Investigation of nutrient sensing in the yeast Saccharomyces cerevisiae

Eckert-Boulet, Nadine; Hobley, Timothy John

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Nadine Eckert-Boulet

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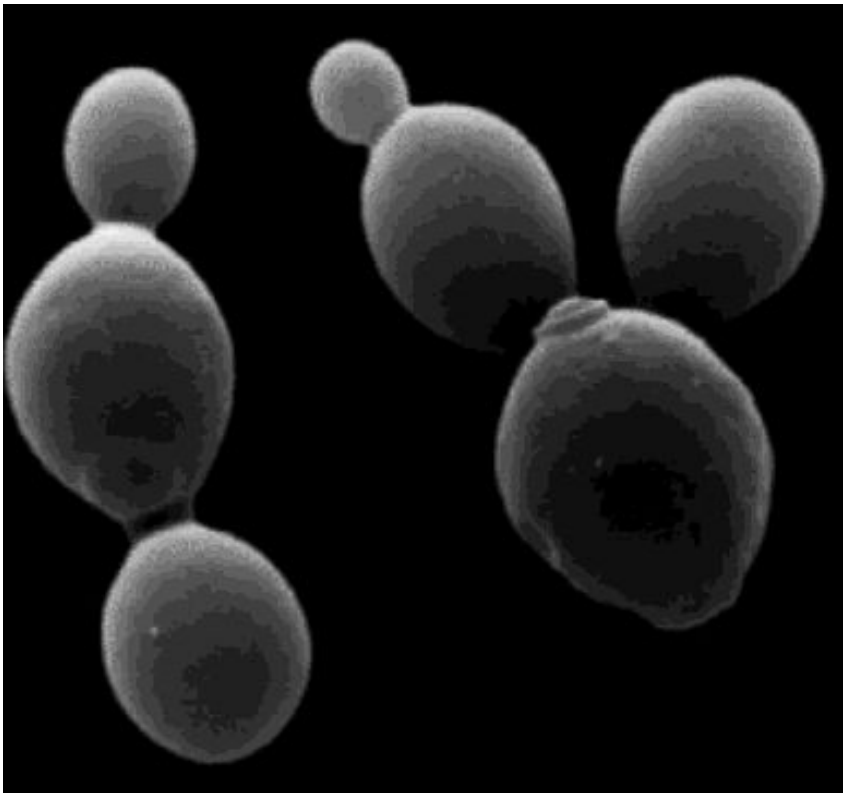

BioCentrum-DTU
TECHNICAL UNIVERSITY OF DENMARK

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La science est l'asymptote de la vérité. Elle approche sans cesse et ne touche jamais.
Victor Hugo (1802-1885)

Preface

The yeast *Saccharomyces cerevisiae* is the best characterised eukaryotic organism to this date. Our understanding of this yeast deepens every day. There are several reasons for this. Baker's yeast has been used by mankind for thousands of years to brew alcoholic beverages and to bake bread, and this has resulted in extensive empirical knowledge on this organism. But *S. cerevisiae* is also used as a model for eukaryotic organisms, partly because it is easy to manipulate genetically. An important application of yeast lies in beer production, and various attempts to minimise production costs led to the awareness that understanding nutrient sensing is important with regard to minimising the formation of unwanted by-products in the brew. Understanding nutrient sensing can, however, also lead to a better understanding of the mechanisms of certain diseases and malfunctions, e.g. diabetes, and in this case *S. cerevisiae* can be used as a model in order to obtain information that will hopefully help developing therapeutic strategies. These two aspects are described in Chapter I.

Glucose is the most important nutrient for most organisms on earth, probably because it is the most abundant. Glucose sensing is discussed in Chapter II. It can be subdivided in two main pathways: glucose repression (of genes coding for enzymes needed for metabolism of other carbon sources) and glucose induction (transcriptional induction of hexose transporter genes). When yeast cells grow on a sugar mixture in which glucose is present, they metabolise each sugar sequentially, and in industrial processes it is desirable to have a parallel consumption of the sugars. The sequential metabolism is due to a mechanism called glucose repression, which ensures that expression of enzymes for metabolising other sugars is repressed at the transcriptional level when glucose is present. This pathway involves the transcriptional repressor Mig1p, together with a number of other proteins. It is coupled to another pathway, called glucose induction, through which glucose induces expression of its transporters. Glucose induction does not require glucose uptake to be active, and the initiating step relies on detection of glucose by two membrane proteins homologous to hexose transporters, Snf3p and Rgt2p. The signalling cascade involves phosphorylation events, followed by turnover or cellular relocation of intermediate factors, before it reaches the nucleus and inactivates transcriptional repression by Rgt1p.

Another crucial nutrient is nitrogen, which is needed for protein synthesis; chapter III presents the main regulatory mechanisms associated with nitrogen sensing: Nitrogen Catabolite Repression, Target Of Rapamycin, and SPS-mediated induction of AAP genes. Amino acids are a possible nitrogen source, and they are taken up by proteins with various substrate ranges and affinities belonging to the family of Amino Acid Permeases (AAPs). The signalling cascade involves a sensor complex, the SPS sensor, located at the plasma membrane, which, when activated by amino acids, causes cellular migration to the nucleus of the transcription factors required for transcriptional induction. Similarly to the expression of hexose transporters being induced by extracellular glucose, transcription of several AAP genes is induced by external amino acids.

