor activity	++	++	123	2
VPS36	Regulator of G-protein signaling activity	+	++	116	2 3 14
HAL9	Specific RNA polymerase II transcription factor activity	+	++	131	2 5
PHO2[a]	Transcription factor activity	++	++	121	2 5 26
EAF3[a]	Histone acetyltransferase activity	++	++	133	2 6 7 19
CUP9[a]	Specific RNA polymerase II transcription factor activity	++	++	120	2 23
ARP5[a]	Actin-related protein	++	++	139	3
ARP8	Actin-related protein	++	++	126	3
LST4	Protein transporter activity	-	+++	116	3

Supplement Table S3 Continued

Gene name	Description of gene product	Growth in solid medium plate with His-Leu		Toxicity of Ala-Eth (% of the wild type)	Functional categories of Biological Processes[b]
		No inducer (Without Trp)	Inducer (With Trp)		
YBT1[a]	Bile acid transporter activity	+	++	156	3
SRN2	Class E vacuolar sorting protein	+	+	109	3
SAC6[a]	Actin filament binding	+	++	118	3 4 5 9 14 21
TPM1	Actin lateral binding	++	++	119	3 4 9 10 14 21
SNF7[a]	Protein sorting in the pre-vacuolar endosome	+++	+++	114	3 4 14
VPS8[a]	Vacuolar sorting	+	++	121	3 4 14
VAM10	Involved in early stages of vacuolar fusion	+	++	107	4
DEF1	Involved in vacuolar import and degradation	++	+++	113	5 12
UBR2	Ubiquitin-protein ligase activity	+++	+++	129	6
DBR1[a]	RNA lariat debranching enzyme activity	+	+++	109	7 27
SFP1[a]	Transcription factor activity	++	++	168	10
KEM1[a]	5'-3' exoribonuclease activity	++	++	135	10 27 28
MRP17[a]	Structural constituent of ribosome	++	++	146	13
MRPS9[a]	Structural constituent of ribosome	++	++	170	13
TIF3	Translation initiation factor activity	+	++	124	13
IES6	Involved in secretion and vacuolar trafficking and maintenance	++	++	132	14
NPR1[a]	Serine/threonine protein kinase	+	+	129	14
KTI12	Enzyme regulator activity	++	+++	109	19
SSD1	Maintenance of cellular integrity	+	++	107	25
TOM72[a]	Minor import receptor of the mitochondrial outer membrane	++	++	108	29
YDR417C	Unknown	++	++	139	29
YFR044C[a]	Unknown	++	++	112	29
YLR114C	Unknown	+	++	143	29
YNL123W	Putative serine protease	+	++	106	29
YNL136W	Unknown	++	++	123	29
YNL295W	Unknown	+	++	106	29
YOR322C	Unknown	-	+	122	29
YPL073C	Unknown	+	++	114	29

[a] These genes encode proteins with more than 30% sequence identity to a human protein.
[b] The functional categories found for each gene according to the gene annotation in SGD Gene Ontology (GO) (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper) are listed. Many genes have multiple listings in GO. The GO functional categories are listed as follows, with the number of genes identified in this screen belonging to each category shown in parentheses taking into account the multiple functional categories found for many genes: 1) Cell cycle (8 genes); 2) Transcription (10 genes); 3) Transport (10 genes); 4) Organelle organization and biogenesis (8 genes); 5) Response to stress (6 genes); 6) Protein modification (3 genes); 7) DNA metabolism (4 genes); 8) Meiosis (6 genes); 9) Cytoskeleton organization and biogenesis (5 genes); 10) Morphogenesis (4 genes); 11) Energy pathways (0 gene); 12) Protein catabolism (1 genes); 13) Protein biosynthesis (4 genes); 14) Vesicle-mediated transport (8 genes); 15) Carbohydrate metabolism (0 gene); 16) Lipid metabolism (0 gene); 17) Signal transduction (2 genes); 18) Cytokinesis (0 gene); 19) Nuclear organization and biogenesis (2 genes); 20) Sporulation (1 gene); 21) Budding (3 genes); 22) Cellular respiration (0 gene); 23) Cell homeostasis (1 gene); 24) Coenzyme and prosthetic group metabolism (0 gene); 25) Cell wall organization and biogenesis (2 genes); 26) Amino acid and derivative metabolism (1 gene); 27) RNA metabolism (2 genes); 28) Ribosome biogenesis and assembly (1 gene); 29) Biological process unknown (9 genes); 30) Membrane organization and biogenesis (0 gene); 31) Electron transport (0 gene); 32) Pseudohyphal growth (0 gene); 33) Vitamin metabolism (0 gene); 34) Conjugation (0 gene).

Supplement Table S4 List of 57 genes whose deletion mutant strain decreased dipeptide utilization. In the solid medium plate, deletion strains were grown in MM+(H-L)HKU with or without Trp. In the toxicity halo assay, deletion strains were grown in MM+HLKU medium supplied with 0.1μmol Ala-Eth in the disk. According to the Gene Ontology (GO) in SGD (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper), these genes cover 25 out of 34 categories of "Biological Processes". A given gene is only listed once, although based on its functions some genes are classified into multiple categories. The genes in bold (*DAL81, STP2, UBC2,* and *PTR1*) were previously identified as being involved in the regulation of *PTR2* transcription.

Gene name	Description of gene product	Growth in solid medium plate with His-Leu		Toxicity of Ala-Eth	Functional categories of Biological Processes[b]
		No inducer (Without Trp)	Inducer (With Trp)		
Wild-type		-	+	100	
TAF14[a]	General RNA polymerase II transcription factor activity	-	-	0 (Fuzzy halo)	1 2 7 19
MCK1[a]	Protein kinases, positive regulator of meiosis and spore formation	-	+	83	1 5 6 7 8 20
MRE11[a]	3'-5' exonuclease activity	-	+	82	1 5 7 8
PHO85[a]	Cyclin-dependent protein kinase activity	-	-	0	1 6 11 15
HFM1[a]	DNA helicase activity	-	-	0 (Fuzzy halo)	1 7 8
SPO21	Structural molecule activity	-	-	0 (Fuzzy halo)	1 8 20
RPN4[a]	Transcriptional activator activity	-	-	0	1 12
BYE1[a]	Transcriptional elongation regulator activity	-	-	85	2
DAL81	Specific RNA polymerase II transcription factor activity	-	-	0	2
STP2[a]	Specific RNA polymerase II transcription factor activity	-	-	61	2
THP1	Protein binding	-	-	88	2 3 4 7 9 10 18
REG1	Protein phosphatase type 1 activity	-	-	0 (Fuzzy halo)	2 11 15
ISA1[a]	Mitochondrial protein required for normal iron metabolism	-	-	0	3
ISA2	Mitochondrial protein required for iron metabolism	-	-	0	3
NPL3[a]	mRNA binding	-	-	90	3
VBM1	Fatty acid elongase activity	-	-	93	3 14 16
ASM4	Structural molecule activity	-	+	0 (Fuzzy halo)	3 19
BUD32[a]	Protein serine/threonine kinase activity	-	-	0 (Fuzzy halo)	4 6 9 10 18
BEM4	Rho-type GTPases	-	-	90	4 9 10 17

Supplement Table S4 Continued

Gene name	Description of gene product	Growth in solid medium plate with His-Leu		Toxicity of Ala-Eth	Functional categories of Biological Processes[b]
		No inducer (Without Trp)	Inducer (With Trp)		
BUD28	Bud site selection	-	-	75	4 9 10 18
UBC2 [a]	Ubiquitin conjugating enzyme activity	-	-	0	5 6 7 12 19
RAD23 [a]	Damaged DNA binding	-	-	84	5 7 12
TSA1 [a]	Thioredoxin peroxidase activity	-	-	88	5 23
PTR1	Ubiquitin-protein ligase activity	-	-	0	6
UBA3 [a]	NEDD8 activating enzyme activity	-	-	0	6
UBP14	Ubiquitin-specific protease activity	-	-	73	6 11 15
ELF1 [a]	Molecular function unknown	-	-	94	10
CSF1	Required for normal growth rate	-	-	90	11
SHP1 [a]	Ubiquitin binding; Enzyme regulator activity	-	-	74	11 12 15 20
ETR1 [a]	Enoyl-[acyl-carrier protein] reductase activity	-	-	0	11 16 22
MCT1	[acyl-carrier protein] S-malonyltransferase activity	-	+	0	11 16 22
OAR1	3-oxoacyl-[acyl-carrier protein] reductase activity	-	-	0	11 16 22
PRD1 [a]	Metalloendopeptidase activity	-	+	84	12
RPL21A [a]	Structural constituent of ribosome	-	-	0	13
RPS9B [a]	Structural constituent of ribosome	-	-	65	13
IPK1	Inositol or phosphatidylinositol kinase activity	-	-	67	15
LAT1 [a]	Dihydrolipoyllysine-residue acetyltransferase activity	-	-	0	15
PDB1 [a]	Pyruvate dehydrogenase (acetyl-transferring) activity	-	-	0	15
HFA1 [a]	Acetyl-CoA carboxylase activity	-	-	0	16
IRA2	Ras GTPase activator activity	-	-	86	17
HOF1 [a]	Cytoskeletal protein binding	-	-	0	18
RMD12	3-hydroxyacyl-[acyl-carrier protein] dehydratase activity	-	-	0	20
NFU1 [a]	Involved in maturation of mitochondrial iron-sulfur proteins	-	-	0	23
PDX1 [a]	Protein binding	-	-	0	24
LPD1 [a]	Dihydrolipoyl dehydrogenase activity	-	-	0	24 26
GCV2 [a]	Glycine dehydrogenase (decarboxylating) activity	-	+	0	26

Supplement Table S4 Continued

Gene name	Description of gene product	Growth in solid medium plate with His-Leu		Toxicity of Ala-Eth	Functional categories of Biological Processes[b]
		No inducer (Without Trp)	Inducer (With Trp)		
GCV3[a]	Glycine dehydrogenase (decarboxylating) activity	-	-	0	26
SHM2[a]	Glycine hydroxymethyltransferase activity	-	-	89	26
ILM1	Mitochondrial DNA maintenance	-	-	85	29
YDR015C	Unknown	-	+	82	29
YDR157W	Unknown	-	-	0	29
YDR290W	Unknown	-	+	85	29
YDR433W	Unknown	-	-	79	29
YJL046W[a]	Unknown	-	-	0	29
YJL175W	Unknown	-	-	0	29
YPL098C[a]	Unknown	-	-	82	29
YPR174C	Unknown	-	+	79	29

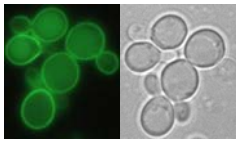
[a] These genes encode proteins with more than 30% sequence identity to a human protein.

[b] The functional categories found for each gene according to the gene annotation in SGD Gene Ontology (GO) (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper) are listed. Many genes have multiple listings in GO. The GO functional categories are listed as follows with the number of genes identified in this screen belonging to each category shown in parentheses taking into account the multiple functional categories found for many genes: 1) Cell cycle (8 genes); 2) Transcription (6 genes); 3) Transport (7 genes); 4) Organelle organization and biogenesis (5 genes); 5) Response to stress (5 genes); 6) Protein modification (8 genes); 7) DNA metabolism (7 genes); 8) Meiosis (4 genes); 9) Cytoskeleton organization and biogenesis (5 genes); 10) Morphogenesis (5 genes); 11) Energy pathways (8 genes); 12) Protein catabolism (5 genes); 13) Protein biosynthesis (2 genes); 14) Vesicle-mediated transport (1 gene); 15) Carbohydrate metabolism (7 genes); 16) Lipid metabolism (5 genes); 17) Signal transduction (2 genes); 18) Cytokinesis (4 genes); 19) Nuclear organization and biogenesis (3 genes); 20) Sporulation (4 genes); 21) Budding (0 gene); 22) Cellular respiration (3 genes); 23) Cell homeostasis (2 genes); 24) Coenzyme and prosthetic group metabolism (2 genes); 25) Cell wall organization and biogenesis (0 gene); 26) Amino acid and derivative metabolism (4 genes); 27) RNA metabolism (0 gene); 28) Ribosome biogenesis and assembly (0 gene); 29) Biological process unknown (10 genes); 30) Membrane organization and biogenesis (0 gene); 31) Electron transport (0 gene); 32) Pseudohyphal growth (0 gene); 33) Vitamin metabolism (0 gene); 34) Conjugation (0 gene).

Supplement Table S5 Halo assay of mutants in components of the ESCRT protein complex.

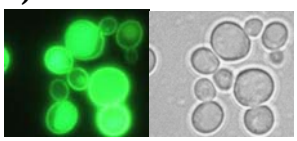
Protein complex	Strains	Halo size (Ala-Eth)	
		0.2µmol	**0.1µmol**
	WT (BY4742)	28	23
	ptr2	0	0
ESCRT I	*vps23*	33	28
	vps28	32	27.5
	srn2	31	23
ESCRT II	*vps22*	33	28.5
	vps25	36	31
	vps36	33	31
ESCRT III	*vps2* (Sub-complexes 1)	34	30
	vps24 (Sub-complexes 1)	33	29
	vps20 (Sub-complexes 2)	38	34
	snf7 (Sub-complexes 2)	32	34

Supplement Figure S1: Ptr2p-GFP expression signal and localization in deletion strains with increased dipeptide utilization. Some strains (underlined) are also shown in Figure 3 as examples of the expression level and localization of Ptr2p-GFP.

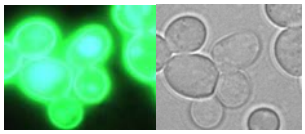


WT

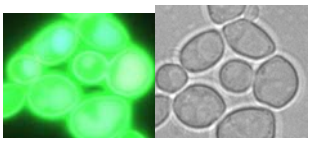
1)



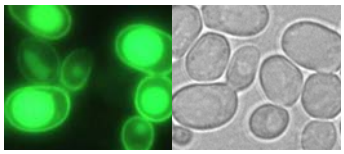
cup9



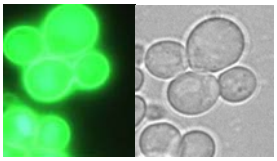
ssn2



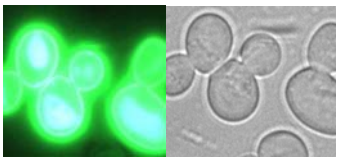
ssn3



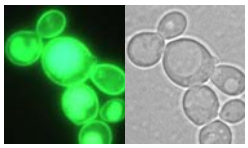
srb8



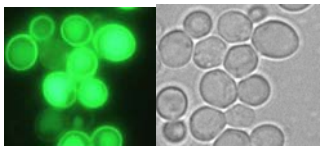
ssn8



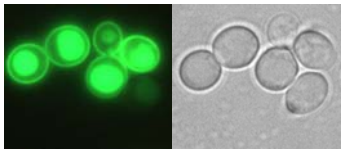
pat1



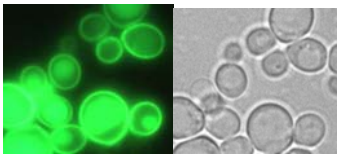
kem1



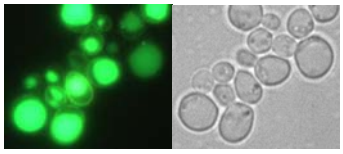
hal9



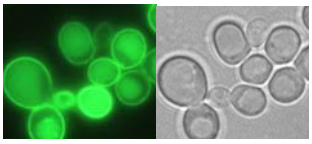
ubr2



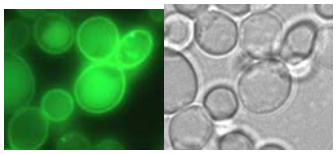
eaf3



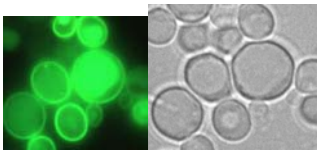
sfp1



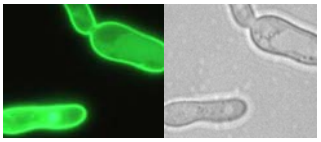
dbr1



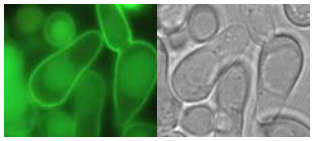
csm1



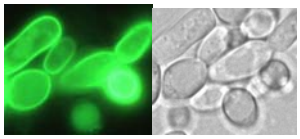
ssd1



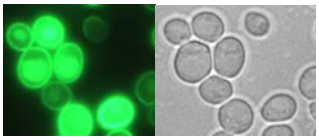
arp5



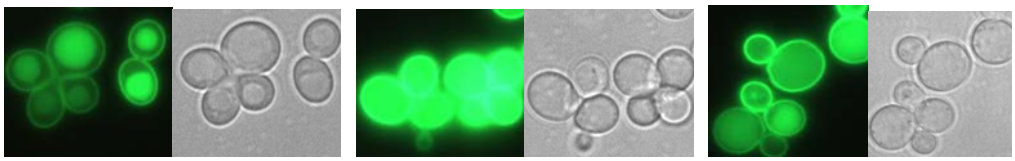
arp8



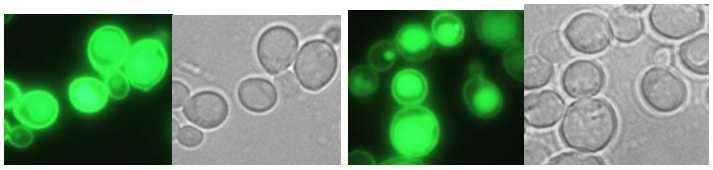
ies6



mrps9

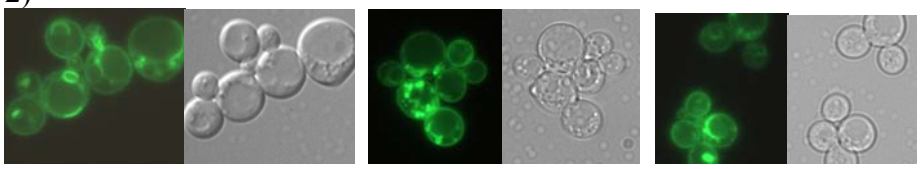


tom72 *yor322c* *ynl123w*

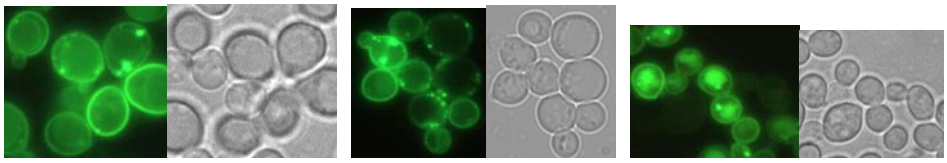


yfr044c *ylr114c*

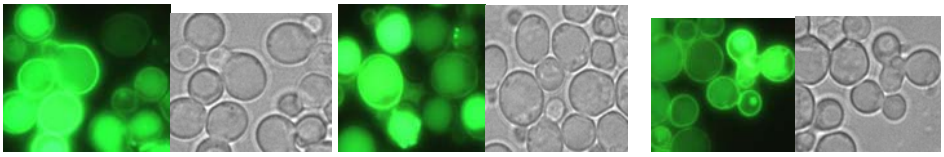
2)



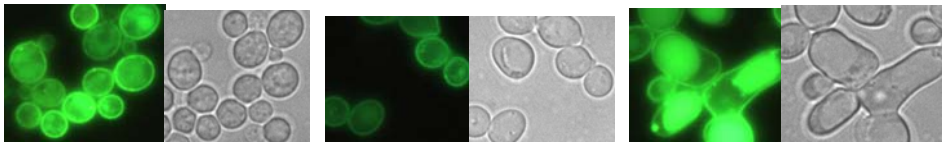
srn2 *vps36* *snf7*



vps8 *eaf7* *npr1*

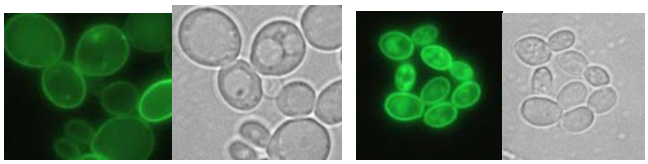


tpm1 *lst4* *bni1*



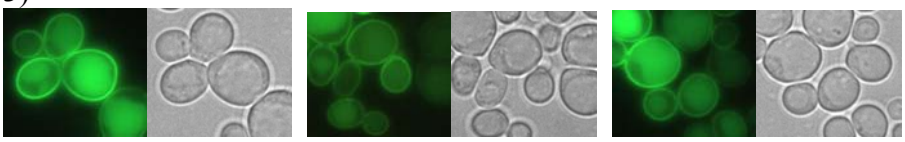
vam10 *sac6* *def1*

Supplement Figure S1 Continued

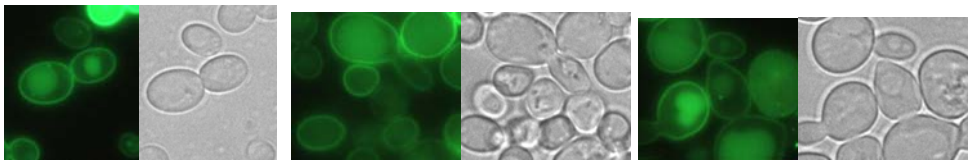


ypl073c *ynl295w*

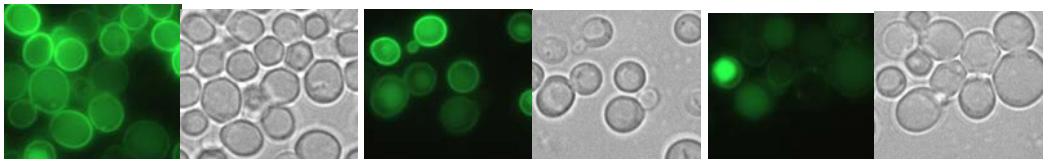
3)



ybt1 *mlh1* *cik1*

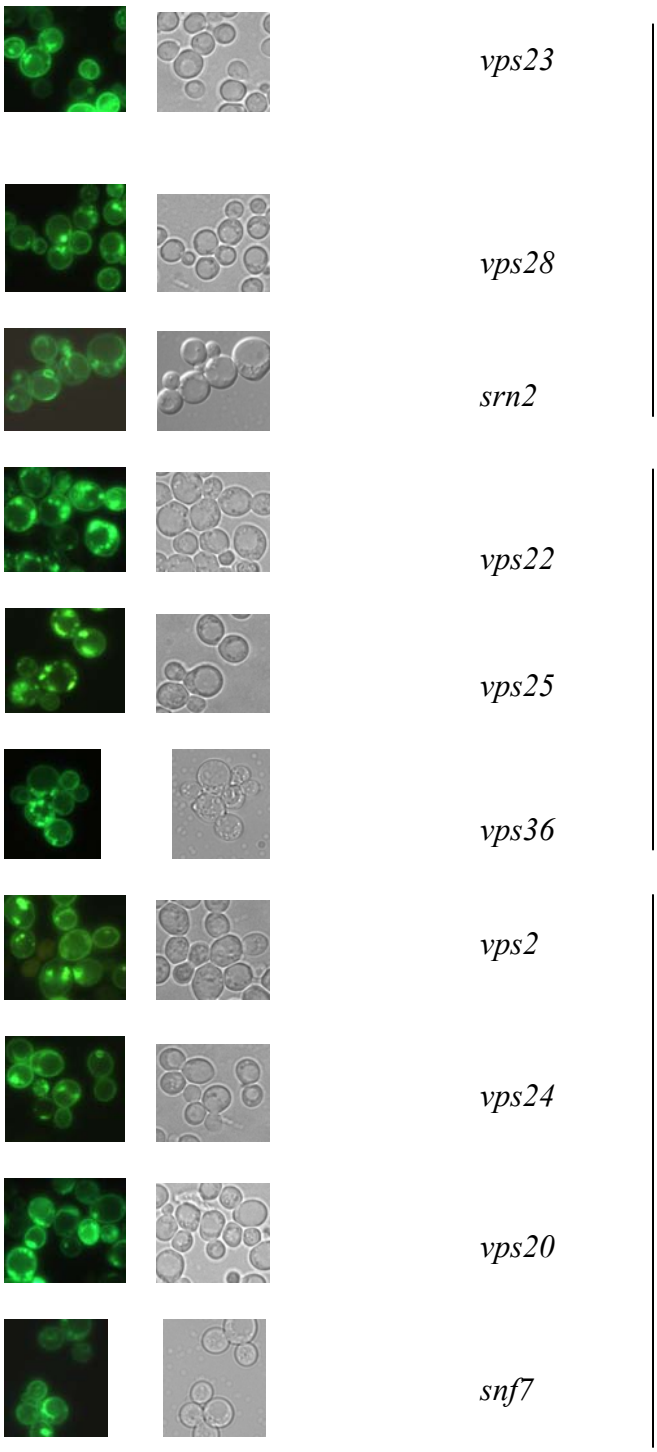


rim101 *kti12* *tif3*



pho2 *mrp17* *ydr417c*

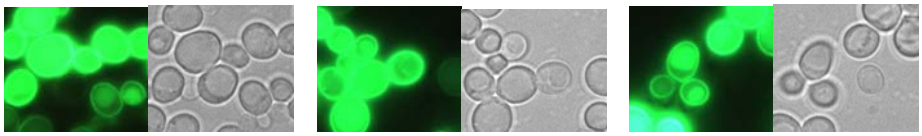
Supplement Figure S1 Continued



Supplement Figure S2 Ptr2p-GFP localization in mutants of the ESCRT complex. Strains *srn2* is also shown in Figure 3.

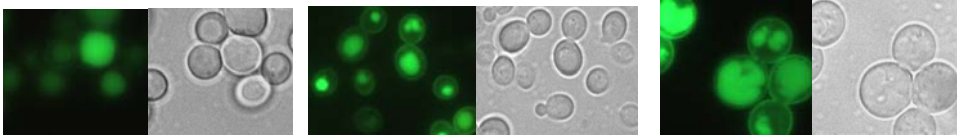
Supplement Figure S3: Ptr2p-GFP expression signal and localization in deletion strains with decreased dipeptide utilization. Some strains (underlined) are also shown in Figure 4 as examples of the expression level and localization of Ptr2p-GFP.

1)

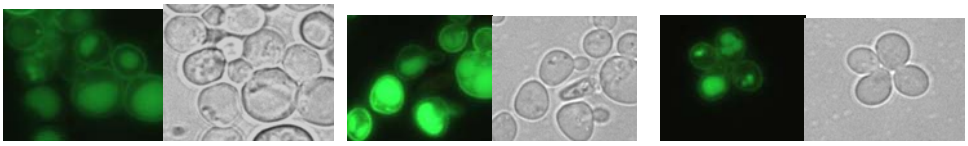


asm4 *ydr015c* *ydr290w*

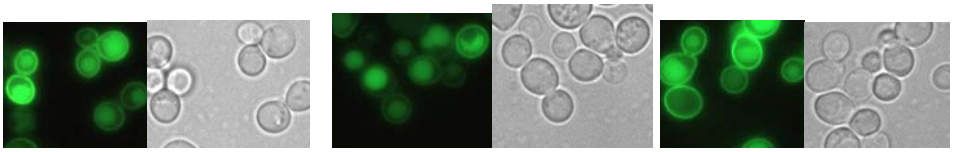
2)



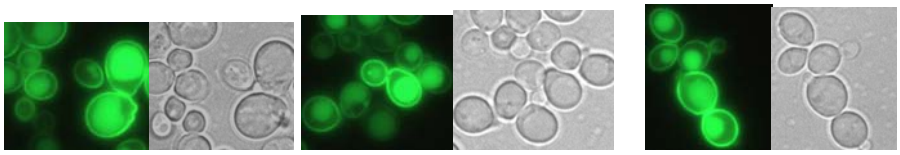
bud32 *reg1* *npl3*



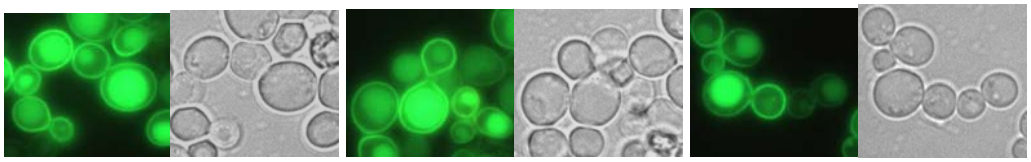
csf1 *shp1* *mck1*



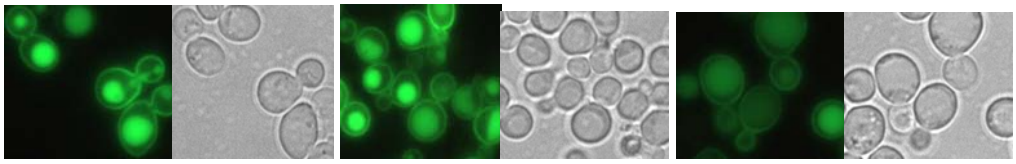
vbm1 *rps9b* *hfm1*



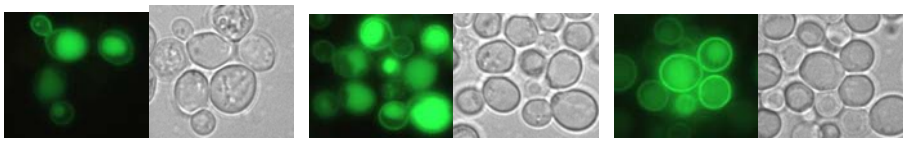
mre11 *bem4* *tsa1*



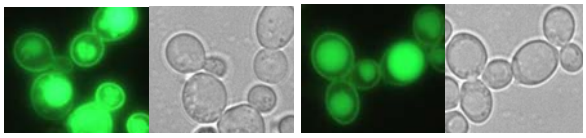
gcv3 *gcv2* *etr1*



rmd12 *ilm1* *isa1*

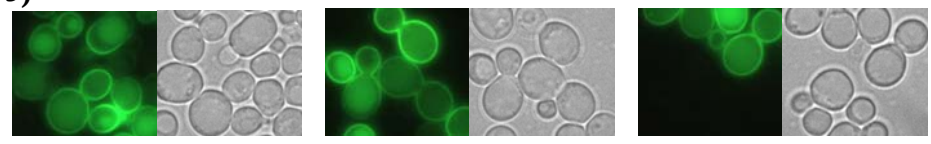


isa2 *ydr433w* *yjl046w*

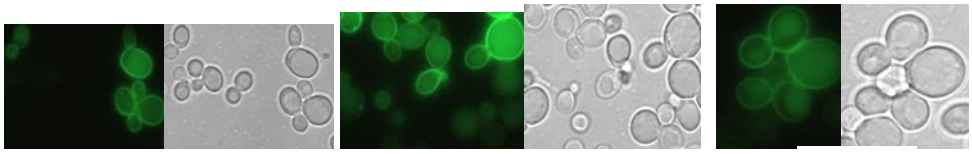


ydr157w *yjl175w*

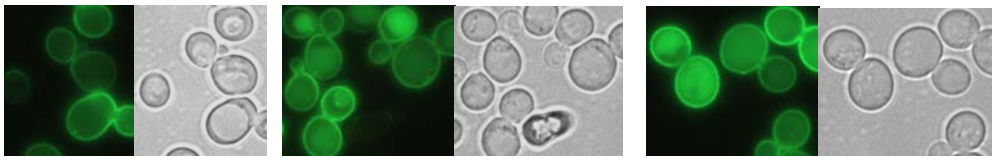
3)



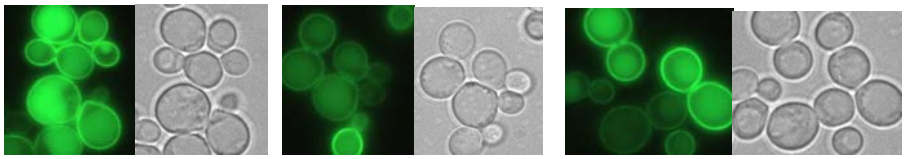
spo21 *prd1* *bye1*



elf1 *rad23* *ira2*

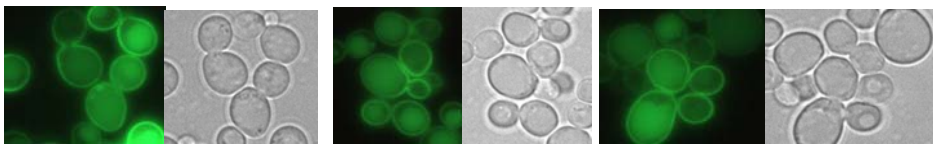


bud28 *pdb1* *lpd1*



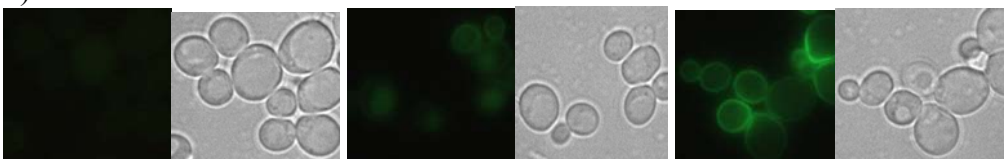
pdx1 *lat1* *oar1*

Supplement Figure S3 Continued

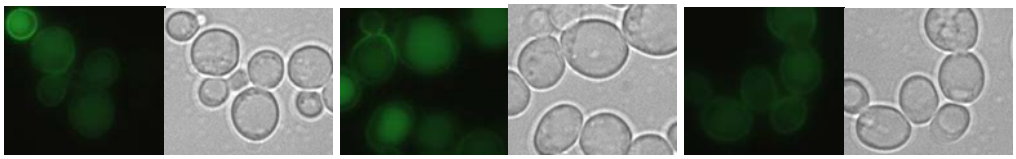


mct1 *hfa1* *ypl098c*

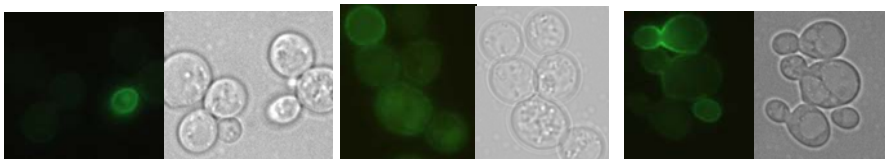
4)



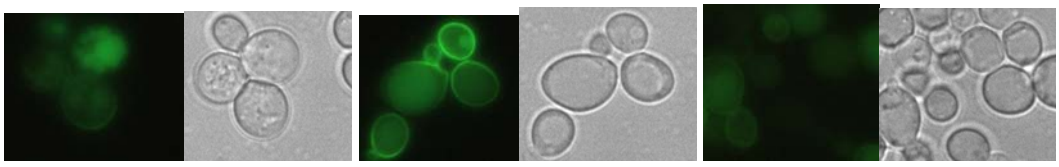
ptr1 *ubc2* *stp2*



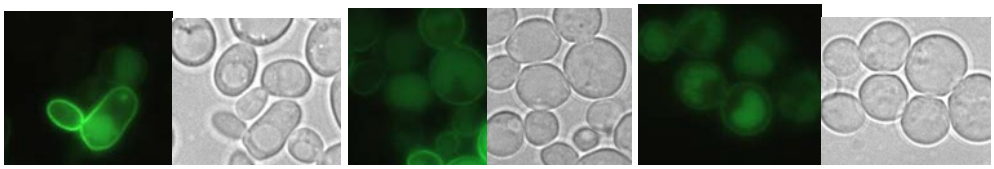
dal81 *rpn4* *nfu1*



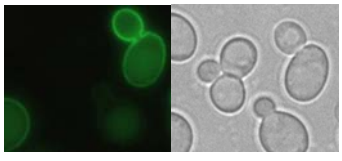
shm2 *hof1* *pho85*



rpl21a *uba3* *ubp14*



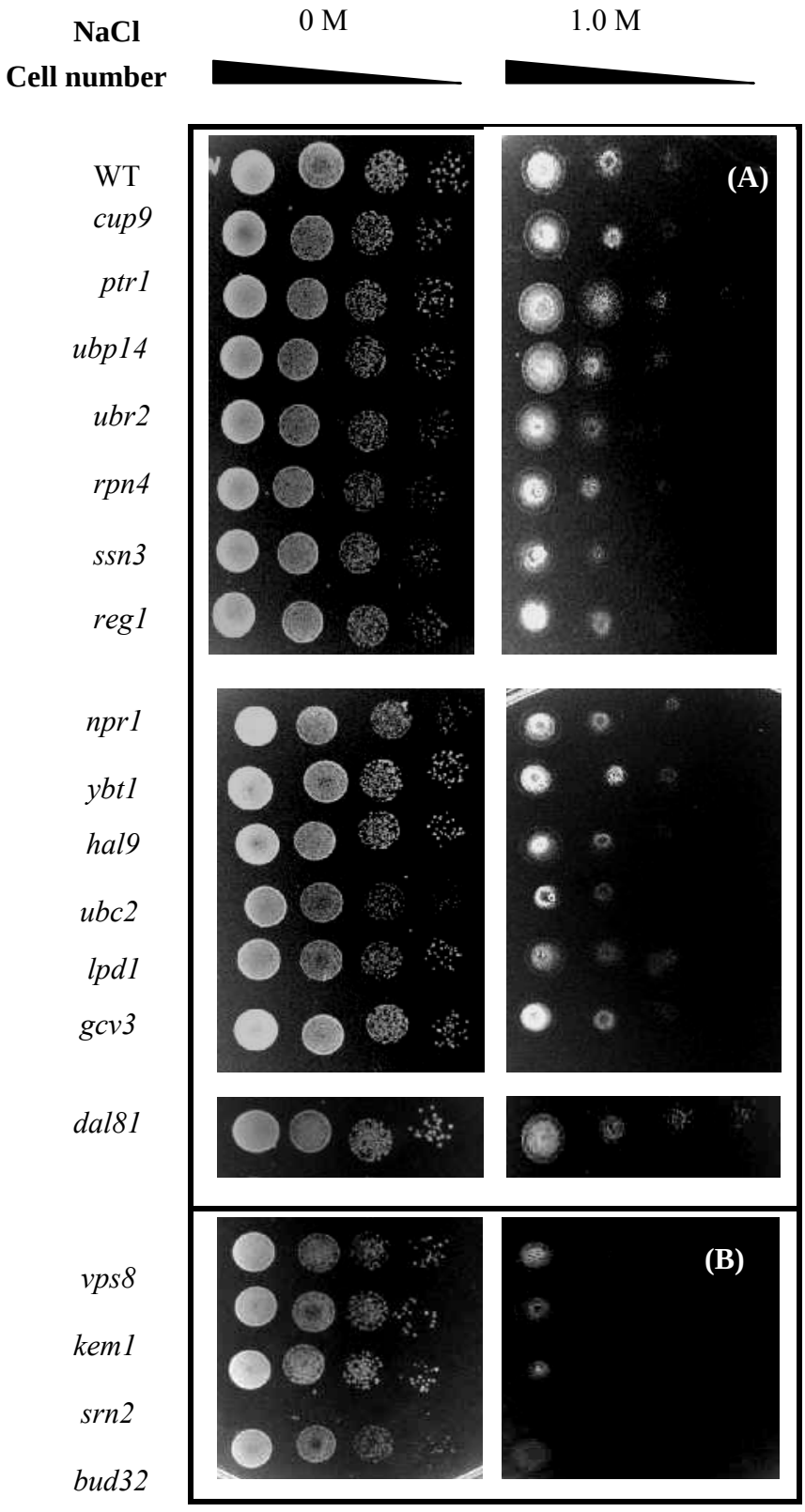
ipk1 *thp1* *ypr174c*



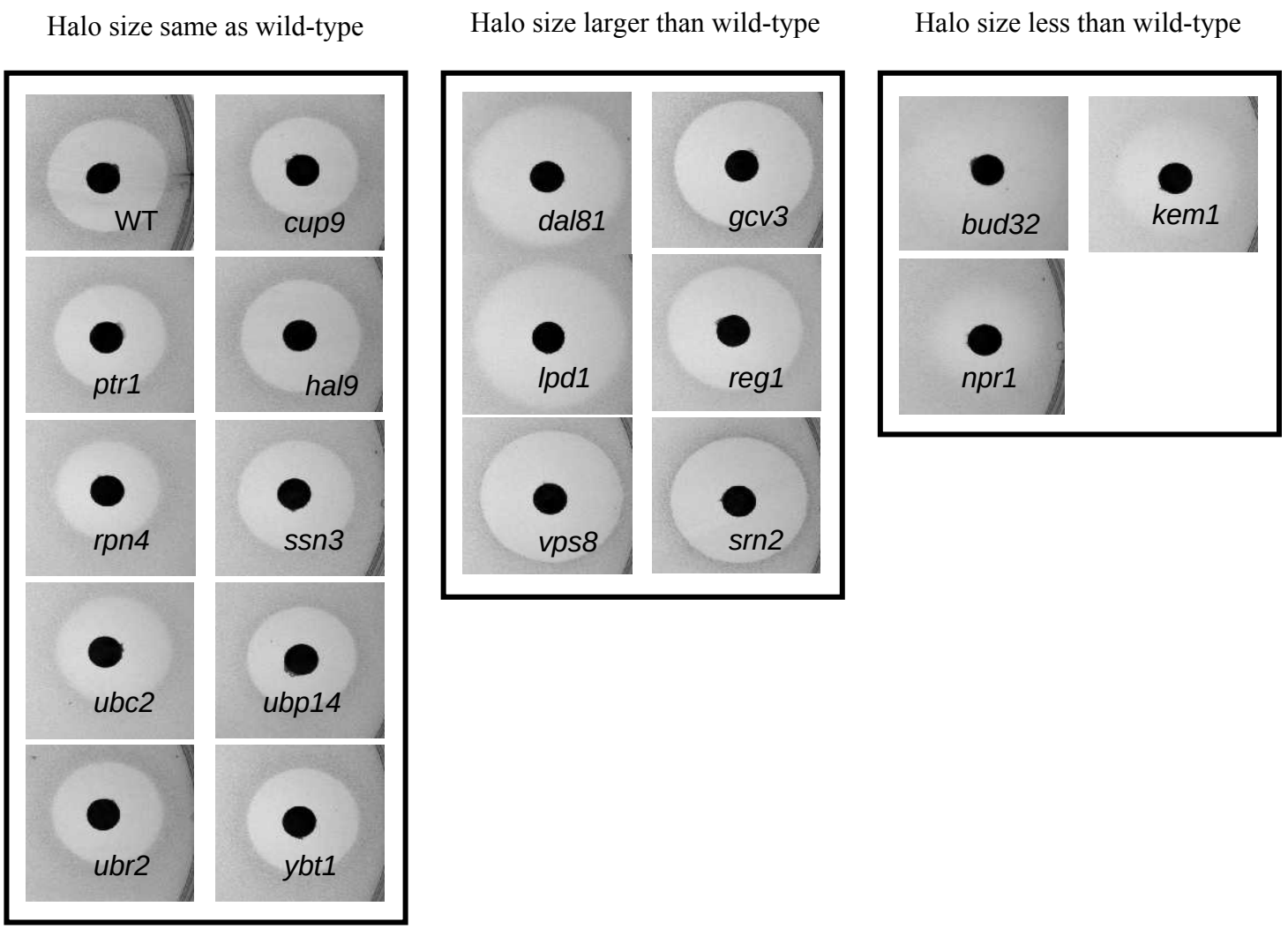
taf14

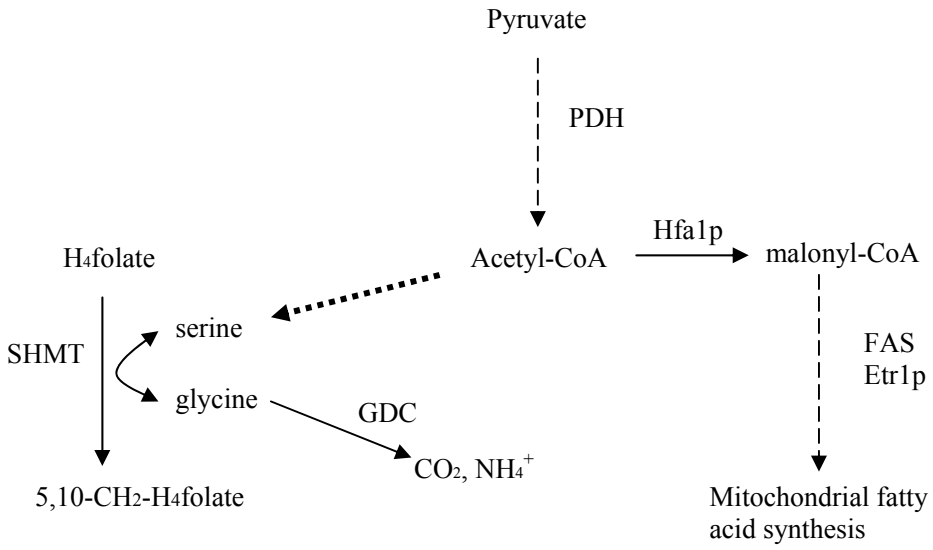
Supplement Figure S3 Continued

Supplement Figure S4 The osmotic sensitivity of selected deletion mutants. The selected strains were grown in either 0 or 1.0 M of NaCl. In panel A, deletion strains showed a similar sensitivity to 1.0 M NaCl in comparison to that of the wild-type strain; in panel B panel, deletion strains *bud32, kem1, srn2,* and *vps8* were more sensitive to 1.0 M NaCl.


NaCl
0 M
1.0 M
Cell number
WT
cup9
ptr1
ubp14
ubr2
rpn4
ssn3
reg1
(A)
npr1
ybt1
hal9
ubc2
lpd1
gcv3
dal81
vps8
kem1
srn2
bud32
(B)

Supplement Figure S5 The canavanine sensitivity to the selected deletion mutants. The selected strains were grown in medium MM+HLKU and canavanine (1.0 μg) was added to each disk. In panel A, nine deletion strains showed a similar sensitivity to canavanine sensitivity in comparison to that of the wild-type. In panel B, the six deletion strains demonstrated a greater sensitivity to canavanine. In panel C, the three deletion strains were less sensitive to canavanine.


Halo size same as wild-type
Halo size larger than wild-type
Halo size less than wild-type
WT
cup9
ptr1
hal9
rpn4
ssn3
ubc2
ubp14
ubr2
ybt1
dal81
gcv3
lpd1
reg1
vps8
srn2
bud32
kem1
npr1



PDH (Pyruvate dehydrogenase complex): Pdx1p, Lpd1p, Pdb1p, and Lat1p
GDC (Glycine decarboxylase multienzyme complex): Gcv2p, Gcv3p, and Lpd1p
FAS (Fatty acid synthase): Oar1p and Mct1p
SHMT (Serine hydroxymethyltransferase): Shm2p

Supplement Figure S6 Proteins involved in cellular metabolic processes impacted in dipeptide utilization. These proteins include the components of pyruvate dehydrogenase complex (Pdx1p, Lpd1p, Pdb1p, and Lat1p), the components of GDC [Glycine decarboxylase multienzyme complex (*GCV2*, *GCV3*, *LPD1*)], the components of FAS [Fatty acid synthase (Oar1p, Mct1p)], SHMT [Serine hydroxymethyltransferase (Shm2p)], Hfa1p, and Etr1p.

PART III

PROBING METABOLIC PATHWAYS OF DI/TRI-PEPTIDE UTILIZATION BASED OB NATURAL DIVERSITY

Part III was published in its entirety as Oliver R. Homann, Houjian Cai, Jeffrey M. Becker, and Susan L. Lindquist 2005. Harnessing Natural Diversity to Probe Metabolic Pathways. *PLoS* Genetics 1(6):715-729. Houjian Cai was responsible for Ptr2p-GFP expression analysis and toxic dipeptide halo assay, and proposed a research on a second peptide transporter related to dipeptide utilization.

ABSTRACT

Analyses of cellular processes in the yeast *Saccharomyces cerevisiae* primarily rely upon a small number of highly domesticated laboratory strains, leaving the extensive natural genetic diversity of the model organism largely unexplored and unexploited. We asked if this diversity could be used to enrich our understanding of basic biological processes. As a test case, we examined a simple trait: the utilization of di/tri-peptides as nitrogen sources. The capacity to import small peptides is likely to be under opposing selective pressures (nutrient utilization versus toxin vulnerability) and may therefore be sculpted by diverse pathways and strategies. Hitherto, dipeptide utilization in *S. cerevisiae* was solely ascribed to the activity of a single protein, the Ptr2p transporter. Using high-throughput phenotyping and several genetically diverse strains, we identified previously unknown cellular activities that contribute to this trait. We find that the Dal5p allantoate/ureidosuccinate permease is also capable of facilitating di/tri-peptide transport. Moreover, even in the absence of Dal5p and Ptr2p, an additional activity - almost certainly the periplasmic asparaginase II Asp3p - facilitates the utilization of dipeptides with C-terminal asparagine residues by a different strategy. Another, as yet unidentified activity, enables the utilization of dipeptides with C-terminal arginine residues. The relative contributions of these activities to the utilization of di/tri-peptides vary among the

strains analyzed, as does the vulnerability of these strains to a toxic dipeptide. Only by sampling the genetic diversity of multiple strains were we able to uncover several previously unrecognized layers of complexity in this metabolic pathway.

INTRODUCTION

Our understanding of the inner workings of eukaryotic cells owes much to the yeast *Saccharomyces cerevisiae*. The application of powerful genetic and molecular tools to this model organism has yielded an extensively annotated proteome. These analyses have benefited greatly from the engineering of experimentally tractable strains of *S. cerevisiae*, but an unintended consequence of this focus has been a tendency to ignore the vast wealth of natural genetic variation found in diverse strains of this organism. Following the "gold rush" ushered in by the sequencing of the *S. cerevisiae* genome, efforts are being made to revisit this natural diversity. Phenotypic analyses of diverse yeast strains (MORTIMER *et al.* 1994; TRUE and LINDQUIST 2000) and the application of microarray technology to the analysis of allelic variation (BREM and KRUGLYAK 2005; BREM *et al.* 2002; STEINMETZ *et al.* 2002; WINZELER *et al.* 1999; WINZELER *et al.* 1998; YVERT *et al.* 2003) and population genetic variation in gene expression (CAVALIERI *et al.* 2000; FAY *et al.* 2004; TOWNSEND *et al.* 2003) are providing new insights into the ecology and diversity of the species. Such analyses should also be applicable to the elucidation of pathways that face strong diversifying selection, such as those governing uptake and metabolism of nutrients. As a test case, we employed high-throughput phenotyping of diverse *S. cerevisiae* strains to dissect the multiple activities contributing to the utilization of di/tri-peptides as a nitrogen source.

The capacity to import small peptides is a ubiquitous cellular function, found in bacteria, fungi, plants, and animals (HAUSER *et al.* 2001; HERRERA-RUIZ and KNIPP 2003; PAYNE and SMITH 1994; STACEY *et al.* 2002; STEINER *et al.* 1995). While small peptides

have clear nutritional value as a source of amino acids, carbon, and nitrogen, they can have additional beneficial functions. For example, studies in bacteria have demonstrated a link between peptide transport and chemotaxis (MANSON *et al.* 1986), sporulation (MATHIOPOULOS *et al.* 1991; PEREGO *et al.* 1991), and the recycling of cell wall peptides (GOODELL and HIGGINS 1987).

Peptide transport systems, however, can also be a source of biological vulnerability. A variety of antimicrobial and antifungal agents utilize the di/tri-peptide transport machinery to gain entry into the cell (RINGROSE 1980; ST GEORGIEV 2000). Thus, the benefits and risks associated with peptide import represent conflicting evolutionary pressures that may help shape the regulation and substrate specificity of peptide transporters. In a similar vein, transporters of small peptides are also of great interest for their medical applications, as routes for delivery of peptidomimetic drugs (BECKER and NAIDER 1995; NIELSEN and BRODIN 2003).

Members of the PTR family of peptide transporters transport a variety of substrates, including nitrates, amino acids, and di/tri-peptides (GRAUL and SADEE 1997; STEINER *et al.* 1995). All PTR members contain 12 predicted transmembrane helices, and transport a variety of substrates by means of proton-motive force (CHIANG *et al.* 2004; MACKENZIE *et al.* 1996). While many organisms, such as humans and plants, contain multiple PTR2 family members, the yeast *S. cerevisiae* contains only one, the di/tri-peptide transporter Ptr2p (Swiss-Prot: P32901).

The regulation of *PTR2* expression in *S. cerevisiae* is strongly influenced by the composition of the extra-cellular environment. *PTR2* expression is induced in the absence of preferred nitrogen sources (ALAGRAMAM *et al.* 1995) - even more so when the

medium contains particular amino acids (ISLAND *et al.* 1987). Import of di/tri-peptides containing basic or bulky hydrophobic N-terminal residues also induces *PTR2* by reducing cellular levels of the *PTR2* repressor Cup9p (Swiss-Prot: P41817). Specifically, these di/tri-peptides serve both as ligands and as regulators of the E3 ubiquitin ligase Ubr1p (Swiss-Prot:P19812), which mediates a protein degradation system that is governed by the identity of N-terminal amino acids (VARSHAVSKY 1996; VARSHAVSKY 1997) (Figure 1). These peptides are too small to serve as targets for degradation by the proteasome and they are assimilated as nutrients via other peptidases in the cells. However, di/tri-peptides with basic (Type 1: Arg, His, or Lys) and bulky (Type 2: Ile, Leu, Phe, Trp, or Tyr) N-terminal residues can compete with larger protein substrates for binding at the type 1 and type 2 Ubr1p substrate binding sites. Upon binding, these peptides allosterically activate Ubr1p-mediated degradation of Cup9p (Swiss-Prot: P41817) by release of the Ubr1p autoinhibitory domain, thus exposing a substrate binding domain that binds an internal degron in Cup9p (DU *et al.* 2002). Relief of Cup9p repression of *PTR2* results in enhanced *PTR2* expression. This initiates a positive regulatory feedback loop in which di/tri-peptide uptake perpetuates Ubr1p-mediated degradation of Cup9p, upregulation of *PTR2*, and additional di/tri-peptide uptake (BYRD *et al.* 1998; DU *et al.* 2002; TURNER *et al.* 2000).