When comparing the mechanisms of extracellular glucose sensing and amino acid sensing, it is striking to see that these two pathways share many components. The final chapter will focus on their similarities and differences.

Publication list

The thesis is based on the following three papers, which are included at the end of the thesis:

I. Eckert-Boulet, N., Nielsen P.S., Friis C., Moreira dos Santos M., Nielsen J., Kielland-Brandt M.C., Regenberg B. (2004) Transcriptional profiling of extracellular amino acid sensing in *Saccharomyces cerevisiae* and the role of Stp1p and Stp2p. *Yeast* **21:** 635-648

II. Eckert-Boulet, N., Regenberg B., Nielsen J. (2005) Grr1p is required for transcriptional induction of amino acid permease genes and proper transcriptional regulation of genes in carbon metabolism of *Saccharomyces cerevisiae*. *Curr. Genet.* **47:** 139-149

III. Eckert-Boulet, N., Larsson K., Wu B., Poulsen P., Regenberg B., Nielsen J., Kielland-Brandt M.C. Deletion of *RTS1*, encoding a regulatory subunit of Protein Phosphatase 2A, results in constitutive amino acid signaling via increased Stp1p processing. *Eukaryot. Cell.* **5:** 174-179

Table of Contents

Abstract

The yeast *Saccharomyces cerevisiae* has developed complex regulatory systems to control expression of nutrient transporters so that these are only produced when needed. This is the case for hexose transporters and amino acid transporters (the latter are known as *amino acid permeases* (AAPs)). Their expression is induced at the transcriptional level following detection of extracellular nutrients, glucose and amino acids, respectively, by sensor proteins located at the plasma membrane. Amino acids are sensed by the SPS (Ssy1p-Ptr3p-Ssy5p) sensor. The resulting signal is transmitted to the homologous transcription factors, Stp1p and Stp2p, which undergo endoproteolytic processing in the cytoplasm and migrate to the nucleus, where they bind an Upstream Activating Sequence (UAS_{aa}) present in the promoter DNA of several AAP genes, thereby inducing transcription.

In order to confirm or identify targets of this pathway, whole-genome transcription profiles of wild-type strains and of strains deleted in either *SSY1* or *STP1* and *STP2* were compared using DNA microarrays, in the absence and presence of the amino acid L-citrulline. L-citrulline cannot be taken up by the strains used, since they are devoid of the General Amino acid Permease Gap1p. The AAP genes *TAT1, BAP2, BAP3* and *PTR2* were confirmed to be under the control of the amino acid induction pathway, while A*GP2* was identified as a new AAP gene target. Global transcription analysis showed that 46 genes were induced by L-citrulline, in a manner dependent on *SSY1* and *STP1/STP2*. Alignment of the promoter sequences allowed a more precise definition of the consensus sequence of the UAS_{aa} identified previously. Besides the effects on AAP genes, *ssy1Δ* and *stp1Δ stp2Δ* mutants exhibited a number of other transcriptional phenotypes, such as increased expression of genes subject to Nitrogen Catabolite Repression and genes involved in stress response.

The F-box protein Grr1p, known for its role in cell cycle regulation and glucose induction of the hexose transporter genes, is required for amino acid induction to occur. Genome-wide transcription analysis of a wild-type strain and of a *grr1Δ* strain, in the absence and presence of L-citrulline, were performed using DNA microarrays. L-citrulline cannot be taken up in the experimental conditions used. Data analysis showed that amino acid induction of the AAP genes *AGP1, BAP2, BAP3, DIP5, TAT1,* and *GNP1* is completely dependent on the presence of *GRR1*. Comparison of the transcriptional profiles of the two strains in the absence of inducer revealed that *GRR1* disruption leads to increased transcription of numerous genes encoding enzymes of the central carbon metabolism. In addition, promoter analysis showed that many of the genes with increased transcription display Mig1p- and/or Msn2p/Msn4p-binding sites. Increased expression of glucose-repressed genes in the *grr1Δ* strain may be an indirect consequence of the reduced glucose uptake expected from reduced expression of several hexose transporter genes in such a strain.

In an attempt to identify novel components of the SPS-mediated pathway, a transposon mutant library was screened for mutants exhibiting (constitutive) transcriptional induction of the AAP genes, i.e. mutants in which the pathway is active even in the absence of inducer. Several transposons were found at the *RTS1* locus. *RTS1* encodes one of the two regulatory subunits of protein phosphatase 2A known in yeast. Deletion of

RTS1 indeed resulted in constitutive activation of the amino acid-inducible *AGP1* and *BAP2* promoters, in a manner that was dependent on *SSY1, PTR3, SSY5, GRR1* or *STP1, STP2* and their homologue *STP3*. Increased transcription from the *AGP1* and *BAP2* promoters in *rts1Δ* cells appeared to occur via increased Stp1p processing.

Through this work, new targets of the SPS-mediated pathway have been identified and the consensus sequence of the UAS_{aa} involved in amino acid induction has been defined more precisely. The involvement of Grr1p has been confirmed and the effects of a *GRR1* deletion at the whole-genome transcriptional levels investigated. In addition, a new component of the pathway, namely protein phosphatase 2A associated with its regulatory subunit Rts1p, has been identified as a down-regulating factor.

Sammenfatning

Gæren *Saccharomyces cerevisiae* har udviklet komplekse regulatoriske systemer til at kontrollere ekspression af de proteiner, der importerer næringsstoffer, således at disse kun bliver produceret, når der er brug for dem. Dette er tilfældet for hexose-transportører samt aminosyre-transportører (disse bliver også kaldt *amino acid permeases* (AAPs)). Deres ekspression induceres på det transkriptionelle niveau efter at ekstracellulære næringsstoffer, henholdsvis glukose og aminosyrer, bliver detekteret af sensor-proteiner, som sidder i plasmamembranen. Aminosyrer bliver detekteret af den såkaldte SPS (Ssy1p-Ptr3p-Ssy5p) sensor, og et signal genereres og bliver transmitteret via de homologe transkriptionsfaktorer Stp1p og Stp2p. Stp1p og Stp2p undergår endoproteolytisk kløvning i cytoplasma og migrerer derefter til cellekernen, hvor de binder til en UAS_{aa} element (*Upstream Activating Sequence*) som findes i promoter-DNA ved flere AAP gener, hvilket medfører transkriptionel induktion.

For at kunne bekræfte eller identificere nye gener som reguleres via SPS-systemet, blev der udført global transkriptionel profilering af vildtype stammer samt af stammer deleterede for enten *SSY1* eller *STP1* og *STP2*, både med og uden aminosyren L-citrullin i mediet. L-citrullin kan ikke optages af disse stammer, da de mangler *GAP1* (General Amino acid Permease). Det blev bekræftet, at AAP generne *TAT1, BAP2, BAP3* og *PTR2* er under kontrol af aminosyre induktionsvejen, mens *AGP2* blev identificeret som et nyt gen under kontrol af denne mekanisme. Den globale transkriptionsanalyse viste at 46 gener induceres af L-citrullin, afhængigt af Ssy1p og Stp1p/Stp2p. Sekvenssammenligning af promoterregionerne muliggjorde en mere præcis definition af den tidligere beskrevet Upstream Activating Sequence (UAS_{aa}). Udover effekten på AAP gener, viste *ssy1Δ* og *stp1Δ stp2Δ* mutanter mange andre transkriptionelle fænotyper, såsom øget ekspression af gener under kontrol af Nitrogen Catabolite Repression og gener involverede i stress respons.

F-box proteinet Grr1p, som også spiller en rolle i regulering af cellecyklus og glukose induktion af heksose transportørgener, er nødvendig for at aminosyre induktion kan finde sted. Hel-genom transkriptionsanalyser af en vildtype stamme og en *grr1Δ* stamme med og uden L-citrullin blev udført. Dataanalyse viste at aminosyre induktion af AAP generne *AGP1, BAP2, BAP3, DIP5, TAT1,* og *GNP1* er helt afhængig af tilstedeværelsen af *GRR1*. Sammenligningen af de to stammers transkriptionelle profiler viste at, når *GRR1* er slået ud, transkription af mange gener til enzymer i den centrale karbon metabolisme øges. Promoteranalyse viste at mange af de gener med øget transkription har mulige Mig1p og/eller Msn2p-Msn4p bindingelementer. Den øgede ekspression af glukose-represserede gener i *grr1Δ* stammen kan være en indirekte konsekvens af den begrænsede glukose optagelse som forventes i denne mutant på grund af formindsket ekspression af flere hexose transportører.

I et forsøg på at identificere nye komponenter i SPS-signaltransduktionsmekanismen, blev en transposon samling screenet for mutanter med (konstitutive) transkriptionel induktion af AAP gener, det vil sige, mutanter, i hvilke signaleringsvejen er aktiv selv uden aminosyre i mediet. Flere transposoner blev fundet i *RTS1* locus. *RTS1* koder en af de to regulatoriske enheder af protein fosfatase 2A som er kendt i gær. *RTS1* blev slået ud, og dette medførte konstitutiv aktivering af *AGP1* og *BAP2* promotorer. Dette var

afhængigt af *SSY1, PTR3, SSY5, GRR1* eller *STP1, STP2* og deres homolog *STP3*. Den forøgede transkription fra *AGP1* og *BAP2* promotorer i *rts1Δ* celler ser ud til at skyldes forøget processering af Stp1p.

Med dette arbejde blev der identificeret nye gener som reguleres via SPS-signalnetværket og konsensussekvensen af UAS_{aa} involveret i aminosyre induktion blev defineret mere præcist. Det er blevet bekræftet at Grr1p er involveret i denne vej og effekterne af *grr1Δ* mutationen blev undersøgt på hel-genom transkriptionelt niveau. Derudover, blev der identificeret en ny komponent af signalnetværket, som er protein fosfatase 2A sammen med sin regulatoriske enhed Rts1p, og som udfører en negativ regulering af vejen.