Investigations of di/tri-peptide import in *S. cerevisiae* have typically been limited to the analysis of a small number of substrates through complementation of auxotrophies or the use of radiolabeled dipeptides. The recent development of the Biolog system of "Phenotype MicroArrays™" (PM; (BOCHNER 2003; BOCHNER *et al.* 2001)) provides the opportunity to dramatically expand the scope of these analyses. The PM system

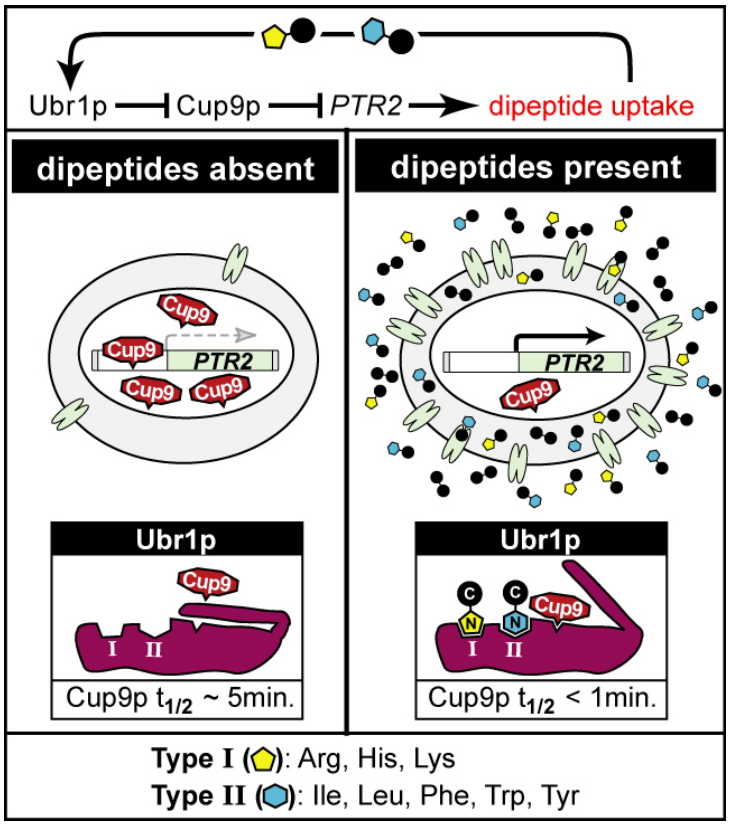


Figure 1 Di/tri-peptide uptake is enhanced by a positive feedback loop governed by the N-end rule pathway. The E3 ubiquitin ligase Ubr1p contains binding sites for proteins containing Type 1 (Arg, His, Lys) and Type 2 (Ile, Leu, Phe, Trp, Tyr) N-terminal amino acids. In the absence of di/tri-peptides, the *PTR2* repressor Cup9p is abundant and *PTR2* expression is minimal. Upon binding of Type 1 and/or Type 2 di/tri-peptides to Ubr1p, an autoinhibitory domain blocking the recognition of an internal Cup9p degron dissociates from the Cup9p binding site, and Cup9p turnover is accelerated. Consequently, *PTR2* repression is relieved and elevated levels of Ptr2p enhance di/tri-peptide uptake, initiating a positive feedback loop. This figure was adapted from the diagram of Turner *et al.* (TURNER *et al.* 2000), with the authors' permission.

facilitates the classification of bacterial and fungal species by generating a characteristic phenotypic profile, which reflects the metabolic capabilities and chemical sensitivities of different species. The strong representation of peptides in the PM assay plates – 284 of the 400 possible dipeptide permutations and 11 tripeptides – provides a unique opportunity to assay the capacity of different *S. cerevisiae* strains to import and utilize dipeptides as nitrogen sources.

Using a combination of high-throughput data from the PM assays, we uncovered extensive variation in dipeptide utilization in different strains of *S. cerevisiae*. We exploited this variation to uncover a diversity of strategies that may serve to balance the opposing selection pressures of nutrient utilization and toxin vulnerability. We find that Ptr2p provides only one of at least four distinct molecular activities that facilitate the utilization of dipeptides in *S. cerevisiae*. The varying strengths of these activities in different strains were key to their identification. No single strain would have revealed the full complexity of dipeptide utilization, underscoring the importance of natural genetic variation for developing a comprehensive understanding of cellular processes.

MATERIALS AND METHODS

Media and reagents: Synthetic defined (SD), YPD, and 5-FOA (BioVectra) media were made using standard formulations (AUSUBEL *et al.* 1988). When needed, G418 (Gibco) was added to the YPD media at 200 μg/ml to select for Kan^r. To select for $HygB^r$, Hygromycin B (American Bioanalytical) was added to YPD at 250 μg/ml. All dipeptides used outside of the PM assays were obtained from Bachem. A minimal media (MM), supplemented with the indicated nitrogen sources, was utilized for the Bioscreen C growth curve assays. MM media contained 2% glucose, 0.17% yeast nitrogen base without $(NH_4)_2SO_4$ and amino acids (Bio101), 18 mg/L uracil (omitted for *URA3* plasmid-bearing strains), and 2% agar (for solid media). The MMA medium used in the halo and GFP assays was identical to the MM media, except that it contained 1 g/L allantoin as a nitrogen source and a slightly different concentration of uracil (20 mg/L). The Biolog PM media contained 50 mM glucose, 1 mM disodium pyrophosphate, 2 mM sodium sulfate, and a proprietary tetrazolium dye mix and "IFY-0" media. IFY-0 is a basal media, lacking nitrogen, carbon, phosphate, and sulfur sources, which are supplemented as needed.

Plasmid construction: Construction of the pMS2 plasmid is described in (HAUSER *et al.* 2005). The plasmid pPTR2-2xGFP-SFH-KanR was constructed by addition of tandem copies of GFP (2xGFP) and a *kan*^{r} selectable marker into plasmid pMS2. First, primers PTR2-FLAG-GFP-F and PTR2-FLAG-GFP-R (all primer sequences are supplied in supplemental Table S1) were used to amplify a 1.5kb fragment containing 2xGFP from plasmid pKW430 (a gift of Dr. Mary Miller, Rhodes University

(STADE *et al.* 1997)) and an additional 40 bp of flanking sequence homologous to pMS2. The plasmid pMS2 was then linearized at the unique restriction site *Age*I, located between the FLAG and His tags. 2xGFP was then inserted between these two tags by homologous recombination, creating pMS4. A *Hin*cII fragment containing *kanMX4* from pFA6a-kanMX4 (WACH *et al.* 1997) was then inserted into the *Nae*I site of pMS4, which lies beyond the stop codon following the 6xHis tag, creating the final pPTR2-2xGFP-SFH-KanR plasmid.

The plasmid pRS426-*DAL5* was created by cloning an *Eco*RV genomic fragment from a plasmid recovered in the genomic screen (see below) into the 2μ vector pRS426 (ATCC#77107; (CHRISTIANSON *et al.* 1992)). This fragment spans a region from -1334 to +2908, relative to the start codon of the *DAL5* gene.

pUG6-HygB was created by replacement of the *kanMX* marker in pUG6 (GULDENER *et al.* 1996) with a *hphMX* cassette from pAG32 (GOLDSTEIN and MCCUSKER 1999). First, a PCR fragment containing the *hphMX* cassette was amplified from pAG32 using primers O-199 and O-200 and digested with *Mlu*I and *Bsm*I. The resulting fragment was then inserted into the similarly digested pUG6.

Strain construction: The source and origins of the strains used in this study are indicated in Table 1. To make all strains MATa *ura3*, some modifications were made to eliminate amino acid auxotrophies, mutate *URA3*, and change mating types (described in detail in Protocol S1). All transformations were conducted using the standard lithium acetate technique (ITO *et al.* 1983).

PTR2 was deleted with a short-flanking homology (SFH) cassette amplified from

Table 1 Strains used in this study

Strain[a]	Genotype	Type	Source(s)
W303-x	*MAT***a** *ura3 can1-100 ho*	laboratory	G. Fink/ R. Rothstein
YAT7-x	*MAT***a** *ura3 ho::MX3*	clinical (peritoneal fluid)	J. McCusker
RM8-x	*MAT***a** *ura3*Δ0 *ho::loxP*	vineyard (California)	B. Garvik/ R. Mortimer
RM3-x	*MAT***a** *ura3*Δ0 *ho::loxP*	vineyard (California)	B. Garvik/ R. Mortimer
S288c-x	*MAT***a** *ura3 ho*	laboratory	B. Garvik
Y55-x	*MAT***a** *ura3 ho::KAN-MX3*	vineyard (France)	J. McCusker
YAT17-x	*MAT***a** *ura3 ho::KAN-MX3*	clinical (broncheolar)	J. McCusker
YAT21-x	*MAT***a** *ura3 ho::KAN-MX3*	clinical (vaginal)	J. McCusker

[a]When additional deletions were introduced into the strains, these modifications were appended to the strain name (*i.e.* "x"= *ptr2*Δ, *cup9*Δ, *dal5*Δ or *ptr2*Δ*dal5*Δ.)

plasmid pFA6a-GFP(S65T)-kanMX6 (WACH *et al.* 1997) (pAG32 (GOLDSTEIN and MCCUSKER 1999) in the case of Y55; the GFP portion of the plasmid was not amplified). This deletion cassette replaced the *PTR2* region spanning +77 to +1781, relative to the start codon, with a *kanMX* marker (*hphMX* in the case of Y55). *CUP9* was deleted with a SFH cassette amplified from plasmid pFA6a-GFP(S65T)-kanMX6, removing the *CUP9* region spanning +41 to +878, relative to the start codon. *DAL5* was deleted with a *hphMX* marker from a SFH cassette amplified from pAG32. This deletion cassette eliminated the *DAL5* region spanning -70 to +1594, relative to the start codon.

Addition of a FLAG-2xGFP-6xHis tag to the C-terminus of endogenous *PTR2* was accomplished with a SFH cassette amplified from plasmid pPTR2-2xGFP-SFH-KanR. Proper integration was ensured by selection on G418 media and verification by colony PCR. Final confirmation of proper integration was obtained by observation of membrane-localized GFP fluorescence in MMA+Leu media.

PM assays of nitrogen source utilization (BOCHNER *et al.* 2001; TANZER *et al.* 2003): A full list of the nitrogen sources tested in the PM assay plates is provided in Dataset S1. Each well of the PM plates contains a very low micromolar amount of amino acids, at a level insufficient to complement auxotrophies or serve as an adequate nitrogen source. All dipeptides are present at the same concentration (between 1-4 mM, the precise concentration is proprietary information), with the exception of the small set of dipeptides present in PM03, which are present at double the concentration of the others (these are notated as "(2x)" when presented in the figures).

Strains were prepared for inoculation into the PM assay plates as follows. Glycerol stocks were streaked to YPD plates and incubated at 30 °C for ~48 h. Next, 2-3

colonies from each strain were restreaked to one section of a fresh YPD plate and incubated overnight at 30 °C. In the morning, cells on each YPD plate were spread over the plate to form a thin layer and incubated an additional 4-6 h to ensure that most cells were actively growing. This technique also served to avoid the late-growth clumping encountered with some wild strains. In the case of strains containing plasmids, standard SD –ura dropout plates were used in place of YPD.

Cells were then inoculated into sterile water in 20x100 mm test tubes and adjusted to a transmittance of 63% (~$5x10^6$ cells/ml). This 48x concentration cell suspension also contained 14.4 mM uracil, except when plasmids were used. The cell suspension was then reduced to 1x concentration by dilution into the Biolog PM media. 100 µl of this mix were then inoculated into each well of the PM assay plates PM03, PM06, PM07, and PM08. The plates were then sealed with sterile Axygen SealPlate film, placed in the OmniLog reader, and incubated for 48 h at 30 °C. The OmniLog reader photographs the plates at 15-min intervals, converting the pixel-density in each well to a signal value reflecting cell growth and dye-conversion.

After completion of the run, the signal data were compiled and analyzed. Signal data were exported from the Biolog software, compiled using Microsoft Excel, and then visually represented using Java TreeView v1.0.8 (SALDANHA 2004) and Adobe Illustrator. We chose to export the average and maximum signal value for each well and then average the two values. In doing so, we were able to represent the full time course by a single number and weight the value towards latter time points. Shadows cast over perimeter wells in the OmniLog machine sometimes raised the basal signal level of these wells. This was accounted for by determining a "baseline" signal level for each well by

calculating the average signal level for the first two hours of the run. This value was then subtracted from the previously calculated signal value, yielding a final signal value. These calculations can be summarized as follows:

Signal value = [((average signal over 48 h) + (maximum signal over 48 h)) / 2)
- (average signal over first 2 h)]

In all cases, a minimum of two replicate PM assay runs were conducted, and the average of the signal values was used. Additionally, when non-wild-type strains were assayed, two independently constructed deletion strains were always tested. The PM assay data used to calculate the signal values presented in this study are provided in Dataset S1. Images of all signal curves are provided in Datasets S2-S4.

To ensure that dye reduction was not occurring in the absence of growth, all PM plates were carefully examined following each run. The plates were inverted upon a light-box such that cells were visible in wells that supported growth. This approach revealed that dipeptides containing lysine, histidine, and cysteine residues, and also the amino acid histidine, sometimes supported reduction of the dye in the absence of growth (dipeptides containing these residues were omitted from the analysis; see Results). Two additional control measures were taken to ensure that the correlation between dye reduction and cell density was robust. First, the PM assay was conducted once with each wild-type strain in the absence of the tetrazolium dye. Second, several PM runs were plated as dilution series to YPD media at the conclusion of the 48h run. These additional

measures confirmed that, with the exceptions noted above, the PM assay signal is a reliable indicator of growth in the presence of the nitrogen sources discussed in this study.

Bioscreen growth curve analysis: A selection of PM assay dipeptide-utilization phenotypes were verified using a Bioscreen C (Growth Curves, USA) plate reader. Strains were cultured as described for the PM assay, but were inoculated at an optical density at 595 nm (OD_{595}) of 0.02 into MM media supplemented with the indicated nitrogen source. The mixture was inoculated into a 100-well plate and incubated in the Bioscreen C at 30 °C with heavy shaking. Measurements of OD_{600} were made at 45-min intervals. Each growth curve was conducted in duplicate wells and was repeated at least once.

Screen for Ptr2p-independent dipeptide utilization: The screen was conducted using a pRS202-based genomic library (2μ; URA3) containing *Sau*3AI-digested genomic DNA with an average insert size of 6-8 kb (gift of Dr. Gerald Fink; originally constructed by Connelly and Hieter from a strain congenic with S288c (CONNELLY and HIETER 1996)). A full description of the screen can be found in the supplemental information (Protocol S2).

Halo assay of Ala-Eth toxicity: The sensitivity of deletion mutants to the toxic dipeptide Ala-Eth was measured as previously described (ISLAND *et al.* 1987). Ethionine (Eth) is an analog of methionine, and utilization of Eth causes cell death. The tested strains were grown overnight in MMA medium. Yeast cells were harvested and washed three times with sterile, distilled water, then counted and adjusted to $5x10^6$ cells/ml. One ml of the cell suspension was added to 0.8% noble agar (3 ml) and plated onto MMA medium with or without leucine (30 μg/ml), an inducer of *PTR2* expression. Four 6-mm

sterile paper disks containing either 0.2, 0.1, 0.05, or 0.025 μmoles of Ala-Eth were placed on the lawn of cells. The size of the halo was measured after two days of incubation at 30 °C. Between two and four replicates were conducted for each assay. Although strains Y55 and RM3 were unable to utilize allantoin in the PM assay (Figure 2E) and in additional Bioscreen C assays using liquid media (0.5 mM allantoin; data not shown), both strains were capable of utilizing allantoin in the solid halo assay media.

Sequencing upstream of *PTR2*: While the region comprising the *PTR2* promoter has yet to be clearly defined, the sequenced span, an 889 bp region immediately upstream of the *PTR2* start codon, should contain the key regulatory elements. The region bound by the Cup9p repressor has been narrowed to the region spanning -897 to -448 (BYRD *et al.* 1998), relative to the *PTR2* start codon. In addition, a predicted amino acid-dependent upstream activator sequence (UAS_{AA}) is located between -777 and -760 (DE BOER *et al.* 1998). Genomic DNA was prepared using the "smash and grab" technique (ROSE *et al.* 1990). A 927 bp fragment containing the desired sequence was amplified with primers O-321 and O-324, using an equal amount of Taq and Vent polymerases. Each reaction mix was split into eight aliquots and repooled following amplification. This approach was taken to help control for the possibility that a single replication error could propagate through the entire sample. PCR products were then purified with a Qiagen PCR purification kit and sequenced in both directions by Northwoods DNA Inc. using oligos O-321, O-322, O-323, and O-324. Sequencing data were analyzed using Sequencher DNA sequencing software.

GFP microscopy: To test the localization of GFP-tagged Ptr2p, yeast strains carrying *PTR2* with a C-terminal FLAG-2xGFP-6xHis tag were grown at 30 °C in MMA,

MMA+Leu, or YPD media. Yeast cells were collected during log-phase and imaged. The GFP signal was observed by fluorescence microscopy with a 470-490 nm excitation wavelength and 515 nm emission filter fitted to an Olympus microscope. Both fluorescent and differential interference contrast (DIC) images were taken with a MicroFire^{TM} camera (Model S99809, Olympus).

RESULTS

To advance our understanding of the processes contributing to nitrogen utilization in *S. cerevisiae*, we studied a panel of strains isolated from several widely differing growth environments (Table 1). We included two related laboratory strains, S288c and W303, two California vineyard isolates, RM3 and RM8, a vineyard isolate thought to be of French origin, Y55 (GREIG *et al.* 2003), and a clinical isolate, YAT7. Because expression of the dipeptide transporter *PTR2* is strongly induced by even micromolar amounts of certain amino acids supplemented in the media (ISLAND *et al.* 1987), we first restored all of these strains to full amino acid prototrophy (see Materials and Methods).

Assaying nitrogen source utilization in diverse strains using Phenotype MicroArrays: We utilized the newly developed PM system to assay the ability of these strains to utilize an extensive selection of dipeptides and other compounds as nitrogen sources. Cells were grown in 96 well plates in which each well contained a different nitrogen source. A proprietary tetrazolium dye solution, which is reduced to the purple compound formazan, was included in each well. Plates were photographed every 15 minutes, generating a growth curve for each well that primarily reflected dye reduction (BOCHNER 1989). The remaining growth signal was attributable to turbidity resulting from cell growth.

As the technology was developed for bacterial cells, we first asked how well the PM signal curves reflect yeast cell growth and respiration, and how reproducible these phenotypes were in standard yeast laboratory media. Reduction of the tetrazolium dye ostensibly results from cellular respiration (BOCHNER 1989), but when we tested petite

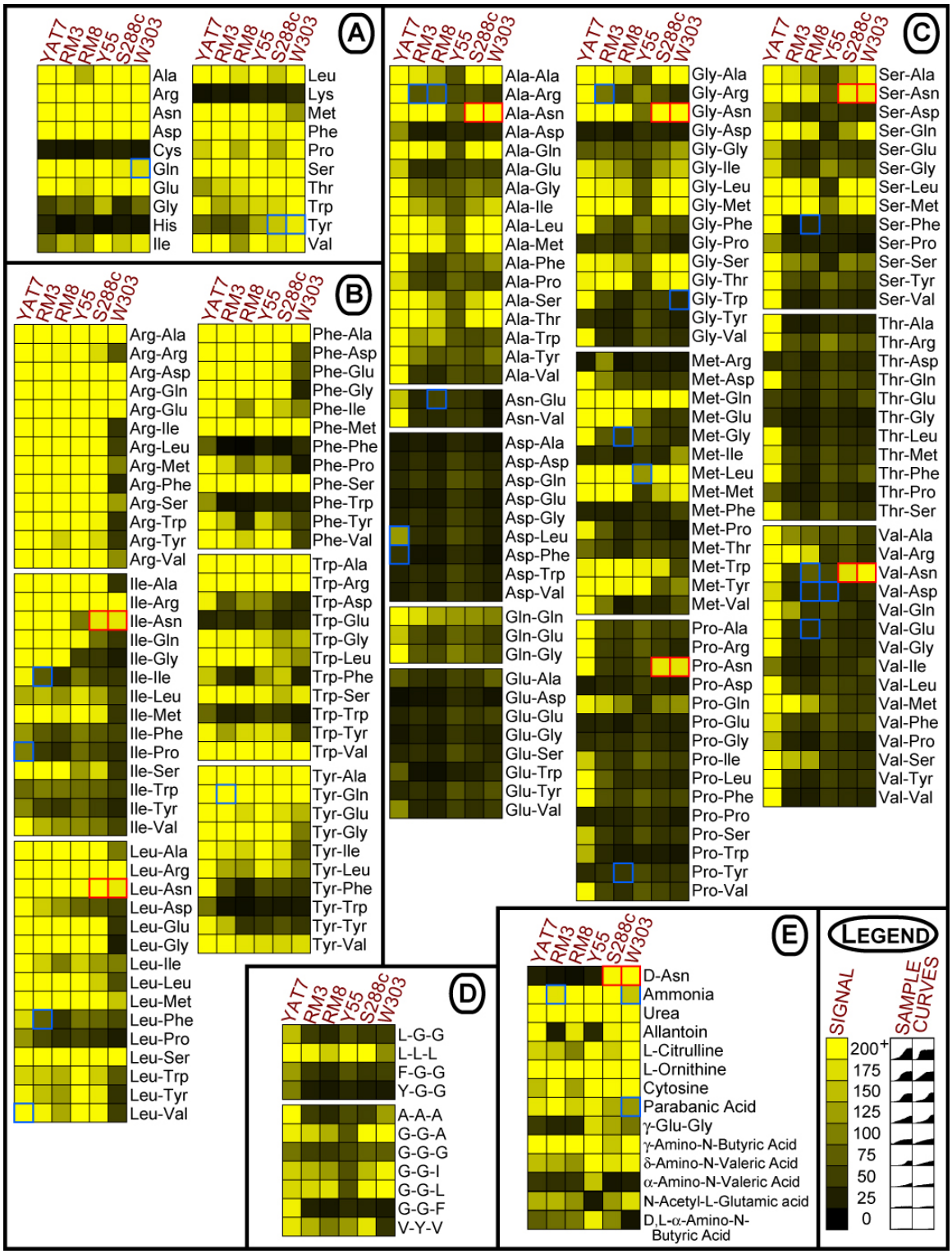
strains of *S. cerevisiae* in the PM assay, they remained capable of robust dye conversion (data not shown). Thus, mitochondrial respiration is not required to produce reductants for this dye. Next, we employed dye-independent measures of growth, such as measuring optical density of PM plates lacking the tetrazolium dye and plating serial dilutions of cells from PM plates to solid media to measure the number of colony forming units in each PM well. Dye-conversion correlated well with growth (see Materials and Methods), except that dipeptides containing the amino acids histidine, lysine, and/or cysteine sometimes caused dye reduction in the absence of growth (data not shown). Interestingly, this growth-independent dye reduction occurred only in the presence of cells. Since it reflects an unknown metabolic process, these dipeptides were omitted from our analyses. It should be noted however, that this growth-independent dye reduction exhibited strong inter-strain variation, suggesting yet another source of metabolic variation for further study.

The PM assay utilizes a viscous medium which supports less vigorous growth than typical laboratory media (data not shown). To determine if growth on diverse substrates in PM plates correlates well with standard laboratory media, we assayed growth on dipeptides as the sole nitrogen source in both solid and liquid standard media (see below), using optical density at 600nm to measure growth in the liquid assays. Given the sensitivity of the dipeptide transport machinery to environmental cues, it is unsurprising that some differences were observed between these platforms. In large part, however, these differing growth conditions provided similar results, and the reproducibility on all tested media was robust.

Shared properties and inter-strain variation in nitrogen source utilization: Next, we asked if the natural diversity in our yeast strains produced phenotypic diversity in nitrogen source utilization. The 48 hour PM growth curves for each strain on each medium are provided in the supplemental materials. To simplify comparisons, each growth curve was assigned a score reflecting the extent of growth, as shown for representative curves in the legend of Figure 2 (see also Materials and Methods). In agreement with an earlier study of the ability of individual amino acids to serve as nitrogen sources in *S. cerevisiae* (LARUE and SPENCER 1967), we found that cysteine, histidine, and lysine did not support growth in any of our strains (Figure 2A). These are the same amino acids that, when present in dipeptides, caused some strains to reduce the tetrazolium dye in the absence of growth. Apart from some inter-strain variation in growth on glycine and tyrosine, the remaining amino acids supported robust growth in all strains tested.

In contrast to the general uniformity in the utilization of individual amino acids, the six strains we analyzed had very different patterns of dipeptide utilization. To assist visualization of the data, dipeptides were separated into two classes, N-end rule dipeptides, which upregulate *PTR2* by enhancing Ubr1p-mediated degradation of the Cup9p repressor (Figure 2B), and non-N-end rule dipeptides (Figure 2C). The clinical isolate YAT7 utilized the broadest spectrum of dipeptides (see Figure 2C in particular) and tripeptides (Figure 2D). Indeed, it grew robustly on many peptides that none of the other strains could utilize well, if at all. (Preliminary analyses of two additional clinical isolates, YAT17 and YAT21, suggest that robust di/tri-peptide transport may be a common feature of strains adapted to the atypical mammalian host environment; data not

Figure 2. Variation in nitrogen source utilization among six strains of *S. cerevisiae*. (A) Amino acids. (B) N-end rule dipeptides. (C) Non-N-end rule dipeptides. (D) Tripeptides; amino acids are represented as single letters. (E) Miscellaneous nitrogen sources. Each square represents the growth of one strain in the PM assay supplied with the indicated nitrogen source. The value reflecting the extent of growth was generated from the 48 h signal curve of the Phenotype MicroArray (PM) assay and represented by the intensity of coloration, as shown in the legend. All signal values exceeding 200 reflected very robust growth, and are therefore represented with the same color. Blue-bordered squares denote nitrogen sources that produced varying results in that strain, exhibiting substantial variation in signal among replicates (σ^2/mean > 10 and at least one replicate with signal > 50 or < 200). Red-bordered squares denote putative substrates of the Asp3p asparaginase.