Résumé

La levure *Saccharomyces cerevisiae* a développé des systèmes de régulation complexes pour contrôler l'expression des transporteurs de nutriments, de sorte que ceux-ci ne sont produits que lorsqu'ils sont nécessaires. Tel est le cas des transporteurs d'hexose et des transporteurs d'acides aminés (ces derniers sont aussi connus sous le nom de perméases d'acides aminés (AAPs : *Amino Acid Permeases*)). Leur expression est induite au niveau de la transcription suivant la détection de nutriments extracellulaires, c'est-à-dire, respectivement, de glucose et d'acides aminés, par des senseurs protéiques localisés à la membrane plasmique. Les acides aminés sont détectés par le senseur SPS (Ssy1p-Ptr3p-Ssy5p). Le signal résultant est transmis aux facteurs de transcription homologues Stp1p et Stp2p, qui subissent alors, dans le cytoplasme, un remaniement endoprotéolytique et migrent ensuite vers le noyau, où ils s'associent à un élément activateur (UAS_{aa}: *Upstream Activating Sequence*) présent dans les promoteurs de plusieurs gènes d'AAPs, induisant ainsi leur transcription.

De manière à confirmer ou identifier de nouvelles cibles de cette voie de signalisation, les profils de transcription à l'échelle de tout le génome de souches sauvages et de souches privées de *SSY1* ou *STP1* et *STP2* ont été comparés en l'absence et en la présence de l'acide aminé L-citrulline. Les souches utilisées ne sont pas capables d'importer la citrulline puisqu'elles sont dépourvues du gène codant pour son transporteur Gap1p. Les gènes d'AAPs *TAT1, BAP2, BAP3* et *PTR2* sont bien sous le contrôle de la voie d'induction par les acides aminés, et *TAT2* a été identifié comme une nouvelle cible de cette voie. L'analyse de transcription globale a montré que 46 gènes sont inductibles par la L-citrulline, et ce de façon entièrement dépendante de Ssy1p et Stp1p/Stp2p. Les séquences de promoteurs ont été alignées, ce qui a permis une définition plus précise de la séquence consensus de l'UAS_{aa} (séquence d'activation en aval). En plus des effets observés sur les gènes d'AAPs, les mutants *ssy1Δ* et *stp1Δ stp2Δ* présentent d'autres phénotypes transcriptionnels, tels qu'une augmentation de l'expression de plusieurs gènes régulés par la répression catabolique par l'azote ainsi que de gènes impliqués dans la réponse au stress.

La protéine F-box Grr1p, connue également pour jouer un rôle dans la régulation du cycle cellulaire et l'induction des gènes de transporteurs d'hexoses par le glucose, est elle aussi nécessaire pour que l'induction par les acides aminés puisse avoir lieu. Les profils de transcription d'une souche sauvage et d'une souche *grr1Δ* ont été comparés à l'échelle du génome en l'absence et en la présence de L-citrulline. Celle-ci ne peut être importée par les cellules dans les conditions expérimentales appliquées. L'analyse des données a révélé que l'induction par les acides aminés des gènes d'AAPs *AGP1, BAP2, BAP3, DIP5, TAT1* et *GNP1* est entièrement dépendante de la présence de *GRR1*. La comparaison des profils de transcription des deux souches en l'absence d'inducteur a montré que la disruption de *GRR1* entraîne une augmentation de la transcription de nombreux gènes codant pour des enzymes du métabolisme central du carbone. L'analyse de promoteurs a révélé que de nombreux gènes dont les niveaux de transcription sont plus élevés dans la souche mutante *grr1Δ* présentent des sites de reconnaissance pour Mig1p et/ou Msn2p/Msn4p. Il est probable que l'expression plus élevée des gènes réprimés par le glucose dans la souche *grr1Δ* est une conséquence indirecte d'une réduction de

l'import de glucose, effet attendu dans une telle souche où l'expression de plusieurs gènes de transporteurs d'hexose est réduite.

Dans le but d'identifier de nouveaux composants de la voie médiée par le SPS, une librairie de transposons mutants a été criblée afin de trouver des mutants présentant une induction transcriptionnelle (constitutive) des gènes d'AAPs, c'est-à-dire des mutants dans lesquels la voie de signalisation est active même en l'absence d'inducteur. Plusieurs transposons ont été trouvés dans le locus de *RTS1*. *RTS1* code pour l'une des deux sous-unités régulatrices de la protéine phosphatase 2A connues chez la levure. La suppression de *RTS1* cause effectivement une activation constitutive depuis les promoteurs d'*AGP1* et de *BAP2*, qui sont inductibles par les acides aminés, et ce de manière entièrement dépendante de *SSY1, PTR3, SSY5, GRR1* et *STP1, STP2* et leur homologue *STP3*. Il apparaît que l'augmentation de la transcription d'*AGP1* et de *BAP2* dans les cellules *rts1Δ* est une conséquence de l'augmentation du remaniement de Stp1p.

Cette étude a permis l'identification de nouvelles cibles de la voie médiée par le SPS ainsi qu'une redéfinition plus précise de la séquence consensus de l'UAS_{aa} impliquée dans l'induction par les acides aminés. Il a été confirmé que Grr1p est un facteur de cette voie et les effets d'une délétion de *GRR1* ont été étudiés à l'échelle du génome. Enfin, un nouveau composant de cette voie, la protéine phosphatase 2A associée à sa sous-unité régulatrice Rts1p, a été identifiée comme un facteur exerçant une régulation négative.

Chapter I

The importance of understanding nutrient sensing

The baker's yeast *Saccharomyces cerevisiae* has been used by mankind for thousand of years. Hieroglyphics show indeed that Egyptians used yeast and fermentation processes more than 5000 years ago. Baker's yeast was used then, as it is now, to leaven bread and produce alcoholic beverages, e.g. beer and wine. In 1861, Louis Pasteur identified yeast as the living microorganism responsible for alcoholic fermentation (Pasteur, 1861). It then became possible to isolate baker's yeast, and this led to its commercial production, which started at the turn of the 20th century.