YAT7
RM3
RM8
Y55
S288c
W303
A
Ala
Arg
Asn
Asp
Cys
Gln
Glu
Gly
His
Ile
Leu
Lys
Met
Phe
Pro
Ser
Thr
Trp
Tyr
Val
B
Arg-Ala
Arg-Arg
Arg-Asp
Arg-Gln
Arg-Glu
Arg-Ile
Arg-Leu
Arg-Met
Arg-Phe
Arg-Ser
Arg-Trp
Arg-Tyr
Arg-Val
Phe-Ala
Phe-Asp
Phe-Glu
Phe-Gly
Phe-Ile
Phe-Met
Phe-Phe
Phe-Pro
Phe-Ser
Phe-Trp
Phe-Tyr
Phe-Val
Ile-Ala
Ile-Arg
Ile-Asn
Ile-Gln
Ile-Gly
Ile-Ile
Ile-Leu
Ile-Met
Ile-Phe
Ile-Pro
Ile-Ser
Ile-Trp
Ile-Tyr
Ile-Val
Trp-Ala
Trp-Arg
Trp-Asp
Trp-Glu
Trp-Gly
Trp-Leu
Trp-Phe
Trp-Ser
Trp-Trp
Trp-Tyr
Trp-Val
Leu-Ala
Leu-Arg
Leu-Asn
Leu-Asp
Leu-Glu
Leu-Gly
Leu-Ile
Leu-Leu
Leu-Met
Leu-Phe
Leu-Pro
Leu-Ser
Leu-Trp
Leu-Tyr
Leu-Val
Tyr-Ala
Tyr-Gln
Tyr-Glu
Tyr-Gly
Tyr-Ile
Tyr-Leu
Tyr-Phe
Tyr-Trp
Tyr-Tyr
Tyr-Val
C
Ala-Ala
Ala-Arg
Ala-Asn
Ala-Asp
Ala-Gln
Ala-Glu
Ala-Gly
Ala-Ile
Ala-Leu
Ala-Met
Ala-Phe
Ala-Pro
Ala-Ser
Ala-Thr
Ala-Trp
Ala-Tyr
Ala-Val
Asn-Glu
Asn-Val
Asp-Ala
Asp-Asp
Asp-Gln
Asp-Glu
Asp-Gly
Asp-Leu
Asp-Phe
Asp-Trp
Asp-Val
Gln-Gln
Gln-Glu
Gln-Gly
Glu-Ala
Glu-Asp
Glu-Glu
Glu-Gly
Glu-Ser
Glu-Trp
Glu-Tyr
Glu-Val
Gly-Ala
Gly-Arg
Gly-Asn
Gly-Asp
Gly-Gly
Gly-Ile
Gly-Leu
Gly-Met
Gly-Phe
Gly-Pro
Gly-Ser
Gly-Thr
Gly-Trp
Gly-Tyr
Gly-Val
Met-Arg
Met-Asp
Met-Gln
Met-Glu
Met-Gly
Met-Ile
Met-Leu
Met-Met
Met-Phe
Met-Pro
Met-Thr
Met-Trp
Met-Tyr
Met-Val
Pro-Ala
Pro-Arg
Pro-Asn
Pro-Asp
Pro-Gln
Pro-Glu
Pro-Gly
Pro-Ile
Pro-Leu
Pro-Phe
Pro-Pro
Pro-Ser
Pro-Trp
Pro-Tyr
Pro-Val
Ser-Ala
Ser-Asn
Ser-Asp
Ser-Gln
Ser-Glu
Ser-Gly
Ser-Leu
Ser-Met
Ser-Phe
Ser-Pro
Ser-Ser
Ser-Tyr
Ser-Val
Thr-Ala
Thr-Arg
Thr-Asp
Thr-Gln
Thr-Glu
Thr-Gly
Thr-Leu
Thr-Met
Thr-Phe
Thr-Pro
Thr-Ser
Val-Ala
Val-Arg
Val-Asn
Val-Asp
Val-Gln
Val-Glu
Val-Gly
Val-Ile
Val-Leu
Val-Met
Val-Phe
Val-Pro
Val-Ser
Val-Tyr
Val-Val
D
L-G-G
L-L-L
F-G-G
Y-G-G
A-A-A
G-G-A
G-G-G
G-G-I
G-G-L
G-G-F
V-Y-V
E
D-Asn
Ammonia
Urea
Allantoin
L-Citrulline
L-Ornithine
Cytosine
Parabanic Acid
γ-Glu-Gly
γ-Amino-N-Butyric Acid
δ-Amino-N-Valeric Acid
α-Amino-N-Valeric Acid
N-Acetyl-L-Glutamic acid
D,L-α-Amino-N-Butyric Acid
LEGEND
SIGNAL
SAMPLE CURVES
200+
175
150
125
100
75
50
25
0

shown). The laboratory strain W303 utilized the fewest dipeptides. It lacked the broad capacity for N-end rule dipeptide utilization characteristic of the other strains (Figure 2B), but it did grow robustly on some of these dipeptides. In contrast, the French vineyard isolate Y55 was growth-limited on the non-N-end rule dipeptides in comparison to the other strains tested (Figure 2C). Each strain also exhibited a unique pattern in the utilization of other nitrogen sources (Figure 2, D and E).

Given the close relationship between W303 and S288c (W303 was derived in part from crosses with S288c; R. Rothstein, personal communication), it was surprising that the PM profiles of S288c more closely resembled those of the California vineyard isolates RM3 and RM8. However, S288c and W303 did share two characteristics: both grew robustly on all of the dipeptides in the array that contained C-terminal asparagine residues (Figure 2, B and C, boxes with red borders), and both grew well on D-asparagine (Figure 2E).

Overexpression of *PTR2* masks inter-strain variation and enhances di/tri-peptide utilization: The diversity of dipeptide-utilization traits suggests profound differences in the underlying mechanism of di-tri/peptide import among our conspecific strains. One likely source of inter-strain variation in dipeptide utilization is differences in *PTR2* regulation. If dipeptide utilization is limited by low levels of Ptr2p, upregulation of *PTR2* should enhance growth. To determine how broadly a lack of *PTR2* expression limits dipeptide utilization, we eliminated the repressor of *PTR2*, Cup9p, in three strains whose wild-type dipeptide-utilization profiles were robust, intermediate, and weak: YAT7, RM8, and W303 respectively.

In all three strain backgrounds, the *CUP9* deletions caused robust growth on most of the di- and tripeptides present in the arrays (Figure 3). Even dipeptides that had not supported growth in any of the wild-type strains, such as dipeptides bearing the N-terminal acidic residues aspartate or glutamate, supported growth upon deletion of *CUP9* (Figure 3B). Of the 197 dipeptides amenable to PM analysis, only a small fraction – most notably Trp-Trp, Trp-Glu, Asp-Asp, and Pro-Pro – supported little growth in the *cup9*Δ strains. These dipeptides may either be refractory to transport or less amenable to use as a nitrogen source following transport. The robust dipeptide utilization phenotype of the *cup9*Δ mutants indicates that Ptr2p is capable of transporting a very broad array of di/tri-peptides, a property that has long been assumed but never addressed with such a large spectrum of dipeptides.

Further, the robust dipeptide-utilization phenotypes of the *cup9*Δ mutant strains indicate that differences in regulatory control of *PTR2* are a key feature of the natural diversity observed among the strain backgrounds. Whereas the wild-type strains exhibited different dipeptide utilization phenotypes (Figure 2, B and C), relief of *PTR2* repression by deletion of *CUP9* eliminated almost all inter-strain variation (Figure 3). Thus, the limited capacity for dipeptide utilization in wild-type W303 relative to RM8 and YAT7 is likely to reflect a difference in *PTR2* regulation rather than a difference in Ptr2p function.

Ptr2p is not required for the utilization of some dipeptides: The only known transporter of di/tri-peptides in *S. cerevisiae* is Ptr2p. Therefore, we expected that *ptr2*Δ

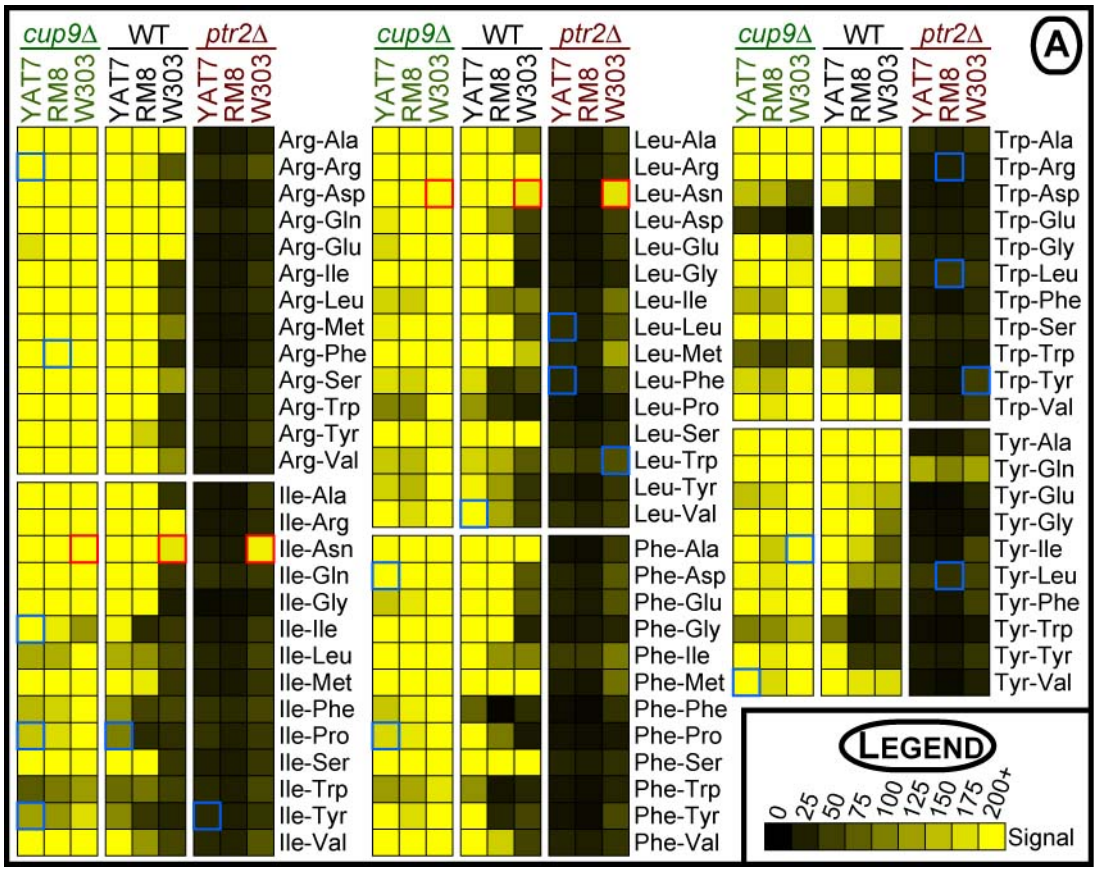


Figure 3 Deletion of *CUP9* and *PTR2* strongly impacts di/tri-peptide utilization in *S. cerevisiae* strains. Elimination of the *PTR2*-repressor Cup9p revealed that Ptr2p can facilitate the utilization of nearly all di/tri-peptides tested in the PM assay, yet Ptr2p was not required for the utilization of a subset of di/tri-peptides as a nitrogen source. (A) N-end rule dipeptides. (B) Non-N-end rule dipeptides. (C) Tripeptides; amino acids are represented as single letters. See Figure 2 for a detailed explanation of the yellow boxes and colored borders.

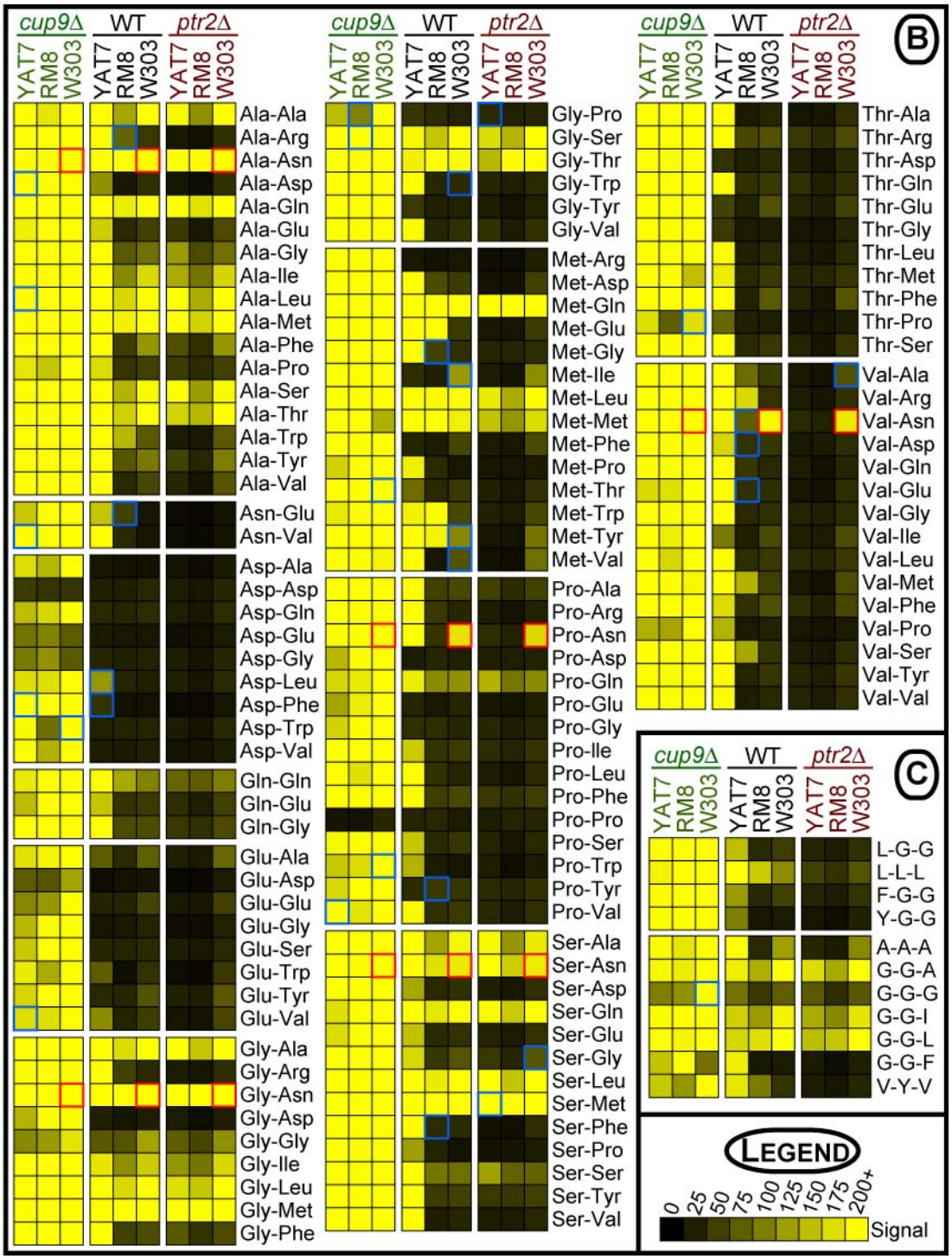


Figure 3 Continued

trains would be unable to utilize any di/tri-peptides in the PM assay. Surprisingly, this was not the case, particularly for non-N-end rule di/tri-peptides (Figure 3, B and C). All three strains tested did not require *PTR2* to utilize a subset of these non-N-end rule dipeptides. Indeed, the W303-*ptr2*Δ mutant grew just as well on the non-N-end rule dipeptides as the wild-type strain.

When examined as a whole, these observations suggested that at least two activities contribute to dipeptide utilization: Ptr2p-mediated transport and an as yet unidentified activity. For N-end rule dipeptides, Ptr2p was the main activity contributing to their utilization, as evidenced by the almost complete abrogation of growth on these nitrogen sources by the *ptr2*Δ deletions (Figure 3A). For non-N-end rule di/tri-peptides, both Ptr2p and the Ptr2p-independent activity played roles in their utilization (Figure 3B). The contribution of Ptr2p to general dipeptide utilization varied from minimal, as observed with W303, to substantial, as observed with the clinical isolate YAT7 (Figure 3, compare wild-type and *ptr2*Δ strains). In contrast, the similar growth properties of the *ptr2*Δ strains suggested that the contribution of the Ptr2p-independent activity was similar in all three strain backgrounds.

Dal5p confers a Ptr2p-independent dipeptide utilization activity: To identify the source of dipeptide utilization in the *ptr2*Δ strains, we took advantage of the robust growth of the W303-*ptr2*Δ strain when Ala-Leu was the sole source of nitrogen in the PM assay. We speculated that the concentration of Ala-Leu needed to support growth would be reduced if the gene allowing its utilization was overexpressed. Accordingly, we screened a high-copy (2μ) genomic library for plasmids that restored growth of

W303-*ptr2*Δ on limiting quantities of Ala-Leu (0.75 mM). After eliminating candidates that contained the *PTR2* ORF, the three remaining plasmids were sequenced. Each plasmid carried the allantoate/ureidosuccinate permease gene *DAL5* (Swiss-Prot: P15365).

To analyze the contribution of Dal5p to basal dipeptide utilization, we constructed *ptr2*Δ, *dal5*Δ, and *ptr2*Δ*dal5*Δ mutants in a variety of strain backgrounds. We began by testing the growth of serially diluted cells on conventional solid media containing different nitrogen sources (Figure 4). These included the putative Dal5p substrate Ala-Leu, as well as Tyr-Ala and Ala-Tyr, which had not supported growth of the *ptr2*Δ mutant in any of the strains tested in the PM assay (Figure 3, A and B). In all strains tested, the *ptr2*Δ*dal5*Δ double-mutant abolished growth on all three assayed dipeptide sources. Thus, utilization of these dipeptides required one of these two systems.

In the case of the dipeptides Ala-Tyr and Tyr-Ala, utilization was mediated by Ptr2p. Growth on these dipeptides was abolished by deletion of *PTR2* alone, whereas deletion of *DAL5* alone had no apparent effect. In contrast, the utilization of Ala-Leu involved both Ptr2p and Dal5p, but the relative contributions of these two activities varied between strains. Similar results were obtained using both solid (Figure 4) and liquid media (2 mM Ala-Leu; Figure S1). Ala-Leu could be utilized in strains RM8 and YAT7 via either Ptr2p or Dal5p, but Ptr2p was necessary and sufficient for wild-type growth levels. Strains W303 and Y55 did not exhibit this functional overlap in Ala-Leu utilization. W303-*ptr2*Δ grew as well as wild-type W303 on Ala-Leu, but W303-*dal5*Δ was completely unable to grow. In Y55, the same deletions created the opposite effect:

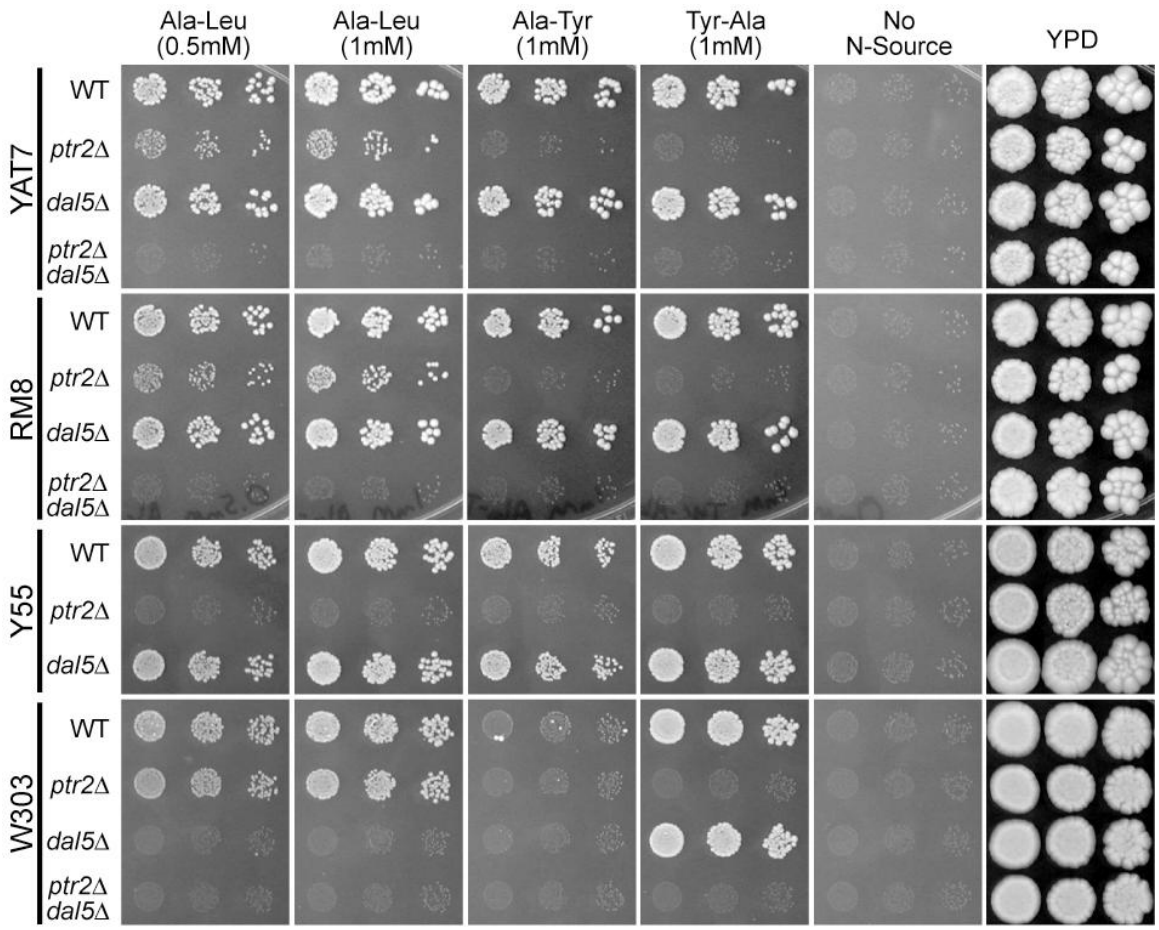


Figure 4 The relative contributions of Dal5p and Ptr2p to dipeptide utilization vary among strains of *S. cerevisiae*. Strains were cultured on YPD plates, as with the PM assays, and then suspended in water at a concentration of ~$3.5x10^6$ cells/ml and plated as 1x, 5x, and 25x dilutions (from left to right). Each plate contained MM media plus the nitrogen source indicated at the top of the column (with the exception of the far right column, in which YPD plates were used). Plates were incubated at 30º C for 4 d and then photographed. A Y55-*ptr2*Δ*dal5*Δ double mutant was not tested, since deletion of *PTR2* alone was sufficient to eliminate dipeptide utilization.

Y55-*ptr2*Δ could not grow, whereas Y55-*dal5*Δ grew as well as wild-type Y55. Thus, Y55 relied upon Ptr2p for utilization of the assayed dipeptides, whereas W303 relied predominantly on Dal5p.

The scope of the contribution of Dal5p to di/tri-peptide utilization: Next, we used the PM assay to ask how many di/tri-peptides could be utilized as nitrogen sources through the activity of the *DAL5* gene. The gene was subcloned into a clean 2μ vector, from the original plasmid that was isolated from the genomic screen, and tested in strain W303-*ptr2*Δ. Unlike the effect of overexpressing *PTR2* (in the *cup9*Δ strain), the multi-copy *DAL5* plasmid only enhanced growth on a small number of di/tri-peptides (Figure 5). With the exception of Tyr-Gln, these di/tri-peptides were all non-N-end rule dipeptides. N-terminal alanine, glycine, and serine residues were particularly favorable to Dal5p-mediated growth.

To determine whether Dal5p activity was redundant or supplemental to that of Ptr2p, the specific contribution of Dal5p to di/tri-peptide utilization was explored using PM assays of the strains Y55-*dal5*Δ and RM8-*dal5*Δ (full data presented in Figure S2). In the case of Y55, deletion of *DAL5* had negligible impact upon dipeptide utilization (Figure S2B and Figure 4). Because the strongest growth defect in wild-type Y55 was in the utilization of those non-N-end rule dipeptides that we have found to depend on Dal5p, we speculated that Y55 might lack Dal5p activity. This hypothesis was verified by a PM assay of Y55-*ptr2*Δ. Unlike our earlier assays of *ptr2*Δ mutants in YAT7, RM8, and W303, deletion of *PTR2* in Y55 completely eliminated growth on all dipeptides and

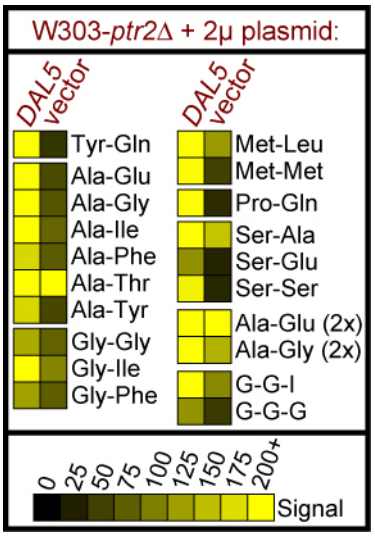


Figure 5 Utilization of a subset of dipeptides is enhanced in W303-*ptr2*Δ upon overexpression of *DAL5*. Strain W303-*ptr2*Δ was transformed with either the vector pRS426 or the plasmid pRS426-*DAL5*, a 2μ plasmid containing a genomic fragment including *DAL5*, and phenotyped using the PM assay. The nitrogen sources shown comprise all instances in which the signal values met our reliability criteria (see Figure 2) and the presence of pRS426-*DAL5* altered the signal value by more than 50 units relative to the vector control. Dipeptides followed by "(2x)" are present in the PM assay plates at twice the concentration of the other dipeptides.

tripeptides (Figure S2). Thus, Y55 relies solely upon Ptr2p for the transport of dipeptides. In contrast, Dal5p contributed substantially to the utilization of dipeptides in RM8. All of the dipeptides that supported more robust growth in the presence of Dal5p belonged to the non-N-end rule category, and the majority contained the small N-terminal residues alanine or glycine (data not shown). Deletion of *DAL5* in strains RM8 and W303 did not significantly affect the utilization of any non-peptide nitrogen sources, except that growth was eliminated on N-acetyl-L-glutamate, an intermediate in arginine biosynthesis (DE DEKEN 1962) (Figure S2C). (The fact that N-acetyl-L-glutamate utilization is an indicator of Dal5p activity explains why wild-type Y55 cannot utilize this substrate; Figure 2E). Clearly, *DAL5* makes an important contribution to dipeptide utilization in yeast, and variations in *DAL5* activity contribute to the naturally occurring variation in nitrogen utilization in diverse strains.

Elimination of Ptr2p and Dal5p uncovers additional dipeptide-utilization activities: To determine whether Ptr2p and Dal5p account for all dipeptide utilization in the PM assays, we analyzed *ptr2*Δ*dal5*Δ double mutants in two strains: W303, which showed the greatest dependence on Ptr2p-independent mechanisms, and RM8, which relied heavily upon both Ptr2p and Dal5p (Figure S2). In both strains, deletion of these two genes abolished all growth on most tested dipeptides and tripeptides in both strains. The most notable exception was that W303-*ptr2*Δ*dal5*Δ, but not RM8-*ptr2*Δ*dal5*Δ, retained the capacity to utilize all dipeptides with C-terminal asparagine residues (Figure S2; boxes with red borders). The obvious candidate for the underlying molecular determinant of this activity is the periplasmic asparaginase II (see Discussion). Since this protein is encoded by four copies of the *ASP3* gene (Swiss-Prot: P11163), interspersed

among rRNA and Ty loci in the genome, the origin of the phenotype was not amenable to genetic analysis.

A second strong exception to the requirement for Ptr2p and Dal5 was the utilization of Met-Arg by RM8, which was actually enhanced by the *dal5*Δ mutation (Figure 6) and remained robust in the *ptr2*Δ*dal5*Δ double mutant (Figure S2). Similar analyses of YAT7, W303, and Y55 mutants (Figure S2B and data not shown) indicated that the ability to robustly utilize Met-Arg in the absence of Ptr2p and Dal5p function was unique to RM8. Closer examination of the PM assay plates revealed that this Ptr2p- and Dal5p-independent activity might affect the utilization of other dipeptides as well. When PM assay plates containing RM8-*ptr2*Δ*dal5*Δ were incubated at 30 °C for an additional one to three days following the PM assay, growth was seen in wells containing other dipeptides - specifically, those with C-terminal arginine residues (data not shown). This delayed growth phenotype did not require the function of Dal5p or Ptr2p and was not observed in plates containing either W303-*ptr2*Δ*dal5*Δ or Y55-*ptr2*Δ. Thus, it represented a distinct fourth dipeptide utilization activity.

Inter-strain variation in sensitivity to the toxic dipeptide Ala-Eth: The benefit of efficient utilization of di/tri-peptides as a nutrient source is likely to be counterbalanced by the vulnerability associated with indiscriminate peptide transport. To assay inter-strain variation in vulnerability to toxic peptides, the panel of diverse *S. cerevisiae* strains was exposed to a dipeptide containing L-ethionine (Eth), a toxic methionine analogue (Table 2). The purine derivative allantoin was provided as a

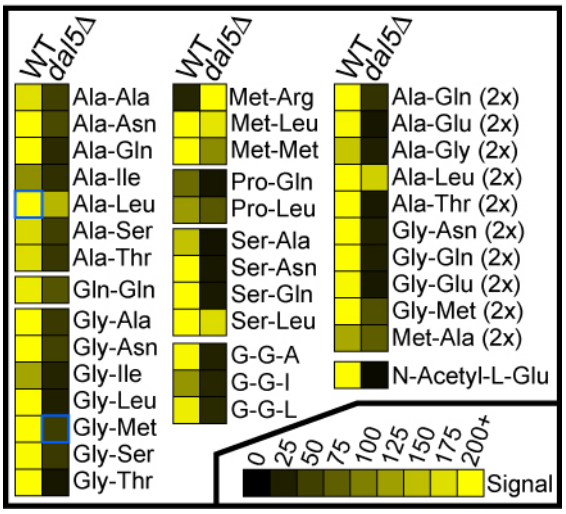


Figure 6 Deletion of *DAL5* in strain RM8 strongly affects the utilization of a subset of assayed dipeptides. All instances in which RM8 and RM8-*dal5*Δ signal values differ by more than 50 units and conform to our reliability criteria (see Figure 2) are presented. Dipeptides followed by "(2x)" are present in the PM assay plates at twice the concentration of the other dipeptides.

Table 2 Halo assay for uptake of the toxic Ala-Eth dipeptide

		Halo size (mm)							
Media:		MMA				MMA+Leu			
Ala-Eth(μmol):		0.2	0.1	0.05	0.025	0.2	0.1	0.05	0.025
Wild-type	YAT7	---	---	---	---	28±2	23±1	19±2	14±1
	RM3	---	---	---	---	23±2	19±3	16±2	11±1
	RM8	---	---	---	---	26±2	22±2	17±2	13±1
	Y55	---	---	---	---	24±2	21±1	16±1	14±1
	S288c	---	---	---	---	37±2	33±3	25±1	20±5
	W303	---	---	---	---	---	---	---	---
	YAT17	13[a]	---	---	---	23±3	19±2	15±3	13±2
	YAT21	18±3	---	---	---	33±2	30±2	25±5	21±4
*cup9*Δ	YAT7	19[a]	17[a]	---	---	30±1	27±0	23±1	17±1
	RM8	25±1	22±2	19±1	16±1	29±0	27±1	22±0	18±1
	W303	32±3	30±2	26±3	22±3	35±2	33±2	29±1	24±1

Cells were plated onto MMA media with or without 30 μg/ml leucine, an inducer of *PTR2* expression; halo diameters in the presence of discs containing the indicated Ala-Eth concentrations are presented as the mean ± S.D.; *ptr2*Δ strains were also tested, and exhibited no halos.

[a]Halo boundary was indistinct, often slowly receding as cells closer to the disc began to grow.

nitrogen source (COOPER 1996), to eliminate the nutritional requirement for dipeptide uptake. Discs containing different concentrations of Ala-Eth were applied to lawns of cells, creating gradients of dipeptide concentration. Thus, the diameter of the halo of growth inhibition surrounding a disc is an indicator of dipeptide import. In the standard halo assay, minimal medium with allantoin is supplemented with 30 μg/ml leucine to induce *PTR2* expression (MMA+Leu media). Under these conditions, wild-type strains varied substantially in sensitivity to Ala-Eth toxicity (Table 2). S288c was the most sensitive to the dipeptide, while W303 was completely resistant. In the absence of the leucine inducer, only the clinical isolates YAT17 and YAT21 were sensitive to Ala-Eth (Table 2).

Deletion of *PTR2* alone was sufficient to confer complete resistance to the toxic dipeptide under the conditions tested (RM8- *ptr2*Δ and YAT7- *ptr2*Δ were tested; data not shown). While this might suggest that Ptr2p is the primary transporter of Ala-Eth, we note that Dal5p may also transport allantoin (CHISHOLM *et al.* 1987; GRETH *et al.* 1977), raising the possibility that allantoin competed with Ala-Eth for Dal5p-mediated uptake. Indeed, Ala-Met, which is structurally similar to Ala-Eth, supports growth in *ptr2*Δ strains (Figure 3B) and *dal5*Δ strains (Figure S2B), suggesting that Dal5p and Ptr2p both contribute to the vulnerability of yeast cells to Ala-Eth.

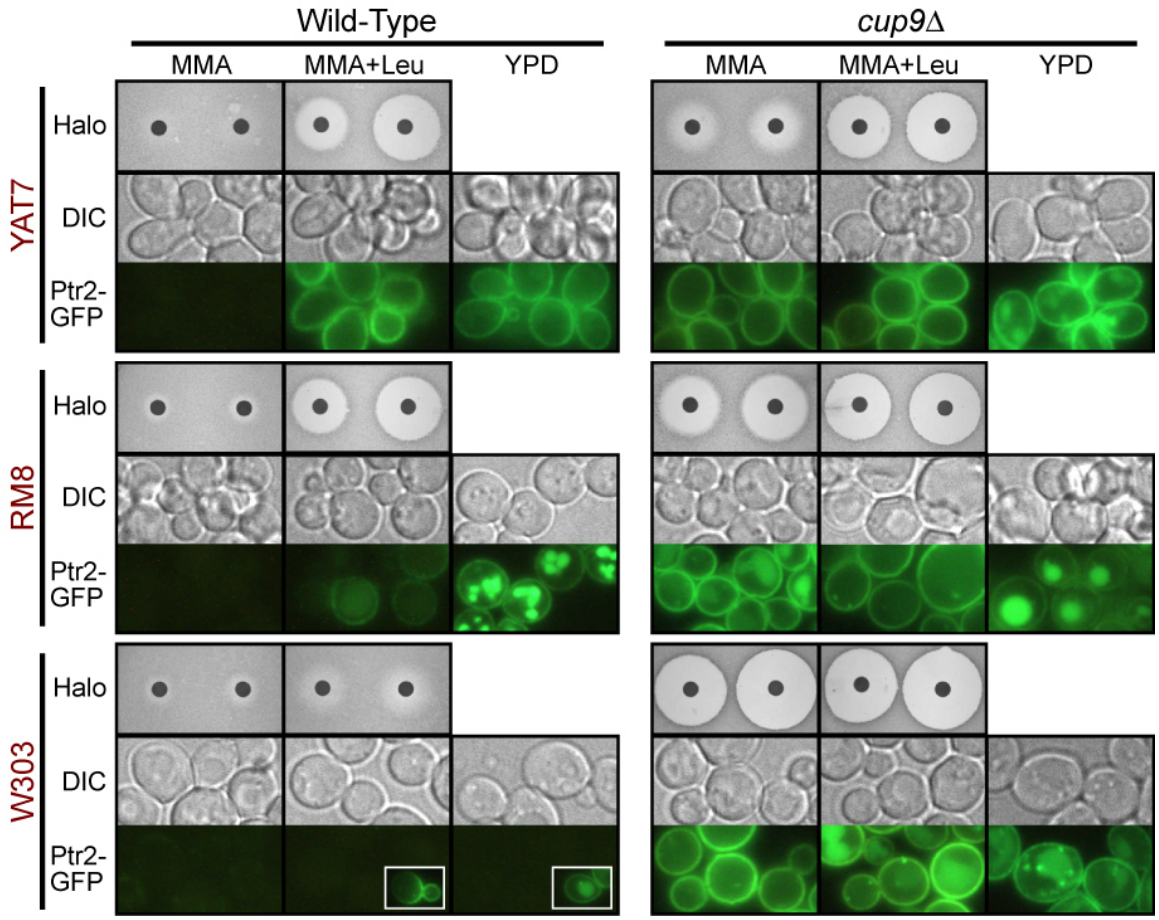
Elimination of the *PTR2* repressor Cup9p enhanced Ala-Eth sensitivity in all strains tested, yet it also revealed additional diversity among the strains (Table 2, *cup9*Δ). The resistance of strain W303 to Ala-Eth was overcome by the *cup9*Δ mutation to the extent that the W303-*cup9*Δ mutant exhibited higher sensitivity to Ala-Eth than *cup9*Δ

mutants of either RM8 or YAT7. All of the *cup9*Δ mutant strains exhibited some sensitivity to Ala-Eth in the absence of the leucine inducer. Curiously, despite the robust dipeptide import characteristic of the YAT7 strain, YAT7-*cup9*Δ produced much smaller halos under these conditions than either W303-*cup9*Δ or RM8-*cup9*Δ (Table 2; see also Figure 7). Some of the inter-strain variation observed in the halo assay might reflect varying sensitivity to ethionine. However, since both changes in media composition and the *cup9*Δ mutations affected strains differently, this diversity is clearly shaped by the complex regulatory controls governing dipeptide import.

Expression and localization of Ptr2p varies substantially among strains: Strain W303 was both highly resistant to the toxic Ala-Eth dipeptide and limited in dipeptide utilization capacity. Therefore, we asked whether *PTR2* expression in this strain differed from that of the other strains tested. To directly visualize the expression of *PTR2*, a C-terminal FLAG-2xGFP-6xHis tag was added to the endogenous *PTR2* gene of strains W303, RM8, and YAT7 by site-specific homologous recombination. These modified strains retained a similar capacity to utilize dipeptides (data not shown). To facilitate comparison of *PTR2* expression with the results of the halo assay, expression of GFP-tagged *PTR2* was assayed in a liquid version of the MMA and MMA+Leu media and fluorescence was measured using a uniform exposure level (Figure 7; halo assay images included for comparison).

The presence of Ptr2p at the plasma membrane correlated well with the extent of Ala-Eth toxicity observed in the halo assay (except that fluorescence was weaker in RM8 than might have been expected from its strong Ala-Eth sensitivity in MMA+Leu). In all

Figure 7 Inter-strain variation in *PTR2* expression and sensitivity to the toxic dipeptide Ala-Eth. Media composition and the absence of Cup9p affect expression of GFP-tagged *PTR2* and Ala-Eth toxicity. Three types of media were tested: MMA, MMA+Leu, and YPD. "MMA" refers to minimal nitrogen media containing 1 mg/ml allantoin as the nitrogen source. "+Leu" refers to the addition of 30 µg/ml leucine, an inducer of *PTR2* expression. YPD is a standard rich *S. cerevisiae* media; the presence of methionine precludes its use for the halo assay. The left and right discs in each halo assay panel contained 0.1 µmoles and 0.2 µmoles of Ala-Eth, respectively. Microscopy was conducted using cells grown to log phase in the indicated media. Three strains were imaged: W303, RM8, and YAT7. In each strain, the endogenous *PTR2* gene contained a C-terminal tag consisting of a FLAG epitope, two copies of GFP, and a 6xHis tag. The insets depict representative cells from the small sub-population of W303 cells that exhibited fluorescence.


Wild-Type
cup9Δ
MMA
MMA+Leu
YPD
MMA
MMA+Leu
YPD
YAT7
RM8
W303
Halo
DIC
Ptr2-GFP

other cases, conditions promoting strong Ala-Eth sensitivity also induced strong *PTR2* expression. Strikingly, wild-type W303, which was resistant to Ala-Eth, exhibited virtually no Ptr2p fluorescence. The *cup9*Δ mutation restored strong *PTR2* expression and also restored Ala-Eth sensitivity. Thus, the resistance of W303 to Ala-Eth in the halo assay resulted from an absence of Ptr2p at the plasma membrane. Moreover, these results confirm that W303 contains a functional *PTR2* gene subject to atypical regulation.

Additional aspects of *PTR2* regulation: Retrograde recycling of membrane-bound Ptr2p is thought to represent an additional layer of regulatory control of dipeptide import (H. Cai and J. Becker, unpublished observations). Although GFP-tagged Ptr2p was typically localized to the membrane, when strain RM8 was grown in YPD, additional fluorescent signal was also observed in the vacuole and what appeared to be large vesicles. The high levels of *PTR2* expression in the rich YPD media was itself surprising, since *PTR2* is thought to be subject to nitrogen catabolite repression in the presence of preferred nitrogen sources (ALAGRAMAM *et al.* 1995). Although YAT7 also exhibited robust *PTR2* expression under these conditions, Ptr2p was confined to the plasma membrane. This inter-strain variation was likely influenced by cellular Ptr2p levels, as *cup9*Δ mutants of RM8, YAT7, and W303 all exhibited robust *PTR2* expression and some intra-cellular Ptr2p localization in YPD media (Figure 7). However, while wild-type RM8 and YAT7 both exhibited strong Ptr2p fluorescence in YPD, only RM8 exhibited abundant intra-cellular compartmentalization of Ptr2p, indicating that retrograde transport of Ptr2p is yet another regulatory process that varies between strains in *S. cerevisiae*.

The regulation of *PTR2* in W303 differed significantly from the other strains analyzed in this study. Considering the possibility that polymorphisms within the *PTR2* promoter region may account for the limited *PTR2* expression in W303, we sequenced an 889 bp region immediately upstream of the *PTR2* start codon in the strains W303, S288c, RM8, and YAT7 (Table S2). The promoter region in W303 differed from that of S288c at only one base, a C→T transition at position -799. However, this polymorphism was shared by the strain RM8, which unlike W303 is sensitive to Ala-Eth and exhibits robust *PTR2*-dependent utilization of N-end rule dipeptides. Thus, differences in promoter sequence are not a likely source for the altered regulation of *PTR2* in W303.

Telomeric regions are common sites of epigenetic regulation, and *PTR2* is located in proximity (~50 kB) to a telomere. In our analyses of GFP-tagged *PTR2*, a small subpopulation (1 in 50 to 1 in 1000, depending on conditions) of strongly fluorescent W303 cells was consistently observed during growth in either the MMA+Leu media or in YPD (Figure 7; inset). W303 cells exhibiting robust *PTR2* expression also arose spontaneously at a high frequency (>0.1%) on solid media. These cells maintain competency for *PTR2* expression following serial propagation on media that relieve selection for active dipeptide transport (J. Manjrekar and S. Lindquist, personal communication). These switching events were far too frequent to reflect mutation, suggesting that an epigenetic switch exists that can stably confer competency for *PTR2* expression in W303 cells.

DISCUSSION

We establish that the rich inter-strain diversity and genetic tractability of *S. cerevisiae* can be combined with high-throughput phenotyping to provide new insights into basic cellular processes. Using this approach, we have uncovered previously unrecognized functions and regulatory systems governing the utilization of dipeptides as a nitrogen source. In addition, we consider dipeptide utilization in the context of an adaptive landscape shaped by opposing selective pressures.

Using high-throughput phenotyping to study di/tri-peptide utilization: The exploration of natural diversity using high-throughput phenotyping techniques has the potential for tremendous synergy with the existing high-throughput technologies that have shaped our understanding of the cellular circuitry of *S. cerevisiae*. The pursuit of an integrated understanding of yeast functional genomics and proteomics (reviewed in (BADER *et al.* 2003)) has been enabled by numerous powerful high-throughput approaches. For example, genetic networks have been elucidated by high-throughput screens for synthetic lethality (TONG *et al.* 2004), protein interaction networks have been mapped using mass spectrometry (GAVIN *et al.* 2002; HO *et al.* 2002), and transcriptional networks have been elucidated by the now ubiquitous technique of transcriptional profiling using microarrays and by the mapping of physical interactions between transcription factors and promoters (LEE *et al.* 2002). The technology for global analysis of growth phenotypes remains largely underdeveloped and unexploited - in spite of the fact that it is phenotype that determines how cellular systems interface with selective pressures. Thus, the broad synthesis of our understanding of genomic, transcriptional,

and phenotypic diversity is contingent upon the development of robust technologies for high-throughput phenotyping.

Here, we have tested one of the first such systems by assaying dipeptide and tripeptide utilization on a previously unfeasible scale. The PM phenotypic assay allows simultaneous testing of ~2000 growth conditions, providing a tremendous savings in time and labor. The technology was typically very robust and provided reliable and reproducible data that were easily manipulated using the accompanying software. However, the PM technology also has several drawbacks that need to be addressed before this technology is broadly suitable for use by the yeast research community. Due to the initial design focus on generalized bacterial phenotyping, many of the available PM assay plates are not optimized for *S. cerevisiae*. Thus, the number of useful growth conditions is considerably less than 2000. This issue is compounded by the high cost of the PM assay plates. Further, different batches of media and PM assay plates provided by the manufacturer often affected reproducibility. Such technical difficulties are not atypical for a new technology, and the power of such tools in exploring the phenotypic diversity of *S. cerevisiae* is clear. Hopefully, the importance of high-throughput phenotyping will lead to reduced costs and enhanced suitability of these technologies.

Uncovering unexpected complexity in di/tri-peptide utilization: Through extensive phenotyping, we found that Ptr2p is but one component of a multifaceted system in *S. cerevisiae* governing the utilization of dipeptides as nitrogen sources. The discovery of these additional components was made possible by the dramatic inter-strain variation observed in the contributions of these activities to dipeptide utilization (summarized in Table 3). We have clearly identified one previously unknown

Table 3. Activities contributing to the utilization of dipeptides as a nitrogen source in each strain background

Activity	YAT7	RM3	RM8	Y55	S288c	W303
Ptr2p	yes[a]	yes	yes	yes	yes	yes[b]
Dal5p	yes	yes	yes	-	yes	yes
X-Asn[c]	-	-	-	-	yes	yes
X-Arg[d]	-	ND	yes	-	ND	-

The contributions of the activities, as indicated by the number of plusses, represent a qualitative summary of the data gathered in this study; -, activity is minimal or absent; ND, not determined due to insufficient data

[a]Ptr2p activity was exceptionally robust in this strain

[b]Ptr2p activity was limited in this strain

[c]Refers to the putative Asp3p activity

[d]Refers to the capacity to utilize dipeptides containing C-terminal arginine residues (particularly Met-Arg) in the absence of Ptr2p and Dal5p activity.

component of this system: the allantoate/ureidosuccinate permease Dal5p, and have defined parameters of two others that suggest mechanistic explanations.

Of these four components, Ptr2p exhibits the broadest dipeptide utilization activity. Relief of *PTR2* repression by deletion of *CUP9* facilitated robust growth on nearly all di/tri-peptides tested. This was even true of W303, which otherwise grew on only a limited subset of the N-end rule dipeptides that supported growth of the other wild-type strains. This restricted utilization of potential Ptr2p substrates provides a window into the complex *PTR2* regulatory system. For example, one can ask why W303 is capable of growing on Trp-Arg, but not Arg-Trp (Figure 3A), even though both are N-end rule ligands composed of identical residues. One possibility is that the N-end rule pathway in W303 is more responsive to Type I ligands (basic N-terminal residues; *e.g.* Arg) than Type II substrates (bulky hydrophobic N-terminal residues; *e.g*. Trp). Alternatively, substrate preference may arise from dipeptidase specificity (HERRMANN *et al.* 1978) resulting in more robust hydrolysis of Trp-Arg upon import. While beyond the scope of this analysis, additional insights and hypotheses can be derived from the wealth of data provided by our high-throughput analysis, and the approach developed here – the application of a diverse panel of strains to the PM assay to analyze di/tri-peptide utilization – can be employed in future studies to enrich our understanding of *PTR2* regulation and Ptr2p function.

Dal5p, a second component of the dipeptide utilization system, was previously identified as a permease for the pyrimidine biosynthetic precursor ureidosuccinate (TUROSCY and COOPER 1987) and the nitrogen source allantoate (CHISHOLM *et al.* 1987) . Expression of *DAL5* is subject to complex regulatory control by the nitrogen catabolite

repression system (CHISHOLM *et al.* 1987; RAI *et al.* 2004; RAI *et al.* 1987). It is therefore fitting that Dal5p also be involved in the utilization of dipeptides as a nitrogen source. With the exception of Tyr-Gln, all Dal5p di/tri-peptide substrates belonged to the non-N-end rule class. Many of the dipeptides supporting growth in RM8-*ptr2*Δ (Figure 3) did not support growth in RM8-*dal5*Δ (Figure 6). Thus, Dal5p-mediated dipeptide transport is not redundant to that of Ptr2p, but rather a complementary activity that enhances the range of dipeptides that can be utilized.

A third component, evident in strains W303 and S288c, governs the utilization of dipeptides containing C-terminal asparagine residues as nitrogen sources (Figure 2). Several lines of evidence support the conclusion that the Asp3p periplasmic asparaginase facilitates the utilization of these substrates. Asp3p activity is closely linked to nitrogen utilization. Nitrogen starvation has been demonstrated to upregulate *ASP3* expression and promote the secretion of asparaginase II (BON *et al.* 1997). Furthermore, the enzymatic activity of asparaginase II hydrolyzes either L- or D-asparagine into ammonia and aspartate, facilitating the use of D-asparagine as a nitrogen source (DUNLOP *et al.* 1978; DUNLOP *et al.* 1976). Of the strains tested, only W303 and the related laboratory strain S288c were able to utilize D-asparaginase in the PM assay (Figure 2E). Genomic analyses utilizing microarrays have noted a lack of detectable *ASP3* in Y55 (LASHKARI *et al.* 1997) and a clinical isolate, YJM789 (WINZELER *et al.* 1999). Neither Y55 nor our clinical isolate, YAT7, were capable of utilizing the putative Asp3p substrates (Figure 2). Most importantly, the asparaginase of the bacterial plant pathogen *Erwinia carotovora* has been shown to deamidate C-terminal, but not N-terminal, asparagine residues of small peptides (HOWARD and CARPENTER 1972). This specificity precisely matches the

dipeptide utilization phenotypes observed for W303 and S288c. Both strains are unable to utilize the two dipeptides with N-terminal asparagine residues present in the PM nitrogen plates (Figure 2B), but are able to utilize all dipeptides containing C-terminal asparagine residues (Figure 2; boxes with red borders). To our knowledge, our study provides the first evidence for such an activity in *S. cerevisiae*, in which mechanisms of extra-cellular peptide processing are rare.

A fourth component of the dipeptide utilization system, evident in strain RM8 but not W303, governs the use of dipeptides containing C-terminal arginine residues, and Met-Arg in particular. Utilization of Met-Arg is clearly enhanced by deletion of *DAL5* (Figure 6) and persists in the *ptr2*Δ*dal5*Δ double mutant (Figure S2), and thus represents another previously unrecognized mechanism of dipeptide utilization. Given the specificity for a nitrogen-rich C-terminal residue, an arginase activity comparable to the asparaginase activity of Asp3p would seem a likely candidate.

Balancing the benefits and potential risks of robust di- and tripeptide import: The striking inter-strain diversity in dipeptide utilization revealed in this study represents a broad phenotypic landscape. Variation in the relative strengths of the activities contributing to dipeptide import can strongly impact the range of dipeptide substrates utilized by the cell. While di/tri-peptides can serve as a valuable source of nutrients, evolutionary pressures opposing indiscriminate import may reinforce mechanisms promoting transport specificity. One such pressure might be the need to avoid import of antifungal agents that exploit the di/tri-peptide transport machinery to gain entry into the cell (reviewed in (ST GEORGIEV 2000)).

Of the di/tri-peptide-utilization activities characterized in this study, the Ptr2p transporter provides the means to transport the broadest spectrum of dipeptide substrates (Figure 3), yet it is also the source of vulnerability to the toxic Ala-Eth dipeptide (Table 2). Thus, strains that exhibit robust *PTR2* expression, such as the clinical isolate YAT7, efficiently utilize a broad spectrum of dipeptides as a nitrogen source (Figure 2), but are also vulnerable to Ala-Eth toxicity (Table 2). In contrast, the limited *PTR2* expression in strain W303 provides protection from Ala-Eth at the cost of a more limited capacity to utilize di/tri-peptides. However, in certain growth conditions a fraction of W303 cells exhibit robust *PTR2* expression, suggesting a possible epigenetic mechanism for employing both high- and low-risk di/tri-peptide utilization strategies in an isogenic population.

The optimal strategy for dipeptide utilization will vary between environments. For instance, in the presence of toxic peptidomimetics that resemble Dal5p substrates, strain Y55 would have a significant fitness advantage over the other strains tested in this study, as it exhibits no detectable Dal5p activity (Figure S2). In environments with a strong risk of exposure to a variety of toxic di/tri-peptides, the risks of Ptr2p- and Dal5p-mediated import may outweigh the potential nutritional benefits. In such cases, the utilization of a limited subset of di/tri-peptides may represent a low-risk mechanism by which some nutritional benefit can be extracted from the available di/tri-peptides. For example, extracellular release of ammonia from C-terminal asparagine residues by Asp3p would serve as a more limited, but risk-free, means of obtaining nitrogen from dipeptides. In contrast, in an environment in which nitrogen sources are scarce and the risk of importing toxic species is minimal, robust expression of *PTR2* would be a beneficial trait.

We note that the clinical isolates YAT7 (Figure 2), YAT17, and YAT21 (data not shown) exhibit the most robust dipeptide utilization of the strains studied, perhaps reflecting the environmental pressures of the strains' atypical growth environment in the human host. Further investigation into the ecology, population structure and population genetics of *S. cerevisiae* would benefit future attempts to link phenotypic diversity to likely environmental pressures.

High-throughput phenotyping and genetic diversity can provide new insights into the genetic determinants governing adaptation to the nutritional and chemical challenges of diverse environments. Our study represents a first case example of how this approach can be utilized to elucidate metabolic processes and provide a broader evolutionary context within which the observed natural diversity can be considered.

Acknowledgements

We thank John McCusker, Barbara Garvik, Gerald Fink, and Rodney Rothstein for providing the strains utilized in this study. We are also grateful to Gerald Fink for the use of the C. Connelly and P. Hieter library and Fred Naider for the gift of Ala-Eth. We also thank Jessica Brown, Leah Cowen, Johannes Manjrekar, and Todd Sanger for their critical reading of the manuscript. This work was supported by the Harold and Leila Y. Mathers Charitable Foundation.

REFERENCES

ALAGRAMAM, K., F. NAIDER and J. M. BECKER, 1995 A recognition component of the ubiquitin system is required for peptide transport in Saccharomyces cerevisiae. Mol Microbiol **15:** 225-234.

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, S. J. G. *et al.* (Editors), 1988 *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

BADER, G. D., A. HEILBUT, B. ANDREWS, M. TYERS, T. HUGHES *et al.*, 2003 Functional genomics and proteomics: charting a multidimensional map of the yeast cell. Trends Cell Biol **13:** 344-356.