S. cerevisiae has been used for decades as a model organism for understanding cellular mechanisms in eukaryotic cells. Besides its classification as a GRAS (Generally Regarded As Safe) organism, it is easy to work with and manipulate genetically, which is also why it was the first eukaryotic organism to be completely sequenced. The sequencing was performed by a world-wide consortium of more than 600 scientists and over 100 laboratories in 1997 (Mewes *et al.,* 1997).

Yeast cells have developed complex nutrient sensing systems, which in turn tightly regulate cell metabolism. Some nutrient mechanisms present in *S. cerevisiae* cells will be detailed in Chapters II and III, but first I will try to illustrate that understanding nutrient sensing mechanisms is of primordial importance and has applications in medicine, e.g. for the treatment of diabetes, but also in industry, e.g. with regard to beer production.

1. Nutrient-induced insulin secretion in mammalian cells

Hormones are important molecules in eukaryotic organisms, as they allow intercellular communication and are key components of many regulatory pathways. Insulin is a hormone that helps mammalian cells to maintain glucose homeostasy in the blood, where glucose concentration, in healthy organisms, is at a constant value of ~ 5 mM (Thorens, 2001). The release of insulin from pancreatic β cells triggers a reduction of glucose production in the liver as well as an increase in glucose utilisation in insulin-dependent tissues. Decreased ability of cells to sense glycemic changes and to produce insulin leads to hyperglycemia, which can cause serious damage to the nerves, the blood vessels and many other systems. There are three types of diabetes. Type 1 diabetes describes the drastically reduced or null ability to produce insulin, while type 2 diabetes refers to the inability to use insulin effectively. The third type of diabetes, gestational diabetes mellitus (GDM), is pregnancy-related. According to the World Health Organisation (WHO), there were 171 million people with diabetes world-wide in 2000, and projections for the future are not predicting any improvement. Direct costs related to diabetes patients range from 2.5 to 15% of annual health care budgets (WHO). This figure underscores the importance of understanding the disease.

1.1. Glucose-stimulated insulin secretion

Cells in type 2 diabetes patients show a decreased ability to sense changes in glycemy. Two pathways are responsible for glucose detection by pancreatic β cells (reviewed in Thorens, 2001). Both require the glucose transporter GLUT2 to initiate the first step of glucose-stimulated insulin secretion (GSIS). The imported glucose enters glycolysis, resulting in ATP generation. The increase in the ATP/ADP ratio leads to depolarisation of the plasma membrane, opening of the Ca^{++} channels, following which Ca^{++} enters the cells and triggers exocytosis of the insulin granules. In this case the absolute flux of glucose is responsible for normal insulin secretion. GSIS is depicted in Figure 1-1.

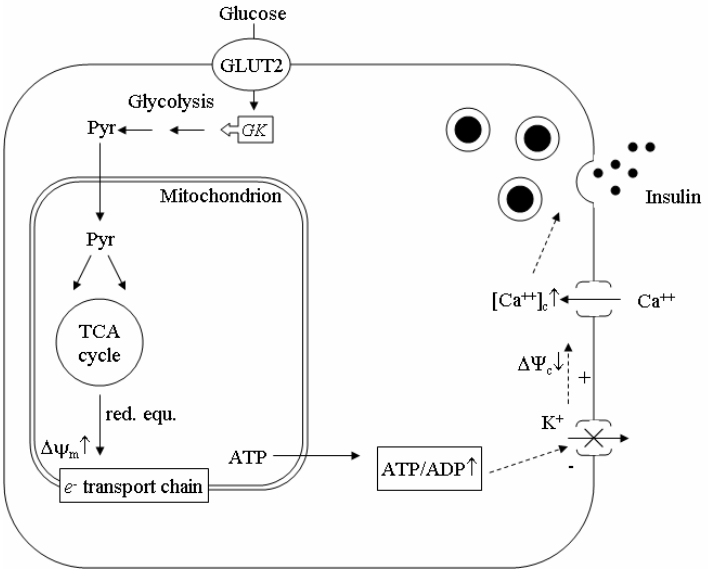

Figure 1-1. Model for glucose-stimulated insulin secretion in β cells. Glucose is taken up by the GLUT2 transporter and is phosphorylated by glucokinase (*GK*). Glucose-6-phosphate enters the glycolysis and is converted to pyruvate (Pyr), which enters the mitochondria and is a substrate for pyruvate dehydrogenase (*PDH*) and pyruvate carboxylase (*PC*).Reducing equivalents (red. equ.) are generated through the TCA cycle and transferred to the electron (e^-) transport chain, leading to hyperpolarisation of the mitochondrial membrane ($\Delta\Psi_m$) and generation of ATP. Transfer of ATP to the cytosol leads to increased ATP/ADP ratio. Subsequently, closure of K_{ATP}-channels depolarizes the cell membrane ($\Delta\Psi_c$). This opens voltage-dependent Ca^{++} channels, increasing cytosolic Ca^{++} concentrations ($[Ca^{++}]_c$), which triggers insulin exocytosis. Figure adapted from Newsholme *et al.,* 2005.