BAUDIN, A., O. OZIER-KALOGEROPOULOS, A. DENOUEL, F. LACROUTE and C. CULLIN, 1993 A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. Nucleic Acids Res **21:** 3329-3330.

BECKER, J. M., and F. NAIDER, 1995 *Fungal Peptide Transport as a Delivery System*. American Chemical Society, Washington DC.

BOCHNER, B. R., 1989 'Breathprints' at the microbial level. ASM News **55:** 536-539.

BOCHNER, B. R., 2003 New technologies to assess genotype-phenotype relationships. Nat Rev Genet **4:** 309-314.

BOCHNER, B. R., P. GADZINSKI and E. PANOMITROS, 2001 Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Genome Res **11:** 1246-1255.

BON, E. P., E. CARVAJAL, M. STANBROUGH, D. ROWEN and B. MAGASANIK, 1997 Asparaginase II of Saccharomyces cerevisiae. GLN3/URE2 regulation of a periplasmic enzyme. Appl Biochem Biotechnol **63-65:** 203-212.

BREM, R. B., and L. KRUGLYAK, 2005 The landscape of genetic complexity across 5,700 gene expression traits in yeast. Proc Natl Acad Sci U S A **102:** 1572-1577.

BREM, R. B., G. YVERT, R. CLINTON and L. KRUGLYAK, 2002 Genetic dissection of transcriptional regulation in budding yeast. Science **296:** 752-755.

BYRD, C., G. C. TURNER and A. VARSHAVSKY, 1998 The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. Embo J **17:** 269-277.

CAVALIERI, D., J. P. TOWNSEND and D. L. HARTL, 2000 Manifold anomalies in gene expression in a vineyard isolate of Saccharomyces cerevisiae revealed by DNA microarray analysis. Proc Natl Acad Sci U S A **97:** 12369-12374.

CHIANG, C. S., G. STACEY and Y. F. TSAY, 2004 Mechanisms and functional properties of two peptide transporters, AtPTR2 and fPTR2. J Biol Chem **279:** 30150-30157.

CHISHOLM, V. T., H. Z. LEA, R. RAI and T. G. COOPER, 1987 Regulation of allantoate transport in wild-type and mutant strains of Saccharomyces cerevisiae. J Bacteriol **169:** 1684-1690.

CHRISTIANSON, T. W., R. S. SIKORSKI, M. DANTE, J. H. SHERO and P. HIETER, 1992 Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119-122.

CONNELLY, C., and P. HIETER, 1996 Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. Cell **86:** 275-285.

COOPER, T. G., 1996 Regulation of allantoin catabolism in Saccharomyces cerevisiae, pp. 139-169 in *The mycota: biochemistry and molecular biology*, edited by G. A. MARZLUF. Springer-Verlag KG, Berlin.

DE BOER, M., J. P. BEBELMAN, P. M. GONCALVES, J. MAAT, H. VAN HEERIKHUIZEN *et al.*, 1998 Regulation of expression of the amino acid transporter gene BAP3 in Saccharomyces cerevisiae. Mol Microbiol **30:** 603-613.

DE DEKEN, R. H., 1962 Pathway of arginine biosynthesis in yeast. Biochem Biophys Res Commun **8:** 462-466.

DU, F., F. NAVARRO-GARCIA, Z. XIA, T. TASAKI and A. VARSHAVSKY, 2002 Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. Proc Natl Acad Sci U S A **99:** 14110-14115.

DUNLOP, P. C., G. M. MEYER, D. BAN and R. J. ROON, 1978 Characterization of two forms of asparaginase in Saccharomyces cerevisiae. J Biol Chem **253:** 1297-1304.

DUNLOP, P. C., R. J. ROON and H. L. EVEN, 1976 Utilization of D-asparagine by Saccharomyces cerevisiae. J Bacteriol **125:** 999-1004.

FAY, J. C., H. L. MCCULLOUGH, P. D. SNIEGOWSKI and M. B. EISEN, 2004 Population genetic variation in gene expression is associated with phenotypic variation in Saccharomyces cerevisiae. Genome Biol **5:** R26.

GAVIN, A. C., M. BOSCHE, R. KRAUSE, P. GRANDI, M. MARZIOCH *et al.*, 2002 Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:** 141-147.

GOLDSTEIN, A. L., and J. H. MCCUSKER, 1999 Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast **15:** 1541-1553.

GOODELL, E. W., and C. F. HIGGINS, 1987 Uptake of cell wall peptides by Salmonella typhimurium and Escherichia coli. J Bacteriol **169:** 3861-3865.

GRAUL, R. C., and W. SADEE, 1997 Sequence alignments of the H(+)-dependent oligopeptide transporter family PTR: inferences on structure and function of the intestinal PET1 transporter. Pharm Res **14:** 388-400.

GREIG, D., M. TRAVISANO, E. J. LOUIS and R. H. BORTS, 2003 A role for the mismatch repair system during incipient speciation in Saccharomyces. J Evol Biol **16:** 429-437.

GRETH, M. L., M. R. CHEVALLIER and F. LACROUTE, 1977 Ureidosuccinic acid permeation in Saccharomyces cerevisiae. Biochim Biophys Acta **465:** 138-151.

GULDENER, U., S. HECK, T. FIELDER, J. BEINHAUER and J. H. HEGEMANN, 1996 A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res **24:** 2519-2524.

HAUSER, M., S. KAUFFMAN, F. NAIDER and J. M. BECKER, 2005 Substrate preference is altered by mutations in the fifth transmembrane domain of Ptr2p, the di/tri-peptide transporter of Saccharomyces cerevisiae. Mol Membr Biol **22:** 215-227.

HAUSER, M., V. NARITA, A. M. DONHARDT, F. NAIDER and J. M. BECKER, 2001 Multiplicity and regulation of genes encoding peptide transporters in Saccharomyces cerevisiae. Mol Membr Biol **18:** 105-112.

HERRERA-RUIZ, D., and G. T. KNIPP, 2003 Current perspectives on established and putative mammalian oligopeptide transporters. J Pharm Sci **92:** 691-714.

HERRMANN, V., I. KNACK and K. H. ROHM, 1978 Yeast dipeptidase: active site mapping by kinetic studies with substrates and substrate analogs. Hoppe Seylers Z Physiol Chem **359:** 47-61.

HO, Y., A. GRUHLER, A. HEILBUT, G. D. BADER, L. MOORE *et al.*, 2002 Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature **415:** 180-183.

HOWARD, J. B., and F. H. CARPENTER, 1972 L-asparaginase from Erwinia carotovora. Substrate specificity and enzymatic properties. J Biol Chem **247:** 1020-1030.

ISLAND, M. D., F. NAIDER and J. M. BECKER, 1987 Regulation of dipeptide transport in Saccharomyces cerevisiae by micromolar amino acid concentrations. J Bacteriol **169:** 2132-2136.

ITO, H., Y. FUKUDA, K. MURATA and A. KIMURA, 1983 Transformation of intact yeast cells treated with alkali cations. J Bacteriol **153:** 163-168.

LARUE, T. A., and J. F. SPENCER, 1967 The utilization of D-amino acids by yeasts. Can J Microbiol **13:** 777-788.

LASHKARI, D. A., J. L. DERISI, J. H. MCCUSKER, A. F. NAMATH, C. GENTILE *et al.*, 1997 Yeast microarrays for genome wide parallel genetic and gene expression analysis. Proc Natl Acad Sci U S A **94:** 13057-13062.

LEE, T. I., N. J. RINALDI, F. ROBERT, D. T. ODOM, Z. BAR-JOSEPH *et al.*, 2002 Transcriptional regulatory networks in Saccharomyces cerevisiae. Science **298:** 799-804.

MACKENZIE, B., D. D. LOO, Y. FEI, W. J. LIU, V. GANAPATHY *et al.*, 1996 Mechanisms of the human intestinal H+-coupled oligopeptide transporter hPEPT1. J Biol Chem **271:** 5430-5437.

MANSON, M. D., V. BLANK, G. BRADE and C. F. HIGGINS, 1986 Peptide chemotaxis in E. coli involves the Tap signal transducer and the dipeptide permease. Nature **321:** 253-256.

MATHIOPOULOS, C., J. P. MUELLER, F. J. SLACK, C. G. MURPHY, S. PATANKAR *et al.*, 1991 A Bacillus subtilis dipeptide transport system expressed early during sporulation. Mol Microbiol **5:** 1903-1913.

MORTIMER, R. K., P. ROMANO, G. SUZZI and M. POLSINELLI, 1994 Genome renewal: a new phenomenon revealed from a genetic study of 43 strains of Saccharomyces cerevisiae derived from natural fermentation of grape musts. Yeast **10:** 1543-1552.

NIELSEN, C. U., and B. BRODIN, 2003 Di/tri-peptide transporters as drug delivery targets: regulation of transport under physiological and patho-physiological conditions. Curr Drug Targets **4:** 373-388.

PAYNE, J. W., and M. W. SMITH, 1994 Peptide transport by micro-organisms. Adv Microb Physiol **36:** 1-80.

PEREGO, M., C. F. HIGGINS, S. R. PEARCE, M. P. GALLAGHER and J. A. HOCH, 1991 The oligopeptide transport system of Bacillus subtilis plays a role in the initiation of sporulation. Mol Microbiol **5:** 173-185.

RAI, R., J. R. DAUGHERTY, J. J. TATE, T. D. BUFORD and T. G. COOPER, 2004 Synergistic operation of four cis-acting elements mediate high level DAL5 transcription in Saccharomyces cerevisiae. FEMS Yeast Res **5:** 29-41.

RAI, R., F. GENBAUFFE, H. Z. LEA and T. G. COOPER, 1987 Transcriptional regulation of the DAL5 gene in Saccharomyces cerevisiae. J Bacteriol **169:** 3521-3524.

RINGROSE, P. S., 1980 Peptides as Antimicrobial Agents, pp. 641-685 in *Microorganisms and Nitrogen Sources*, edited by J. W. PAYNE. John Wiley & Sons Ltd.

ROSE, M. D., F. WINSTON and P. HIETER (Editors), 1990 *Methods in Yeast Genetics: a Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

SALDANHA, A. J., 2004 Java Treeview--extensible visualization of microarray data. Bioinformatics **20:** 3246-3248.

SIKORSKI, R. S., and P. HIETER, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics **122:** 19-27.

ST GEORGIEV, V., 2000 Membrane transporters and antifungal drug resistance. Curr Drug Targets **1:** 261-284.

STACEY, G., S. KOH, C. GRANGER and J. M. BECKER, 2002 Peptide transport in plants. Trends Plant Sci **7:** 257-263.

STADE, K., C. S. FORD, C. GUTHRIE and K. WEIS, 1997 Exportin 1 (Crm1p) is an essential nuclear export factor. Cell **90:** 1041-1050.

STEINER, H. Y., F. NAIDER and J. M. BECKER, 1995 The PTR family: a new group of peptide transporters. Mol Microbiol **16:** 825-834.

STEINMETZ, L. M., H. SINHA, D. R. RICHARDS, J. I. SPIEGELMAN, P. J. OEFNER *et al.*, 2002 Dissecting the architecture of a quantitative trait locus in yeast. Nature **416:** 326-330.

TANZER, M. M., H. N. ARST, A. R. SKALCHUNES, M. COFFIN, B. A. DARVEAUX *et al.*, 2003 Global nutritional profiling for mutant and chemical mode-of-action analysis in filamentous fungi. Funct Integr Genomics **3:** 160-170.

TONG, A. H., G. LESAGE, G. D. BADER, H. DING, H. XU *et al.*, 2004 Global mapping of the yeast genetic interaction network. Science **303:** 808-813.

TOWNSEND, J. P., D. CAVALIERI and D. L. HARTL, 2003 Population genetic variation in genome-wide gene expression. Mol Biol Evol **20:** 955-963.

TRUE, H. L., and S. L. LINDQUIST, 2000 A yeast prion provides a mechanism for genetic variation and phenotypic diversity. Nature **407:** 477-483.

TURNER, G. C., F. DU and A. VARSHAVSKY, 2000 Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. Nature **405:** 579-583.

TUROSCY, V., and T. G. COOPER, 1987 Ureidosuccinate is transported by the allantoate transport system in Saccharomyces cerevisiae. J Bacteriol **169:** 2598-2600.

VARSHAVSKY, A., 1996 The N-end rule: functions, mysteries, uses. Proc Natl Acad Sci U S A **93:** 12142-12149.

VARSHAVSKY, A., 1997 The N-end rule pathway of protein degradation. Genes Cells **2:** 13-28.

WACH, A., A. BRACHAT, C. ALBERTI-SEGUI, C. REBISCHUNG and P. PHILIPPSEN, 1997 Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast **13:** 1065-1075.

WACH, A., A. BRACHAT, R. POHLMANN and P. PHILIPPSEN, 1994 New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast **10:** 1793-1808.

WINZELER, E. A., B. LEE, J. H. MCCUSKER and R. W. DAVIS, 1999 Whole genome genetic-typing in yeast using high-density oligonucleotide arrays. Parasitology **118 Suppl:** S73-80.

WINZELER, E. A., D. R. RICHARDS, A. R. CONWAY, A. L. GOLDSTEIN, S. KALMAN *et al.*, 1998 Direct allelic variation scanning of the yeast genome. Science **281:** 1194-1197.

YVERT, G., R. B. BREM, J. WHITTLE, J. M. AKEY, E. FOSS *et al.*, 2003 Trans-acting regulatory variation in Saccharomyces cerevisiae and the role of transcription factors. Nat Genet **35:** 57-64.

APPENDIX

Supplement Table S1 Oligos used in this study

Oligo	Sequence	Purpose
O-007	CAATTCAACGCGTCTGTGAGG	Verify SFH deletions
O-008	GATTCTTGTTTTCAAGAACTTG	Verify SFH deletions
O-138	TATACGCATATGTGGTGTTG	Verify *URA3* deletion
O-139	CGACCGAGATTCCCGGGTAA	Verify *URA3* deletion
O-203	ATGGTTTGTGAACAGATTGA	Verify *HSC82* deletion
O-204	TTGAATGGACGCCGAATTGC	Verify *HSC82* deletion
O-206	GTATCGATAAGCTTGATATC	Sequencing genomic library plasmid insert
O-223	ATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGT TTACCAATGCTTAATCAGTGAG	Amplify *URA3* SFH deletion cassette
O-224	TTAGTTTTGCTGGCCGCATCTTCTCAAATATGCTTCCCAGCCTGC GCAACAACGTTGCGCAAACTAT	Amplify *URA3* SFH deletion cassette
O-248	TAATACGACTCACTATAGGG	Sequencing genomic library plasmid insert
O-252	GTGGCATGTGAACTGCCTACCGTAAGTGACATGAACACATGCATT CAGCTGAAGCTTCGTACGCTGC	Amplify lox-flanked *HSC82* SFH deletion cassette
O-253	GGCGAAAAAAGAGATTAATCAACTTCTTCCATCTCGGTGTCAGCT GCATAGGCCACTAGTGGATCTG	Amplify lox-flanked *HSC82* SFH deletion cassette
O-278	CGCAAGTCCTGTTTCTATGC	Verify KanMX excision from *HO*
O-279	CTACGTTGCCTCCATCG	Verify KanMX excision from *HO*
O-286	CAGGACGAAAAGCAAGGCGACTTCCCGGTCATCGAAGAGGGCCTC GTCCCCGCCGGGTCA	Amplify *PTR2* SFH deletion cassette
O-287	ACTTTCCATTGGTTCTAATATTTCAATATCGTTAGCTTTACACTG GATGGCGGCGTTAGT	Amplify *PTR2* SFH deletion cassette
O-288	GGGCTAACGGCGTCCGCAGA	Verify *PTR2* deletion

Supplement Table S1 Continued

Oligo	Sequence	Purpose
O-289	AAGAGAAAGTGTGGTCACAC	Verify *PTR2* deletion
O-290	ATGAATTATAACTGCGAAATACAAAACAGGAACAGTAAGAGCCTC GTCCCCGCCGGGTCA	Amplify *CUP9* SFH deletion cassette
O-291	ATTCATATCAGGGTTGGATAGCTTTTTCAATTCTTCCAGCCACTG GATGGCGGCGTTAGT	Amplify *CUP9* SFH deletion cassette
O-292	GCGTGCTTCCTCACACTGGC	Verify *CUP9* deletion
O-293	GAAAGAGAAGATGATAACTA	Verify *CUP9* deletion
O-314	CGAATTTGATCTCAATCCAATTTCCGCACC	SFH integration of FLAG-2xGFP-6xHIS/kanMX4 at C-terminus of *PTR2*
O-315	AATATGGACCTCTCTTCACAAATTGTTCTATAATACAATATATAT TCTGGGCAGATGATGTCGAG	SFH integration of FLAG-2xGFP-6xHIS/kanMX4 at C-terminus of *PTR2*
O-316	ATTGATCTTTGCTGGTAAGC	Verify FLAG-2xGFP-6xHIS/KanR integration
O-321	ATCTGAGCCTTGGCTGGGAT	*PTR2* promoter sequencing (+30 to +11)
O-322	CCGTCCTTTTCACTTCACGT	*PTR2* promoter sequencing (-466 to -447)
O-323	CCGTACAGATAAGAAACCCA	*PTR2* promoter sequencing (-368 to -387)
O-324	GATAAGCTTGGGGTAACGCA	*PTR2* promoter sequencing (-897 to -878)
PTR2-FLAG-GFP-F	GATCCACCACCAAATATGACTACAAGGACGACGATGACAAGCTTG ATATCAACAAGACAGGG	Amplify GFP for insertion into pMS2
PTR2-FLAG-GFP-R	GATGATGATGACTGCTGCCGCTGCCGCGCGGCACACCGGT CCTCGAGCCGCTTTTCTTGTAC	Amplify GFP for insertion into pMS2

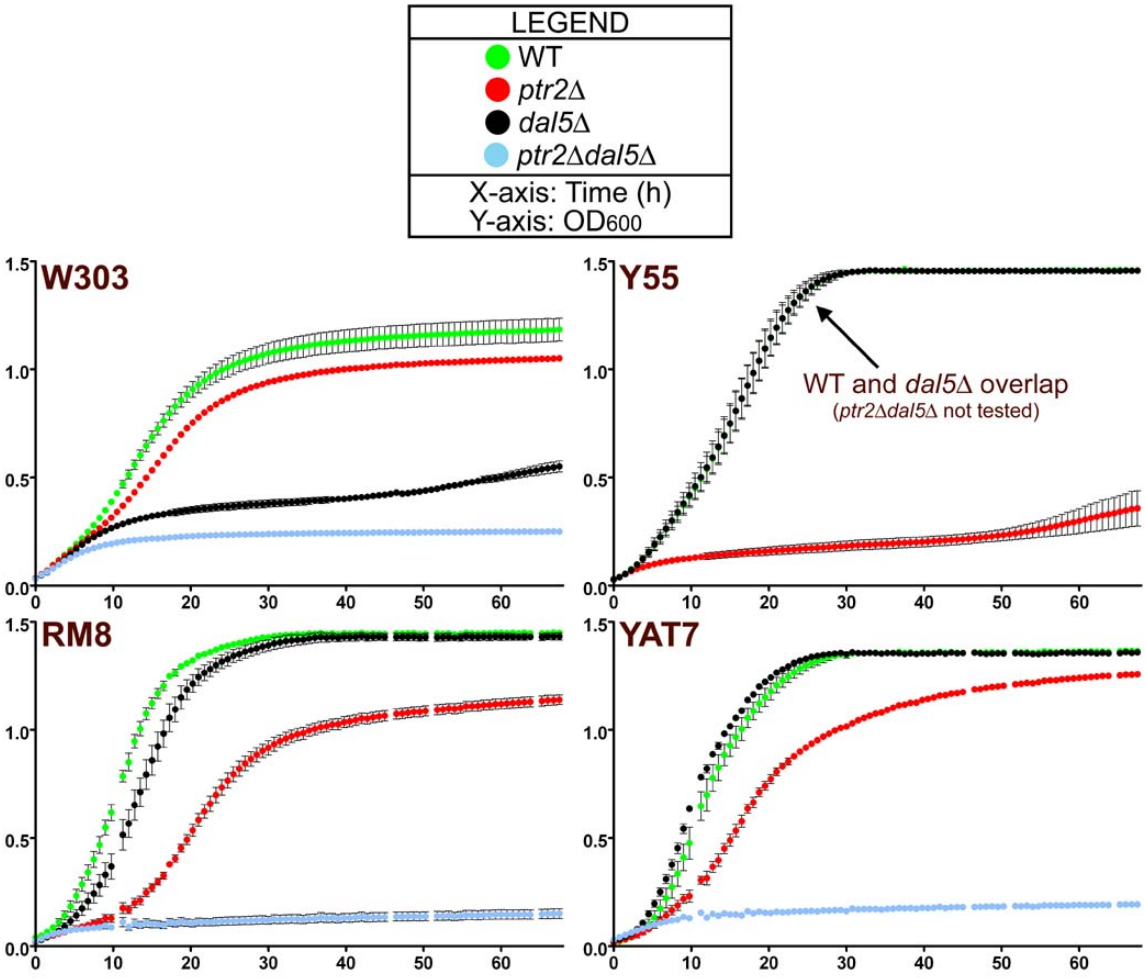
Supplement Table S2 Polymorphisms in the region upstream of *PTR2*, relative to the standard S288c sequence

Strain	C-799T	C-582T	A-555G	A-496G	T-458G	G-385C	T-297C	T-152C	ins-46T	del-32CTT
S288c	--	--	--	--	--	--	--	--	--	--
W303	+	--	--	--	--	--	--	--	--	--
RM8	+	+	+	+	--	+	+	+	--	--
YAT7	--	--	--	+	+	--	--	--	+[a]	+[b]

A region encompassing the bases spanning from positions -899 to +3 relative to the *PTR2* stop codon was sequenced in the indicated strains.

[a] "insT-46" refers to the expansion of a string of 11 "T"'s spanning from -46 to -36 by an additional "T".

[b] "del-32CTT" refers to a deletion of the bases "CTT" spanning from -32 to -30 of the published S288c sequence.



Supplement Figure S1 Dal5p and Ptr2p exhibit strain-dependent contributions to the utilization of Ala-Leu as a nitrogen source. Wild-type, *dal5*Δ, *ptr2*Δ and *ptr2*Δ*dal5*Δ versions of the indicated strains were grown at 30°C in liquid MM media containing 2 mM Ala-Leu as the sole nitrogen source. A few timepoints were omitted because of technical difficulties with the Bioscreen C growth curve machine.

Supplement Figure S2 Additional PM signal profiles reveal the varied contribution of Dal5p to di/tri-peptide import in strains RM8, W303, and Y55. Refer to Figure 2 for details on data analysis and presentation. The column label "*ptr2*Δ (vector)" refers to strain W303-*ptr2*Δ transformed with the high-copy (2μ) vector pRS426. The column label "*ptr2*Δ (*DAL5*)" refers to strain W303-*ptr2*Δ transformed with plasmid pRS426-*DAL5*. Note that the PM assays presented in this figure utilized a more recent manufacturing lot of the PM nitrogen plates than those presented in the other figures. Different lots can produce subtle differences in signal magnitude. The wild-type RM8 and Y55 PM data presented here were derived from the same lot to facilitate direct comparison.