Experiments performed on mice showed that deficient glucose detection via the GLUT2 glucose transporter in pancreatic β cells results in a diabetic-like phenotype. $GLUT2^{-/-}$ mice have a very low life expectancy, due to deficient glucose-stimulated insulin secretion, as proven by the fact that generating transgenic mice in which the expression of GLUT2 or GLUT1 is under the control of the rat insulin promoter RIP gives rise to mice that can grow and breed normally (Thorens, 2001).

1.2. Amino acid-stimulated insulin secretion

Insulin secretion can be stimulated by amino acid metabolism, underscoring the importance of enzymes and transporters involved in amino acid uptake and metabolism in β cells (reviewed in Newsholme *et al.,* 2005). Four amino acids stand out as particularly potent inducers of insulin secretion: L-leucine, L-isoleucine, L-alanine and L-arginine, albeit via different mechanisms. In the case of the positively charged arginine, its uptake in the presence of glucose results in a depolarisation of the plasma membrane at neutral pH, subsequent activation of the voltage-dependent Ca^{++} channels, through which Ca^{++} enters the cells and triggers insulin exocytosis. L-leucine is believed to act as an allosteric activator of the glutamate dehydrogenase, leading to increased mitochondrial metabolism. Moreover, L-leucine metabolism results in increased ATP production and subsequent membrane depolarisation.

2. *Industrial applications of nutrient sensing in* Saccharomyces cerevisiae

Besides the applications in the medical field, investigating nutrient sensing can lead to improvement of various industrial processes. In *S. cerevisiae*, glucose repression strongly reduces catabolism of alternative carbon sources when glucose is present, thereby increasing processing times on the cheap, complex substrates, which are industrially interesting to convert to ethanol. Another important application involves the understanding of amino acid sensing, since amino acid metabolism in yeast is responsible for the production of several undesirable flavour and off-flavour compounds, the removal of which is a time-consuming process in beer production.

2.1. Glucose repression and bioethanol production on complex substrates

Ethanol production by microorganisms for use as a fuel is an attractive process since it would meet the increasing energy needs of our society with a renewable energy source. However several issues remain to be addressed before bioethanol production can be cheap enough to become a realistic substitution to fossil fuel. Reducing processing times would naturally be followed by a decrease in production costs. In this aspect, engineering of the glucose repression pathway in *S. cerevisiae* is an attractive possibility. Glucose repression is responsible for repressing expression of the genes encoding enzymes for the utilisation of carbon sources other than glucose when the latter is present. This means that, when growing on a sugar mixture, *S. cerevisiae* uses each sugar sequentially, which significantly increases processing times. *S. cerevisiae* strains have been successfully engineered that are able to consume simultaneously glucose and galactose (Klein *et al.*, 1998; Østergaard *et al.,* 2000), or in which xylose consumption rates were significantly increased (Roca *et al.,* 2004). Also recombinant *Escherichia coli* strains were constructed that are able to convert lignocellulosic substrates to ethanol by co-fermenting pentoses and hexoses (Nichols *et al.,* 2001; Hernández-Montalvo *et al.,* 2001).

2.2. Amino acid sensing and beer production

Insulin is not the only compound, the secretion of which can be triggered by amino acids. Indeed, amino acid metabolism in lager yeast, used in beer fermentation, leads to production of several important flavour and off-flavour compounds. L-α-amino acids are the main source of nitrogen in beer fermentation. Biosynthesis or degradation of the branched-chain amino acids isoleucine, leucine and valine, gives rise to many by-products, e.g. diacetyl (butanedione) and 2,3-pentanedione. These two vicinal diketones are the most important off-flavour compounds in beer. Both are produced non-enzymatically outside the cells from their precursors, acetolactate and acetohydroxybutyrate, respectively, which leak from the cells. They are formed during the main fermentation, and their amounts are reduced during beer maturation, which is a time-consuming, and thus expensive, step in beer production.

The lager brewing yeast *S. carlsbergensis* is an amphiploid organism with one genome closely related to *S. cerevisiae*, and another genome specific to lager yeast (Gjermansen *et al.,* 1988). Genetic manipulation of this lager strain is thus extremely laborious. An attempt was made to reduce diacetyl formation in lager yeast by blocking acetolactate formation (reviewed in Gjermansen *et al.,* 1988). Diacetyl is formed from acetolactate, which originates from the conversion of pyruvate by the acetolactate synthase, encoded by *ILV2,* during the first step of valine biosynthesis (Figure 1-2). Deletion of *ILV2* is not lethal in *S. cerevisiae* and cell growth is normal if the medium is supplemented with isoleucine, leucine and valine. However, this strategy fails with brewing yeast, where *ilv2* mutants show poor growth, even when branched-chain amino acids are supplied. Leucine uptake is similar in lager yeast and in *S. cerevisiae*, whereas isoleucine and especially valine uptake is poorer in lager yeast (Kielland-Brandt *et al.,* 1990). This result indicates that branched-chain amino acid uptake is involved in the poor growth exhibited by *ilv2* lager yeast mutants.