Figure S2A

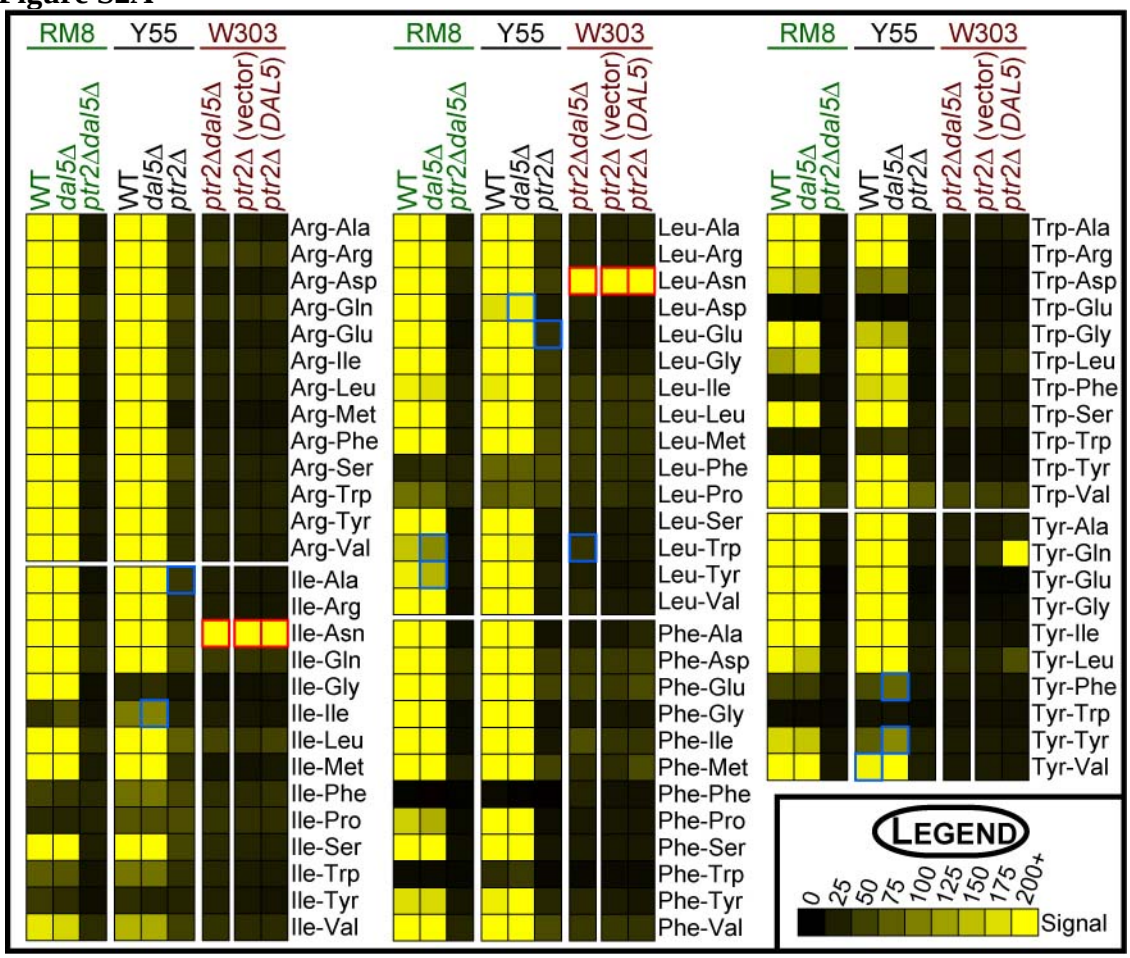
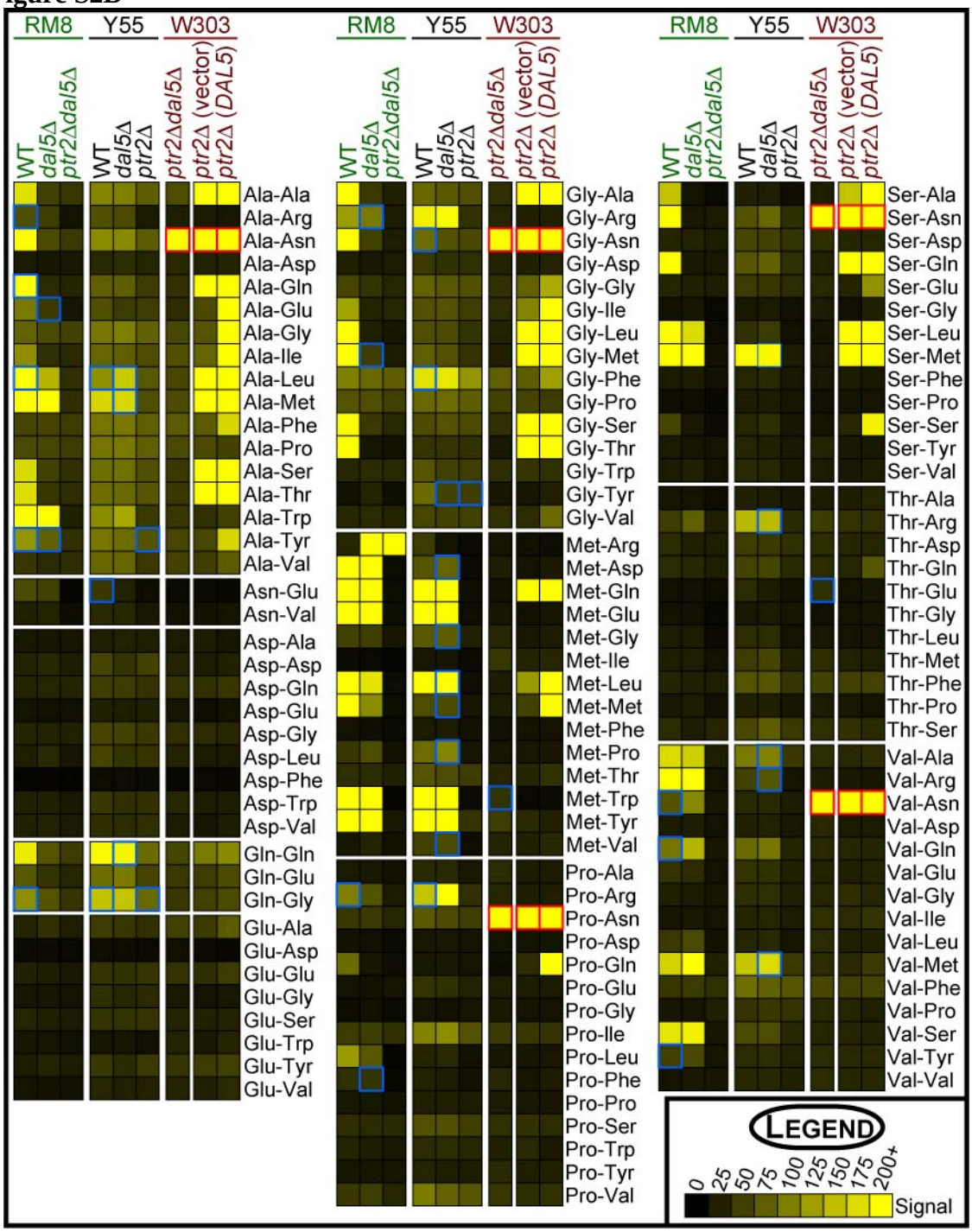
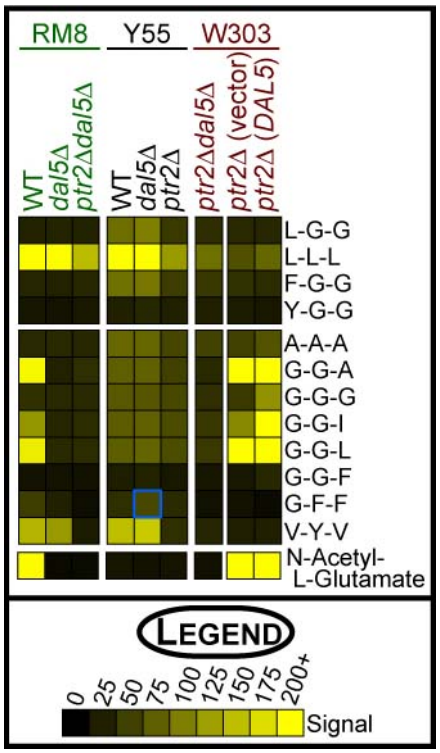

RM8
Y55
W303
WT
dal5Δ
ptr2Δdal5Δ
WT
dal5Δ
ptr2Δ
ptr2Δdal5Δ
ptr2Δ (vector)
ptr2Δ (DAL5)
Arg-Ala
Arg-Arg
Arg-Asp
Arg-Gln
Arg-Glu
Arg-Ile
Arg-Leu
Arg-Met
Arg-Phe
Arg-Ser
Arg-Trp
Arg-Tyr
Arg-Val
Ile-Ala
Ile-Arg
Ile-Asn
Ile-Gln
Ile-Gly
Ile-Ile
Ile-Leu
Ile-Met
Ile-Phe
Ile-Pro
Ile-Ser
Ile-Trp
Ile-Tyr
Ile-Val
Leu-Ala
Leu-Arg
Leu-Asn
Leu-Asp
Leu-Glu
Leu-Gly
Leu-Ile
Leu-Leu
Leu-Met
Leu-Phe
Leu-Pro
Leu-Ser
Leu-Trp
Leu-Tyr
Leu-Val
Phe-Ala
Phe-Asp
Phe-Glu
Phe-Gly
Phe-Ile
Phe-Met
Phe-Phe
Phe-Pro
Phe-Ser
Phe-Trp
Phe-Tyr
Phe-Val
Trp-Ala
Trp-Arg
Trp-Asp
Trp-Glu
Trp-Gly
Trp-Leu
Trp-Phe
Trp-Ser
Trp-Trp
Trp-Tyr
Trp-Val
Tyr-Ala
Tyr-Gln
Tyr-Glu
Tyr-Gly
Tyr-Ile
Tyr-Leu
Tyr-Phe
Tyr-Trp
Tyr-Tyr
Tyr-Val
LEGEND
0
25
50
75
100
125
150
175
200+
Signal

Figure S2B



Supplement Figure S2 Continued

Figure S2C



Supplement Figure S2 Continued

PART IV

SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

Di/tri-peptides are important to organisms as amino acids, nitrogen, and carbon sources, and Ptr2p as the major di/tri-peptide transporter is one of the proteins required for the utilization of di/tri-peptides. In order to identify genes affecting di/tri-peptide utilization, I have performed a systematic, functional examination of dipeptide utilization in a haploid, non-essential, single-gene deletion mutant library in *Saccharomyces cerevisiae*. I have identified 103 candidate genes: 57 genes whose deletion decreased dipeptide utilization and 46 genes whose deletion enhanced dipeptide utilization. The 103 genes were distributed among most of the Gene Ontology functional categories indicating a very wide regulatory network involved in the utilization of dipeptides in yeast. In addition, to exploit the various strategies of importing and utilizing di/tri-peptides as nitrogen sources, we used the Biolog system of "Phenotype MicroArrays™" (PM) (BOCHNER 2003; BOCHNER *et al.* 2001) which represents 284 of the 400 possible dipeptide and 11 tripeptide permutations to investigate the difference of growth phenotype in these arrays for different strains of *S. cerevisiae*. Multiple gene products contributing to di/tri-peptide utilization have been revealed ranging from the initial processes of importing di/tri-peptides to the metabolic pathways of di/tri-peptide degradation. Taken together, the identified genes are involved in different biological pathways contributing to both direct and indirect regulation of di/tri-peptide utilization.

Ptr2p is the key contributor for di/tri-peptide utilization:

Ptr2p is the only known gene of the PTR family involved in di/tri-peptide uptake at the plasma membrane in yeast. After analyzing the preference for any of 284

dipeptides and 11 tripeptides as a sole nitrogen source, we demonstrated that Ptr2p is essential for many, but all yeast strains, for the utilization of most di/tri-peptides and exhibits the broad substrate recognition. For a limited number of peptides and for three out of the seven strains tested, another transporter (Dal5p) was able to substitute for Ptr2p's role as peptide transporter. Relief of *PTR2* repression by the deletion of *CUP9* facilitated robust growth on nearly all di/tri-peptides tested. In contrast, the deletion of *PTR2* resulted in the almost complete abrogation of growth on N-end rule dipeptides, which contain bulky or basic amino acid in the N-terminus, suggesting Ptr2p contributes solely to the utilization of N-end rule dipeptides. For non-N-end rule di/tri-peptides, both Ptr2p and Dal5p played roles in peptide utilization.

Several gene products contribute to di/tri-peptide utilization by regulating *PTR2* expression:

I have identified genes affecting di/tri-peptide utilization by the regulation of *PTR2* expression or *PTR2* mRNA stability, which considerably expands our understanding of the regulation of *PTR2* expression. First, I have identified gene products negatively regulating *PTR2* transcription via a modification of histones. For example, deletion of Arp5p, Arp8p, Ies6p, or Taf14p, the components of the chromatin remodeling INO80 protein complex, or deletion of Eaf3p, a component of the NuA4 histone acetyltransferase complex, results in an increase in *PTR2* transcription with a subsequent increase of dipeptide utilization. The INO80 protein complex preferentially interacts with histones H3 and H4 and is involved in the modification of the nucleosome, while Eaf3p is involved in maintaining the acetylation of histone H3 and H4 (REID *et al.* 2004; SANDERS

et al. 2002). Second, I have confirmed or identified four gene products with transcription factor activity (Cup9p, Dal81p, Stp2p, and Rpn4p) that regulate *PTR2* expression. Cup9p is the known repressor of *PTR2* transcription, and binds to the region between -488 and -897 upstream of the *PTR2* start codon (BYRD *et al.* 1998). Deletion of this gene product leads to an increase of di/tri-peptide utilization. Dal81p, Stp2p, and Rpn4p are positive regulators of *PTR2* transcription, and deletion of these gene products results in a decrease of di/tri-peptide utilization. Dal81p is a transcriptional activator essential for *PTR2* expression induced by the SPS signal pathway (BERNARD and ANDRE 2001). Stp2p is proteolytically processed by truncation of the N-teminus and is translocated into the nucleus in response to amino acid induction via the SPS system (ANDREASSON and LJUNGDAHL 2002; ANDREASSON and LJUNGDAHL 2004). Dal81p and Stp2p might act synergistically in *PTR2* transcription since similar synergistic regulation between Dal81p and Stp1p is also proposed in *AGP1* regulation in response to amino acid induction (ABDEL-SATER *et al.* 2004). Rpn4p may bind between -610 and -603 due to the existence of the Rpn4p binding consensus sequence (5'-GGTGGAAA-3') present at this site (MANNHAUPT *et al.* 1999). The regulation of *PTR2* by Rpn4p was identified in my studies.

I have also identified or corroborated that several gene products proposed to regulate transcriptional activators are involved in dipeptide utilization. For example, Ubr2p, Ptr1p, and Ubc2p modulate the stability of Rpn4p and Cup9p, and deletion of these regulators causes an alteration of di/tri-peptide utilization. Ubr2p has been shown to be involved in the degradation of Rpn4p; Ptr1p and Ubc2p are involved in Cup9p

degradation through the ubiquitination pathway (DU *et al.* 2002; WANG *et al.* 2004). Since Ubr2p has a high amino acid sequence similarity to Ptr1p, this would presumably allow competition with Ptr1p for the stabilization of Cup9p (BARTEL *et al.* 1990). Therefore, Ubr2p likely regulates *PTR2* transcription by influencing the stability of both Rpn4p and Cup9p. The stability of Cup9p is also dependent on N-end rule dipeptides with N-terminal residues containing basic (Type 1: Arg, His, or Lys) and bulky (Type 2: Ile, Leu, Phe, Trp, or Tyr) amino acids. Dipeptides serve as ligands to bind to Ptr1p substrate binding sites, and binding these peptides cause a release of the Ubr1p autoinhibitory domain and allosteric activation of Ubr1p, which leads to the degradation of Cup9p. Relief of Cup9p repression of *PTR2* results in enhanced *PTR2* expression and ultimately di/tri-peptide uptake. This initiates a positive regulatory feedback loop for di/tri-peptide utilization (BYRD *et al.* 1998; DU *et al.* 2002; TURNER *et al.* 2000).

Third, I have identified the components of an RNA polymerase mediator (Ssn3p/Srb10p, Ssn8p/Srb11p, Ssn2p/Srb9p, and Srb8p) that are involved in *PTR2* transcription, thus regulating dipeptide utilization. This protein complex belongs to a cyclin-dependent serine/threonine protein kinase (KORNBERG 2005). The involvement of this protein complex is not specific for *PTR2* transcription since this protein complex also affects the expression of *PMA1* encoding a proton transporter. The deletion of *SSN3* results in a change of both *PMA1* and *PTR2* expression.

Fourth, I have identified genes involved in the stability of *PTR2* mRNA which affects dipeptide utilization. Both Kem1p and Pat1p belong to an mRNA decay protein

complex (BONNEROT *et al.* 2000; BOUVERET *et al.* 2000), and the amount of *PTR2* mRNA is considerably higher in the *kem1* strain than in wild type. It is likely that *PTR2* mRNA is more stable in *kem1* and *pat1* strains, which results in more Ptr2p being synthesized leading to increased dipeptide utilization. However a deletion of *KEM1* could affect expression of many different genes, resulting in a non-specific affect on dipeptide utilization. Another gene product, Thp1p, is involved in mRNA export from the nucleus to the cytoplasm (FISCHER *et al.* 2002), and deletion of this gene might interrupt *PTR2* mRNA translocation.

Several gene products contribute to di/tri-peptide utilization by regulating Ptr2p trafficking

I have identified genes affecting di/tri-peptide utilization by regulation of the Ptr2p trafficking system. Interruption of Ptr2p targeting to the plasma membrane leads to an alteration of di/tri-peptide uptake. I have shown that ESCRT-I (endosomal sorting complex required for transport), ESCRT-II, and ESCRT-III protein complexes are involved in Ptr2p trafficking since deletion of any component of these protein complexes caused a defect in Ptr2p-GFP trafficking from the endosome to the vacuole. For example, deletion strains *srn2*, *vps36*, *snf7, eaf7* and *vps8* demonstrated a defect in Ptr2p-GFP localization and increased dipeptide utilization. Since ubiquitination of membrane proteins is essential for sorting by the ESCRT protein system, I infer that Ptr2p ubiquitination is required for its turnover. Ubiquitinated Ptr2p has been identified by a mass spectrometer analysis supporting this hypothesis (HITCHCOCK *et al.* 2003). In addition to the ESCRT protein complexes, several gene products, such as *NPR1, LST4,*

BUD32, and *REG1* showed an accumulation of Ptr2p-GFP in vesicles. They are also likely involved in the Ptr2p trafficking system. Strains *npr1* and *lst4* showed an increase of dipeptide utilization while *bud32* and *reg1* showed a decrease of dipeptide utilization.

Several gene products contribute to di/tri-peptide utilization via Ptr2p-independent manner

I have identified an important gene product involved in di/tri-peptide utilization independent of Ptr2p in yeast. Dal5p is a plasma membrane protein, which does not belong to PTR system but imports di/tri-peptides from the extracellular environment. Dal5p was previously identified as a permease for the pyrimidine biosynthetic precursor ureidosuccinate (TUROSCY and COOPER 1987) and the nitrogen source allantoate (CHISHOLM *et al.* 1987) . Expression of *DAL5* is subject to complex regulatory control by the nitrogen catabolite repression system (CHISHOLM *et al.* 1987; RAI *et al.* 2004; RAI *et al.* 1987). With the exception of Tyr-Gln, all Dal5p di/tri-peptide substrates belong to the non-N-end rule class. For example, many of the dipeptides supporting growth in the RM8-*ptr2*Δ strain did not support growth in RM8-*dal5*Δ. Since Ptr2p can transport the majority of di/tri-peptides, the spectrum of Ptr2p substrates overlaps with that of Dal5p but the substrate specificity is strain dependent. In the RM8 strain, Dal5p-mediated dipeptide transport is not redundant to that of Ptr2p, but rather a complementary activity that enhances the range of dipeptides that can be utilized.

Ptr2p-independent di/tri-peptide utilization is also governed by the activity of Asp3p, a periplasmic asparaginase, which facilitates the utilization of dipeptides

containing C-terminal asparagine residues as nitrogen sources. For example, W303-*ptr2*Δ *dal5*Δ strain could utilize X(Ala, Gly, Pro, Val, Ser, Ile, Leu)-Asn dipeptides as the nitrogen source. Nitrogen starvation has been demonstrated to up-regulate *ASP3* expression and promote the secretion of asparaginase II (BON *et al.* 1997). Furthermore, the enzymatic activity of asparaginase II hydrolyzes either L- or D-asparagine into ammonia and aspartate, facilitating the use of D-asparagine as a nitrogen source (DUNLOP *et al.* 1978; DUNLOP *et al.* 1976).

Ptr2p-independent di/tri-peptide utilization also includes the extracellular peptide degradation that is related to the utilization of dipeptides containing C-terminal arginine residues, particularly Met-Arg. Since the utilization of Met-Arg is enhanced by the deletion of *DAL5* and persists in the *ptr2*Δ*dal5*Δ double mutant, it is likely that an arginase activity comparable to the asparaginase activity of Asp3p could contribute to this dipeptide utilization.

Several cellular metabolic processes are involved in dipeptide utilization independent of Ptr2p activity since no significant alteration of expression or localization of Ptr2p-GFP was observed. These cellular processes are: 1) the conversion of pyruvate into acetyl-CoA which requires pyruvate dehydrogenase complex (Pdx1p, Lpd1, Pdb1p, and Lat1p); 2) the conversion of acetyl-CoA to malonyl-CoA, which involved in Hfa1p; 3) fatty acid synthesis which is involved in fatty acid synthase complex (Oar1p, Mct1p, and Etr1p); 4) glycine degradation in (Gcv2p, Gcv3p, and Lpd1p); and 5) a peptidase activity (Prd1p) (BUCHLER *et al.* 1994; NAGARAJAN and STORMS 1997; SCHNEIDER *et al.*

1997; STOOPS *et al.* 1997). Since the accumulation of dipeptides is not impaired in these mutants as indicated from the non-visible change in Ptr2p-GFP and accumulation of [^{3}H]Leu-Leu, the decrease in dipeptide utilization probably results from the inability to release amino acids from accumulated dipeptides or from blocking metabolic pathways in using the released amino acids for protein biosynthesis.

Other cellular processes might be involved in dipeptide utilization, such as found in the *ybt1* mutant. Ybt1p belongs to the ABC transporter family exhibiting ATP-dependent bile acid transport (ORTIZ et al. 1997; PAULSEN et al. 1998). I speculate that this protein might be involved in the transport of dipeptides into the vacuole. In the deletion mutant the defect in the uptake of dipeptides into the vacuole would lead to an increase of dipeptide availability in the cytoplasm. It is also possible that the deletion of a gene might result in a de-repression or change in function of other transport systems that may mediate dipeptide utilization in the mutant without alteration of *PTR2* expression or Ptr2p function.

I have identified 15 unknown strains with deletions in genes of unknown function that had an alteration of dipeptide utilization, seven strains exhibiting increased and eight strains showing decreased dipeptide utilization. Characterization of the phenotype of these genes should lead to a further understand their cellular roles. In addition, other genes such as *UBP14*, ubiquitin-specific protease activity, require additional study to understand the mechanism of how these genes are involved in dipeptide utilization.

To date, molecular components have been reported in the regulation of the human *PTR2* homologs PEPT1 and PEPT2 (DANIEL 2004). In our research, I have identified 52 genes encoding proteins with more than 30% identity to human genes, which might be involved directly or indirectly in the utilization of dipeptides in yeast. Investigation of these genes may promote understanding of the regulation of dipeptide utilization in mammalian systems (the complete list of genes is in Table 1). These genes include those encoding transcription factors and mRNA processing proteins (14 genes), proteins involved in cellular metabolism (12 genes), protein kinases (4 genes), DNA damage repair proteins (4 genes), ribosomal proteins (4 genes), actin binding proteins and proteins involved in sorting (4 genes), proteins related to ubiquitination (3 genes), peptidases (2 genes), a transporter (1 gene), a protein involved in cell cycle regulation (1 gene), a DNA helicase (1 gene), a protein related to growth regulation (1 gene) and a protein of unknown function (1 gene).

Suggestions for future experiments

My research has considerably expanded the understanding of the regulation of dipeptide utilization by *PTR2* expression, Ptr2p trafficking, and other *PTR2* independent mechanisms. To further explore the role of these gene products, I propose the following experiments.

1. I have identified that Dal5p is involved in dipeptide utilization by importing dipeptides by showing that some of non-N-end rule dipeptide substrates can provide a nitrogen

Table 1 Description of the function of yeast genes and their mammalian counterparts

Yeast gene name	Description of yeast gene product	mammalian gene counterpart	Description of human gene product
TRANSCRIPTION AND MRNA PROCESSING (14)			
BYE1	Transcriptional elongation regulator activity	DIDO1	Death associated transcription factor 1, contains nuclear localization signals and transactivation domains, may have a role in cell death during development, overexpression inhibits limb outgrowth
CUP9	Specific RNA polymerase II transcription factor activity	PKNOX1	PBX knotted 1 homeobox 1, a member of the homeodomain family of DNA binding proteins that regulate gene expression and control cell differentiation, heterodimerizes with PBX proteins to form Urokinase Enhancer Factor 3 (UEF3) DNA binding complexes
DBR1	RNA lariat debranching enzyme activity	DBR1	RNA lariat debranching activity for processing of introns
EAF3	Histone acetyltransferase activity	Morf4l2	Protein with high similarity to mortality factor 4 (human MORF4), which is a predicted transcriptional repressor that binds transcriptional corepressors human SIN3A and human TLE1 and negatively regulates cell proliferation
KEM1	5'-3' exoribonuclease activity	XRN1	5'-3' Exoribonuclease, a putative exoribonuclease that localizes to the cytoplasm, and is expressed ubiquitously
NPL3	mRNA binding	SYNCRIP	Synaptotagmin binding cytoplasmic RNA interacting protein, component of the ApoB mRNA editosome involved in apolipoprotein B (APOB) mRNA editing, may bind RNA, may play a role in the regulation of mRNA splicing or transport from the nucleus
PHO2	Transcription factor activity	SHOX2	Short stature homeobox 2, a member of the paired-related family of homeodomain-containing transcription factors, may have a role in craniofacial, brain, heart, and limb development, involved in skeletal defects of Turner syndrome
RIM101	Specific transcriptional repressor activity	Egr3	Early growth response 3, member of the Egr family of immediate early transcription factors; deletion of the corresponding gene results in gait ataxia, increased frequency of perinatal mortality, scoliosis, resting tremors, and ptosis
SFP1	Transcription factor activity	DPF2	D4 zinc and double PHD fingers family 2 (requiem), a member of the d4 domain family of zinc finger proteins, may be required for the induction of apoptosis by extracellular signals

Table 1 Continued

Yeast gene name	Description of yeast gene product	mammalian gene counterpart	Description of human gene product
SSN3	Cyclin-dependent protein kinase activity	CDK8	Cyclin dependent kinase 8, forms a complex with cyclin C that phosphorylates cyclin H (CCNH) and the C terminus of POLR2A, plays a role in the regulation of transcription through Notch signaling and as component of the RNA polymerase II holoenzyme
SSN8	Cyclin-dependent protein kinase regulator activity	CCNK	Cyclin K, member of the transcription cyclin family, binds and activates CDK9 leading to phosphorylation of the RNA polymerase II large subunit, promotes RNA polymerase II transcription elongation, overexpression suppresses glioblastoma cell proliferation
STP2	Specific RNA polymerase II transcription factor activity	HKR1	GLI-Kruppel family member HKR1, putative transcription factor whose expression in lung cancer cell lines is induced in response to cisplatin, may be involved in a signal transduction pathway that regulates platinum resistance or lung cancer progression
RPN4	Transcriptional activator activity	ZNF473	Zinc finger protein 473, an snRNA binding protein in the snRNP U7 complex, involved in mRNA processing
TAF14	General RNA polymerase II transcription factor activity	MLLT1	Myeloid-lymphoid or mixed-lineage leukemia translocated to 1, a transcription factor with activator and putative repressor activities, binds CBX8; gene translocation causes B-cell, myeloid, and acute lymphocytic forms of leukemia
KINASE (4)			
BUD32	Protein serine/threonine kinase activity	TP53RK	a protein serine-threonine kinase that binds to and phosphorylates p53 (TP53) and regulates p53-mediated transcriptional control, may be involved in the cell cycle and apoptosis
MCK1	Protein kinases, positive regulator of meiosis and spore formation	GSK3B	Glycogen synthase kinase 3 beta, serine/threonine kinase that regulates beta-catenin (CTNNB1) stability and binds presenilin 1 (PSEN1), associated with Alzheimer disease, bipolar disorder, schizophrenia and various neoplasms
NPR1	Serine/threonine protein kinase	CHEK1	Checkpoint homolog 1 (S. pombe), protein kinase, required for mitotic G2 checkpoint in response to radition-induced DNA damage, inhibits mitotic entry after DNA damage via mechanism involving CDC25, alternative form is associated with lung cancer
PHO85	Cyclin-dependent protein kinase activity	CDK2	Cyclin-dependent protein kinase 2, interacts with cyclins to regulate kinase activity and cell cycle progression, regulates DNA replication; decreased expression or inhibition may be therapeutic for many types of cancer

Table 1 Continued

Yeast gene name	Description of yeast gene product	mammalian gene counterpart	Description of human gene product
METABOLISM (12)			
ETR1	Enoyl-[acyl-carrier protein] reductase activity	MECR	Nuclear receptor binding factor 1, reduces trans-2-enoyl-CoA to acyl-CoA with chain length from C6 to C16 in an NADPH-dependent manner with preference to medium chain length substrate, plays a role in mitochondrial respiratory function
GCV2	Glycine dehydrogenase (decarboxylating) activity	GLDC	Glycine decarboxylase (glycine dehydrogenase, P-protein), part of the glycine cleavage system that catalyzes the decarboxylation of glycine; mutation of the corresponding gene causes nonketotic hyperglycinemia
GCV3	Glycine dehydrogenase (decarboxylating) activity	GCSH	Glycine cleavage system protein H, H-protein component of the glycine cleavage system, catalyzes transfer of the methylamine group of glycine from P-protein (GLDC) to T-protein (AMT); mutational inactivation results in atypical nonketotic hyperglycinemia
HFA1	Acetyl-CoA carboxylase activity	ACACA	Acetyl-Coenzyme A carboxylase alpha, catalyzes the rate-limiting step in long-chain fatty acid biogenesis; deficiency leads to defects in fatty acid synthesis
ELF1	Molecular function unknown	ELOF1	Protein containing a DUF701 putative zinc binding domain, has very strong similarity to uncharacterized mouse Elof1
LAT1	Dihydrolipoyllysine-residue acetyltransferase activity	DLAT	Dihydrolipoamide S-acetyltransferase E2 component of pyruvate dehydrogenase complex, binds coenzyme and pyruvate dehydrogenase kinase 2 (PDK2); mutation may cause pyruvate dehydrogenase deficiency; may be associated with primary biliary cirrhosis
LPD1	Dihydrolipoyl dehydrogenase activity	DLD	Dihydrolipoamide dehydrogenase, E3 component of pyruvate dehydrogenase complex, also a component of alpha-ketoglutarate dehydrogenase and alpha-ketoacid dehydrogenase complexes; mutation in the corresponding gene causes lipoamide dehydrogenase deficiency
PDB1	Pyruvate dehydrogenase (acetyl-transferring) activity	PDHB	E1 beta subunit of pyruvate dehydrogenase complex, oxidatively decarboxylates pyruvate to acetyl-CoA
PDX1	Pyruvate dehydrogenase complex protein	DLAT	Dihydrolipoamide S-acetyltransferase E2 component of pyruvate dehydrogenase complex, binds coenzyme and pyruvate dehydrogenase kinase 2 (PDK2); mutation may cause pyruvate dehydrogenase deficiency; may be associated with primary biliary cirrhosis