ILV5 encodes the acetohydroxyacid reductoisomerase responsible for the conversion of acetolactate to dihydroxyisovalerate, during the second step of valine biosynthesis (Figure 1-2). Thus another strategy to reduce diacetyl formation is to increase turnover of its precursor, acetolactate, by increasing activity of this enzyme (reviewed in Gjermansen *et al.,* 1988). Indeed introduction of extra copies of the *ILV5* gene on a 2µ-based multi-copy vector in *S. cerevisiae* was followed by a 50% decrease in diacetyl formation. The *ILV5* gene product is also a key target in the attempts for reducing 2,3-pentanedione formation, as it catalyses the conversion of acetohydroxybutyrate, its precursor, to dihydroxymethylvalerate. The strategy described above also resulted in decreased 2,3-pentanedion formation.

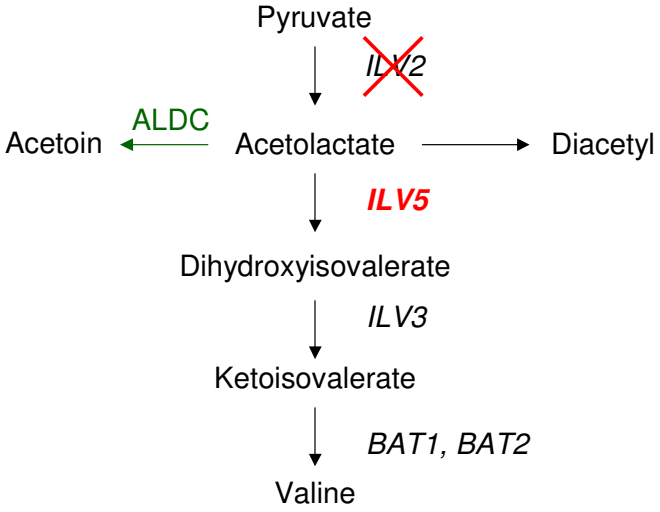

Figure 1-2. Possible targets for reducing diacetyl formation during beer fermentation: deletion of *ILV2*, overexpression of *ILV5* or heterologous production of ALDC to increase acetolactate turnover. See text for details.

An alternative approach to reduce the amount of precursors to the vicinal diketones is to express heterologous enzymes able to convert them rapidly. The gene encoding the acetolactate dicarboxylase (ALDC) from *Acetobacter aceti* ssp. *xylinum* was thus introduced on a Yep-type plasmid, placed under the control of the glyceraldehydes-3-phosphate dehydrogenase promoter, and transformed into brewer's yeast (Yamano *et al.,* 1994). ALDC converts acetolactate and acetohydroxybutyrate to flavour-less compounds (Figure 1-2). This strategy allowed reduction of diacetyl formation, and also, to a lesser extent, of 2,3-pentanedione formation. Others have constructed a strain in which three copies of the *ILV5* gene are integrated in the genome. The resulting strain was stable in the absence of selective pressure and diacetyl production was reduced (Mithieux and Weiss, 1995).

3. Conclusions

In this section I have tried to emphasise the medical and industrial applications that can result from understanding nutrient sensing in *S. cerevisiae*. Insulin secretion can be induced by glucose but also by some amino acids, and recent clinical assessments showed that administrating amino acids to long-term type 2 diabetes patients nearly tripled insulin secretion (van Loon *et al.,* 2003), suggesting new therapeutic possibilities. In beer production, costs could be greatly reduced if the maturation step, aiming at reducing diacetyl and 2,3-pentanedione amounts, could be skipped. The use of *S. cerevisiae* as a model organism to understand how cells react to extracellular amino acids is relevant since genetic manipulation of lager yeast is extremely laborious.

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Chapter II

Glucose sensing in *Saccharomyces cerevisiae*

Glucose is the preferred carbon source of most organisms on Earth, probably because it is the most common sugar. Thus organisms have developed efficient ways of utilising this substrate. For cells to be able to metabolise glucose, they must be able to detect its presence in the environment and to take it up. In *S. cerevisiae*, glucose can be sensed extracellularly, independently from its uptake, and intracellularly, after its conversion to glucose-6-phosphate.

1. Glucose repression and the role of Mig1p

Presence of glucose in the extracellular environment leads to transcriptional repression of numerous genes encoding proteins involved in metabolism of alternative carbon sources and respiration e.g. (reviewed in Gancedo, 1998; Carlson, 1999; Rolland *et al.,* 2001). This mechanism is known as **glucose (catabolite) repression**.

Glucose repression involves the Mig1p transcription repressor (Carlson *et al.,* 1984; Nehlin and Ronne, 1990). Mig1p recruits the Ssn6-Tup1 co-repressor via interaction with Ssn6p, resulting in repressed transcription when glucose levels are high (Treitel and Carlson, 1995). Mig1p has been shown to bind to the promoters of the *GAL1*, *GAL4* and *MAL62* glucose-repressed genes (Wang *et al.,* 1997; Frolova *et al.,* 1999). The phosphorylation status of Mig1p appears to correlate with its subcellular localisation: phosphorylated Mig1p is cytoplasmic, dephosphorylated Mig1p is nuclear (Figure 2-1; De Vit *et al.,* 1997). *MIG2* and *MIG3* encode two homologues of Mig1p (Luftiyya and Johnston, 1996; Luftiyya *et al.,* 1998). Mig2p is involved in glucose repression, while no physiological role was found for Mig3p so far (Luftiyya *et al.,* 1998). Unlike Mig1p, the subcellular localisation of Mig2p does not appear to be glucose-regulated and *MIG2* expression is not repressed by glucose. Mig1p and Mig2p bind similar sites but with different affinities. Thus Mig1p and Mig2p appear to be redundant proteins that respond differently to glucose (Luftiyya *et al.,* 1998).