Table 1 Continued

Yeast gene name	Description of yeast gene product	mammalian gene counterpart	Description of human gene product
SHM2	Glycine hydroxymethyltransferase activity	SHMT1	Soluble serine hydroxymethyltransferase, catalyzes the tetrahydrofolate-dependent interconversion of glycine and serine, source of folate-activated one-carbon units required for purine, thymidine, and methionine synthesis
TSA1	Thioredoxin peroxidase activity	PRDX2	Peroxiredoxin 2, an antioxidant enzyme, acts in the response to lipid hydroperoxide and may play a role in protection of cells from reactive oxygen species, expression is altered in neurodegenerative diseases and breast cancer
YJL046W	Unknown	LIPT1	Lipoyltransferase, putative enzyme that transfers the lipoyl group from lipoyl AMP to the lysine residue of lipoate-dependent enzymes; tissue expression correlates well with expression of lipoate-requiring proteins
PEPTIDASE (2)			
PRD1	Metalloendopeptidase activity	THOP1	Thimet oligopeptidase 1, a metalloendopeptidase that processes neuropeptides and other polypeptides, degrades beta amyloid precursor protein (APP), acts in degrading peptides released by proteasomes to limit the extent of MHC class I antigen presentation
YFR044C	Unknown	CNDP2	Cytosolic nonspecific dipeptidase 2, member of the M20 metalloprotease family, a homodimeric enzyme that requires magnesium ions for full dipeptidase activity and is inhibited by bestatin, expressed ubiquitously
DNA DAMAGE REPAIRMENT (4)			
MLH1	DNA binding; ATPase activity	hMLH1	MutL (E. coli) homolog 1, a DNA mismatch repair protein that may play a role in DNA recombination; decreased expression is associated with various cancers; increased expression may be therapeutic for colorectal cancer
MRE11	3'-5' exonuclease activity	MRE11A	Meiotic recombination (S. cerevisiae) 11 homolog A, an endo- and exonuclease that functions in DNA double-strand break repair; mutation of corresponding gene is associated with an ataxia-telangiectasia-like disorder, breast cancer, and malignant lymphoma
RAD23	protein that binds to the 26S proteasome subunits and is involved in ubiquitin-dependent protein degradation	RAD23B	RAD23 homolog B (S. cerevisiae), a damaged DNA binding protein that also binds XPC to form a DNA damage recognition protein and functions in global genome nucleotide excision repair, may be associated with Machado Joseph disease or xeroderma pigmentosum

Table 1 Continued

Yeast gene name	Description of yeast gene product	mammalian gene counterpart	Description of human gene product
TOM72	Minor import receptor of the mitochondrial outer membrane	PPP5C	Protein phosphatase 5 catalytic subunit, a protein serine-threonine phosphatase that promotes cell proliferation, plays a role in DNA damage response and several signaling pathways, may promote tumor growth, downregulated in Alzheimer disease brain
RIBOSOMAL PROTEINS (4)			
MRP17	Structural constituent of ribosome	Mrps6	Mitochondrial ribosomal protein S6
MRPS9	Structural constituent of ribosome	MRPS9	Member of the ribosomal protein S9 or S16 family, has low similarity to C. elegans F09G8.3, which is involved in embryogenesis and positive growth regulation
RPL21A	Structural constituent of ribosome	RPL21	Ribosomal protein L21, component of the large 60S ribosomal subunit; shows altered expression in colorectal carcinogenesis
RPS9B	Structural constituent of ribosome	RPS9	Ribosomal protein S9, a component of the small 40S ribosomal subunit, gene expression is altered in human colorectal carcinoma cells and in pancreatic adenocarcinoma
ACTIN BINDING AND PROTEIN SORTING (4)			
ARP5	Actin-related protein	Actr5	Member of the actin family, which are major cytoskeletal proteins
HOF1	Cytoskeletal protein binding	SH3RF1	SH3 multiple domains 2, a ubiquitin ligase that functions in protein sorting at the trans Golgi network, plays a role in targeting of HIV 1 to the plasma membrane
SAC6	Actin filament binding	PLS3	Plastin 3 (T isoform), an actin-binding protein involved in actin filament bundling, cell invasion by bacteria, and possibly UV protection, expression is altered in cancer and Sezary syndrome, autoantibodies are linked to systemic lupus erythematosous
SNF7	Protein sorting in the pre-vacuolar endosome	CHMP4B	Chromosome 20 open reading frame 178 (chromatin modifying protein 4b, charged multivesicular body protein 4b), a member of the CHMP family of yeast class E SNF7-Vps protein homologs, binds Alix (PDCD6IP), may act in protein sorting
UBIQUITINATION (3)			
SHP1	Ubiquitin binding; Enzyme regulator activity	NSFL1C	Protein with very strong similarity to Nsfl1c protein (rat Nsfl1c), which is a spermatocyte XY body component that acts in membrane fusion and regrowth of Golgi cisternae following mitosis, member of the UBX (ubiquitin-regulatory) domain containing family

Table 1 Continued

Yeast gene name	Description of yeast gene product	mammalian gene counterpart	Description of human gene product
UBA3	NEDD8 activating enzyme activity	UBE1C	Ubiquitin-activating enzyme E1C, subunit of an E1 (ubiquitin-activating enzyme)-like complex, interacts with the amyloid precursor protein binding protein (APPBP1) to activate the ubiquitin-like protein NEDD8 and target CUL4A for NEDD8 modification
UBC2	Ubiquitin conjugating enzyme activity	UBE2B	Ubiquitin-conjugating enzyme E2B, predicted to be involved in the conjugation of ubiquitin to cellular proteins marking them for proteasomal degradation, involved in DNA repair; activity is increased in multiple disease states
TRANSPORTER (1)			
YBT1	Bile acid transporter activity	ABCC1	ATP-binding cassette subfamily C member 1 (multiple drug resistance protein 1), a transporter and multidrug efflux pump that confers resistance to lipophilic drugs and chemotherapeutic agents, upregulation correlates with severity of breast cancer
CELL CYCLE REGULATION (1)			
NFU1	Involved in maturation of mitochondrial iron-sulfur proteins	HIRIP5	HIRA interacting protein 5, interacts with histone cell cycle regulation defective homolog A (HIRA) which is involved in the pathogenesis of DiGeorge syndrome and the velocardiofacial syndrome
DNA HELICASE (1)			
HFM1	DNA helicase activity	ASCC3	Member of the Sec63 domain containing, helicase conserved C-terminal domain containing, and type III restriction enzyme res subunit families, contains two DEAD/DEAH box helicase domains, has moderate similarity to human ASCC3L1, which binds ATP
GROWTH REGULATION (1)			
ISA1	Mitochondrial protein required for normal iron metabolism	HBLD2	Protein with high similarity to C. elegans Y39B6A.3, which is involved in positive growth regulation and larval development, member of the HesB-like domain containing family
UNKNOWN (1)			
YPL098C	Unknown	C20orf52	Protein of unknown function, has very strong similarity to uncharacterized mouse 2010100O12Rik

source for the growth of a *PTR2* deletion mutant, while a double deletion of *PTR2* and *DAL5* lose the capability to utilize all dipeptides. At least three independent microarray analyses carried out by other workers showed that the expression of *DAL5* is regulated by Ssy1p under different growth conditions. First, *DAL5* expression decreased three-fold in a wild-type strain when Leu was added to the medium. In addition, the expression of *DAL5* in a *SSY1* deletion mutant resulted in an increase of four-fold compared with the wild type in medium containing Leu (FORSBERG *et al.* 2001). Second, the expression of *DAL5* in a *SSY1* deletion mutant increased six-fold compared with the wild-type in rich (YPD) medium (KODAMA *et al.* 2002), and increased two-fold in minimal medium (ECKERT-BOULET *et al.* 2004). Based on these data, I hypothesize that under inducing conditions such as the addition of Leu, yeast cells utilize dipeptides in a *PTR2*-dependent manner, while in non-inducing conditions yeast cells utilize certain dipeptides in a *PTR2*-independent manner. Further, I postulate that in response to amino acid inducing conditions, the SPS system (Ssy1p-Ptr3p-Ssy5p) increases *PTR2* expression but represses *DAL5* expression. In our toxic dipeptide assay (Ala-Eth), preliminary results indicated that the *PTR2* deletion mutant (BY4742 genetic background) remained sensitive to a high Ala-Eth concentration (0.4µmol), suggesting that the sensitivity to high concentration of Ala-Eth might result from the activity of Dal5p function due to the fact that Dal5p has a low affinity for importing some dipeptides. I suggest the following experiments (a, b, and c) to test the above hypotheses:

a) Confirm Dal5p imports dipeptide in our lab strain. This could be done by creating a *PTR2/DAL5* double knockout in the BY4742 background; the double knockout should not be sensitive to high concentrations of Ala-Eth, if Dal5p imports Ala-Eth.

Furthermore, using non-N-end rule substrates, such as Ala-Leu or Ala-His in a growth assay would demonstrate whether dipeptides could satisfy the auxotrophic requirement of His in a *ptr2* strain. Since Dal5p is a low-affinity dipeptide transporter, high concentrations of dipeptides should be used for the growth assay.

b) Explore how Leu, an inducer for the SPS system, changes the expression level of *DAL5* and *PTR2* by using Northern analysis or real time PCR. The wild type strain BY4742 will be grown in minimal medium (using proline as the nitrogen source) with Leu or without Leu, and total RNA will be extracted. I expect that the expression level of *DAL5* will be higher in medium without Leu than that with Leu, but the expression level of *PTR2* will be opposite.

c) Since the *SSY1* deletion mutant is lethal in the BY4742 background, studying the Ssy1p signaling effect should be performed in the FY2 genetic background. A series of knockout strains, *ssy1*, *ptr3*, *ssy5*, *dal5*, *ptr2*, and double knockout mutant strains *ssy1 dal5*, *ssy1 ptr2*, *ptr3 ptr2*, and *ptr3 dal5* should be created. Growth assays, toxic dipeptide assays, and expression of *PTR2* and *DAL5* under the induction of Leu could be performed in these backgrounds. I expect that the expression level of *DAL5* in *ssy1*, *ptr3*, and *ssy5* strains will be higher than that of the wild-type in the medium containing Leu, but the expression level of *PTR2* will have an opposite effect in the above conditions. I should also be able to demonstrate the contribution of *DAL5* to dipeptide utilization by comparing the growth assay and toxic dipeptide assays in *ssy1/ ptr2* and *ptr3/ ptr2* strains with that of *ssy1/ dal5* and *ptr3 /dal5* strains in minimal medium with or without Leu.

2. Dal81p, Rpn4p, Ubp14p, and Ubr2p played a role in the regulation of *PTR2* expression. How these genes fit into the SPS signal transduction pathway should be experimentally determined. The following experiments could be performed:

a) Epistasis experiments will address where these genes are in the SPS signal transduction pathway. Several gene double knockouts need to be created. For example, in order to understand the role of Rpn4p in the SPS signal transduction pathway, a *ptr3/ ubr2* double knockout strain will be created. If the phenotype such as the sensitivity to toxic dipeptide in the *ptr3/ ubr2* double knockout is the same as that in *ptr3*, it suggests that Ptr3p is upstream of Ubr2p. If the phenotype of the double knockout is the same as that in *ubr2*, it suggests that Ubr2p is upstream of Ptr3p. However if the phenotype in the double knockout is additive, it suggests that Ptr3p and Ubr2p are in independent regulation pathways.

b) Since Ubr2p showed homology to Ubr1p/Ptr1p and impacted the stability of Rpn4p, how Ubr2p impacts the positive regulator Rpn4p and repressor Cup9p on *PTR2* transcription should be tested. The relative amount of Cup9p and Rpn4p could be measured in these deletion mutants by Western analysis.

c) Dal81p has transcriptional activity, and Ubp14p has ubiquitin-specific protease activity. Deletion of either of these two genes showed a decrease of the expression level of *PTR2*. Since both genes are involved in the transcriptional regulation of *PTR2*, it is necessary to exam whether these gene products are related to Cup9p repression. The stability of Cup9p in these two deletion mutants could be tested by western analysis. This experiment will answer whether Dal81p and Ubp14p are involved in *PTR2* transcriptional regulation via regulation of Cup9p.

3. The gene products impacting Ptr2p trafficking showed a significant effect on di/tri-peptide utilization. Since ESCRT I, II, III were shown to be involved in Ptr2p trafficking, it is very likely that ubiquitination of Ptr2p is required. In addition, Ptr2p ubiquitination was shown by mass spectrum analysis. Further experiments should be performed to demonstrate the ubiquitination site(s).

a) Screening the lysine positions in Ptr2p to show which position(s) are the ubiquitination site(s). Point mutations of Ptr2p could be generated to change lysine to alanine in Ptr2p-GFP background plasmid (pMS4 or pMS2). The point mutations in Lys sites should lead to reduction of Ptr2p turnover and increase the amount of Ptr2p in the plasma membrane and lead to an increased uptake of dipeptides.

b) The enzymes involved in the ubiquitination of Ptr2p should be identified and tested. The Ptr2p ubiquination process likely follows a similar pattern to that of other membrane proteins since involvement of ESCRT protein complex is a ubiquitous process for a number of membrane proteins. Npi1p/Rsp5p, Npi2/Doa4p, and Bul1p/2 Ptr2p are good candidates for the ubiquitination of Ptr2p since these components are also involved in the Gap1p trafficking system.

c) Npr1p and Lst4p were shown be involved in the Ptr2p trafficking. Deletion of *NPR1* and *LST4* led to accumulation of Ptr2p containing vesicles. Npr1p is a Ser/Thr kinase and played a role in phosphorylation of Bap2p, a branched-chain amino acid permease and is involved in the degradation process. Npr1p might play a similar role in Ptr2p post-translational regulation. First, the plasmid with Flag tagged Ptr2p (pMS2) will be transformed into the wild type and *npr1* strain.

The degradation of Ptr2p will be examined under NH_4^+-induced conditions. I expect that the degradation of Ptr2p will be reduced in *npr1* strain by western analysis. Second, in order to find out the phosphorylation sites in Ptr2p, point mutations at Ser/Thr sites of Ptr2p will be generated. The contribution of Npr1p to the phosphorylation of Ptr2p will be determined by antibodies that react specifically with phorphorylated residues.

d) To further finely define the subcellular localization of Ptr2p in those deletion mutants showing changes in Ptr2p-GFP localizaion, the analysis of Ptr2p localization by confocal microscopy will be performed. From my research, I found deletion mutants of gene product encoding protein sorting such as *srn2*, *snf7* and *vps36* showed higher accumulation of dipeptide Leu-Leu but no significant Ptr2p-GFP expression at the cytoplasmic membrane. One outcome of these experiments might lead to an explanation for those mutants that exhibit higher Leu-Leu accumulation. In addition, these experiments will also show the detail location of Ptr2p-GFP in the endosomal vesicles.

4. A number of metabolic genes, such as the components of pyruvate dehydrogenase complex (Pdx1p, Lpd1p, Pdb1p, and Lat1p), are involved in dipeptide utilization. The roles of these gene products should be further explored. Deletion of these gene products does not influence di/tri-peptide accumulation. In order to examine how these deletion mutants impact di/tri-peptide utilization, the degradation process from di/tri-peptide into amino acids should be tested. Our preliminary data indicated that the tested dipeptide Leu-Leu was degraded into Leu and an unknown metabolite after Leu-Leu is taken up

into cells. This unknown compound is more hydrophilic than Leu, and might be an intermediate product formed in the process of Leu-Leu degradation or a leu derivation product. Identifying this compound will require application of techniques such as HPLC and mass spectrometry.

5. Fifteen genes of unknown function were identified to be involved in di/tri-peptide utilization. Characterization of these genes will help understand their roles in yeast biology. I propose to start with two gene deletion mutants that showed a strong phenotype in the regulation of *PTR2* expression and Ptr2p trafficking. High induction of *PTR2* expression was found in a *yor323c* mutant, and accumulation of Ptr2p vesicles in a *ypl073c* mutant. In order to characterize the function of *YOR323C*, first, the expression level of *PTR2* will be examined by Northern analysis or Real Time PCR. Because a deletion in yor323c leads to an increase in dipeptide utilization and a higher Ptr2-GFP signal in the cell membrane, I expect to see a higher expression level of *PTR2* in *yor323c* strain in comparison to that of the wild-type. Second, bioinformatic analysis should be performed for the protein sequence encoded by this gene to see whether there are any known protein domains whose function is known. Third, the localization of this gene product should also be tested by tagging this gene with GFP. Further an examination of the interaction databases such as the synthetic lethal screens can determine whether there are other known gene products interact with Yor323p. Another example for testing is YPL073c since this gene is involved in Ptr2p trafficking. Whether this gene product interacts with other trafficking related proteins should be tested. The two hybrid experiment to detect protein-protein interaction between this gene product and ESCRT

proteins or other vesicle sorting proteins will be tested. In addition, the localization of this protein should also be tested by tagging with GFP, and Ptr2p turnover should be examined by Western analysis in the ypl073 deletion strain.

In summary, the identified genes regulating dipeptide utilization represent a very wide regulatory network involved in transport and utilization of dipeptides in yeast. This is the first such study of a membrane transport system in any organism and initiates a systems biology approach to understanding membrane transport in cells. Further experiments of characterizing these identified genes could provide valuable information of how these genes of different pathways are involved in di/tri-peptide utilization.

REFERENCES

ABDEL-SATER, F., I. IRAQUI, A. URRESTARAZU and B. ANDRE, 2004 The external amino acid signaling pathway promotes activation of Stp1 and Uga35/Dal81 transcription factors for induction of the AGP1 gene in Saccharomyces cerevisiae. Genetics **166:** 1727-1739.

ANDREASSON, C., and P. O. LJUNGDAHL, 2002 Receptor-mediated endoproteolytic activation of two transcription factors in yeast. Genes Dev **16:** 3158-3172.

ANDREASSON, C., and P. O. LJUNGDAHL, 2004 The N-terminal regulatory domain of Stp1p is modular and, fused to an artificial transcription factor, confers full Ssy1p-Ptr3p-Ssy5p sensor control. Mol Cell Biol **24:** 7503-7513.

BARTEL, B., I. WUNNING and A. VARSHAVSKY, 1990 The recognition component of the N-end rule pathway. Embo J **9:** 3179-3189.

BERNARD, F., and B. ANDRE, 2001 Ubiquitin and the SCF(Grr1) ubiquitin ligase complex are involved in the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. FEBS Lett **496:** 81-85.

BOCHNER, B. R., 2003 New technologies to assess genotype-phenotype relationships. Nat Rev Genet **4:** 309-314.

BOCHNER, B. R., P. GADZINSKI and E. PANOMITROS, 2001 Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Genome Res **11:** 1246-1255.

BON, E. P., E. CARVAJAL, M. STANBROUGH, D. ROWEN and B. MAGASANIK, 1997 Asparaginase II of Saccharomyces cerevisiae. GLN3/URE2 regulation of a periplasmic enzyme. Appl Biochem Biotechnol **63-65:** 203-212.

BONNEROT, C., R. BOECK and B. LAPEYRE, 2000 The two proteins Pat1p (Mrt1p) and Spb8p interact in vivo, are required for mRNA decay, and are functionally linked to Pab1p. Mol Cell Biol **20:** 5939-5946.

BOUVERET, E., G. RIGAUT, A. SHEVCHENKO, M. WILM and B. SERAPHIN, 2000 A Sm-like protein complex that participates in mRNA degradation. Embo J **19:** 1661-1671.

BUCHLER, M., U. TISLJAR and D. H. WOLF, 1994 Proteinase yscD (oligopeptidase yscD). Structure, function and relationship of the yeast enzyme with mammalian thimet oligopeptidase (metalloendopeptidase, EP 24.15). Eur J Biochem **219:** 627-639.

BYRD, C., G. C. TURNER and A. VARSHAVSKY, 1998 The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. Embo J **17:** 269-277.

CHISHOLM, V. T., H. Z. LEA, R. RAI and T. G. COOPER, 1987 Regulation of allantoate transport in wild-type and mutant strains of Saccharomyces cerevisiae. J Bacteriol **169:** 1684-1690.

DANIEL, H., 2004 Molecular and integrative physiology of intestinal peptide transport. Annu Rev Physiol **66:** 361-384.

DU, F., F. NAVARRO-GARCIA, Z. XIA, T. TASAKI and A. VARSHAVSKY, 2002 Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. Proc Natl Acad Sci U S A **99:** 14110-14115.

DUNLOP, P. C., G. M. MEYER, D. BAN and R. J. ROON, 1978 Characterization of two forms of asparaginase in Saccharomyces cerevisiae. J Biol Chem **253:** 1297-1304.

DUNLOP, P. C., R. J. ROON and H. L. EVEN, 1976 Utilization of D-asparagine by Saccharomyces cerevisiae. J Bacteriol **125:** 999-1004.

ECKERT-BOULET, N., P. S. NIELSEN, C. FRIIS, M. M. DOS SANTOS, J. NIELSEN *et al.*, 2004 Transcriptional profiling of extracellular amino acid sensing in Saccharomyces cerevisiae and the role of Stp1p and Stp2p. Yeast **21:** 635-648.

FISCHER, T., K. STRASSER, A. RACZ, S. RODRIGUEZ-NAVARRO, M. OPPIZZI *et al.*, 2002 The mRNA export machinery requires the novel Sac3p-Thp1p complex to dock at the nucleoplasmic entrance of the nuclear pores. Embo J **21:** 5843-5852.

FORSBERG, H., C. F. GILSTRING, A. ZARGARI, P. MARTINEZ and P. O. LJUNGDAHL, 2001 The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Mol Microbiol **42:** 215-228.

HITCHCOCK, A. L., K. AULD, S. P. GYGI and P. A. SILVER, 2003 A subset of membrane-associated proteins is ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery. Proc Natl Acad Sci U S A **100:** 12735-12740.

KODAMA, Y., F. OMURA, K. TAKAHASHI, K. SHIRAHIGE and T. ASHIKARI, 2002 Genome-wide expression analysis of genes affected by amino acid sensor Ssy1p in Saccharomyces cerevisiae. Curr Genet **41:** 63-72.

KORNBERG, R. D., 2005 Mediator and the mechanism of transcriptional activation. Trends Biochem Sci **30:** 235-239.

MANNHAUPT, G., R. SCHNALL, V. KARPOV, I. VETTER and H. FELDMANN, 1999 Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett **450:** 27-34.

NAGARAJAN, L., and R. K. STORMS, 1997 Molecular characterization of GCV3, the Saccharomyces cerevisiae gene coding for the glycine cleavage system hydrogen carrier protein. J Biol Chem **272:** 4444-4450.

ORTIZ, D. F., M. V. ST PIERRE, A. ABDULMESSIH and I. M. ARIAS, 1997 A yeast ATP-binding cassette-type protein mediating ATP-dependent bile acid transport. J Biol Chem **272:** 15358-15365.

PAULSEN, I. T., M. K. SLIWINSKI, B. NELISSEN, A. GOFFEAU and M. H. SAIER, JR., 1998 Unified inventory of established and putative transporters encoded within the complete genome of Saccharomyces cerevisiae. FEBS Lett **430:** 116-125.

RAI, R., J. R. DAUGHERTY, J. J. TATE, T. D. BUFORD and T. G. COOPER, 2004 Synergistic operation of four cis-acting elements mediate high level DAL5 transcription in Saccharomyces cerevisiae. FEMS Yeast Res **5:** 29-41.

RAI, R., F. GENBAUFFE, H. Z. LEA and T. G. COOPER, 1987 Transcriptional regulation of the DAL5 gene in Saccharomyces cerevisiae. J Bacteriol **169:** 3521-3524.

REID, J. L., Z. MOQTADERI and K. STRUHL, 2004 Eaf3 regulates the global pattern of histone acetylation in Saccharomyces cerevisiae. Mol Cell Biol **24:** 757-764.

SANDERS, S. L., J. JENNINGS, A. CANUTESCU, A. J. LINK and P. A. WEIL, 2002 Proteomics of the eukaryotic transcription machinery: identification of proteins associated with components of yeast TFIID by multidimensional mass spectrometry. Mol Cell Biol **22:** 4723-4738.

SCHNEIDER, R., B. BRORS, F. BURGER, S. CAMRATH and H. WEISS, 1997 Two genes of the putative mitochondrial fatty acid synthase in the genome of Saccharomyces cerevisiae. Curr Genet **32:** 384-388.

STOOPS, J. K., R. H. CHENG, M. A. YAZDI, C. Y. MAENG, J. P. SCHROETER *et al.*, 1997 On the unique structural organization of the Saccharomyces cerevisiae pyruvate dehydrogenase complex. J Biol Chem **272:** 5757-5764.

TURNER, G. C., F. DU and A. VARSHAVSKY, 2000 Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. Nature **405:** 579-583.

TUROSCY, V., and T. G. COOPER, 1987 Ureidosuccinate is transported by the allantoate transport system in Saccharomyces cerevisiae. J Bacteriol **169:** 2598-2600.

WANG, L., X. MAO, D. JU and Y. XIE, 2004 Rpn4 is a physiological substrate of the Ubr2 ubiquitin ligase. J Biol Chem **279:** 55218-55223.

Vita

Houjan Cai was born September 16, 1967, in Wenzhou, Zhejiang Province, P.R.China. After graduating from the Biology Department, Wenzhou Teachers' College P.R. China in 1987, Houjian Cai worked as a biology instructor for two years in a primary school in WenZhou, his hometown. He pursued his first Master Degree in Environmental Biology in Nanjing University in 1989. After that, he worked as a research associate in Nanjing Institute of Geography & Limnology of the Chinese Academy of Sciences in 1992. Houjian Cai started his adventure life outside P. R. China in 1996 by starting to pursue his second Master Degree in Environmental Biology in Delft University of Technology, the Netherlands. After finishing his study in 1998, he worked at the Institute of Terrestrial Ecology, Swiss Federal Institute of Technology (ETH Zurich), Switzerland as a research assistant. He left Switzerland and pursued his Ph.D study in Rutgers, the state University of New Jersey in 1999, and transferred his study to Microbiology Department, University of Tennessee, Knoxville in 2001.

Houjian Cai is interested in Stem Cell research and currently he is searching for a postdoctoral position.