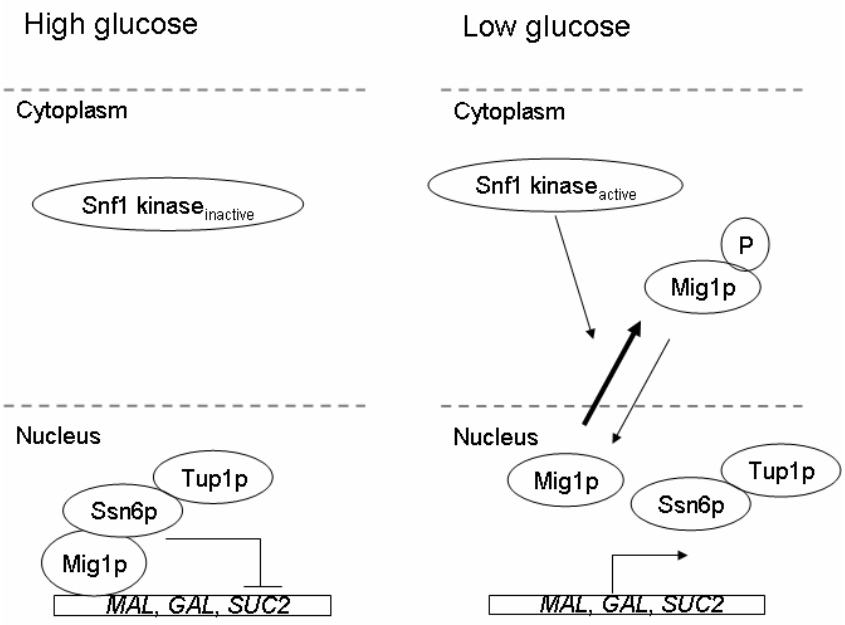

Figure 2-1. Simplified model of Mig1p regulation by Snf1 kinase.

The Snf1 kinase complex is formed by the Snf1 (Sucrose Non Fermenting) kinase protein (α subunit), the Snf4 activating subunit (γ subunit) and either of three scaffolding proteins (β subunits), Sip1p, Sip2p and Gal83p, which are not essential for Snf1p activity (reviewed in Carlson, 1999). In the presence of glucose, the regulatory domain of Snf1p inhibits its catalytic kinase domain; when glucose is absent, Snf4p binds to the regulatory domain of Snf1p, preventing autoinhibition (Jiang and Carlson, 1996). Snf1p and Mig1p interact in vivo, and the Snf1 kinase complex is able to phosphorylate Mig1p in vitro (Treitel *et al.,* 1998). The β subunits appear to be responsible for targeting of the Snf1 kinase complex to different subcellular compartments. In glucose-grown cells, Gal83p, Sip1p and Sip2p, tagged with the Green Fluorescent Protein (GFP), are all cytoplasmic. In the absence of glucose, however, Gal83p-GFP was predominant in the nucleus, Sip2p-GFP in the cytoplasm and Sip1p-GFP in the vacuole (Vincent *et al.,*, 2001).

Snf1p is activated by phosphorylation (Wilson *et al.,* 1996). Three Snf1 kinase kinases have been identified so far: Tos3p, Elm1p and Pak1p (Sutherland *et al.,* 2003; Nath *et al.,* 2003; Kim *et al.,* 2005). Recent work showed that the phosphorylation status of Snf1p regulates its cellular localisation (Hedbacker *et al.,* 2004). Pak1p seems to play a determinant role in that context: it phosphorylates Snf1p associated with Gal83p, resulting in migration of Snf1-Gal83p to the nucleus, suggesting that Mig1p phosphorylation by Snf1p is a nuclear event (Hedbacker *et al.,* 2004). Others have investigated whether there was a one-to-one correspondence between the three Snf1 kinase kinases and the three Snf1 kinase isoforms and found that this is not the case, although each isoform exhibits different, stress-dependent upstream kinase preferences (McCartney *et al.,* 2005).

The protein phosphatase 1, of which Glc7p is the catalytic subunit and Reg1p the regulatory subunit, interacts with the catalytic domain of active Snf1p via Reg1p (Ludin *et al.,* 1998). Reg1p is one of the regulatory subunits that target Glc7p to its substrates.

The interaction of Reg1p with Snf1p is glucose-dependent. The Snf1 kinase remains inactive in a *reg1Δ* mutant regardless of glucose presence. These findings led to the proposal that Glc7p dephosphorylates a component of the Snf1 kinase complex after Reg1p binds Snf1 in response to glucose (Ludin *et al.,* 1998). Snf1 kinase phosphorylates Reg1p in the absence of glucose, while Glc7p dephosphorylates Reg1p when glucose is present, suggesting that phosphorylation of Reg1p by the Snf1 kinase is responsible for dissociation of Reg1-Glc7 from the kinase complex (Sanz *et al.,* 2000). Hexokinase 2 (Hxk2p), which converts glucose to glucose-6-phosphate, is known to be involved in glucose repression, although its role is still poorly understood (Carlson, 1999). Hxk2p is dephosphorylated by Reg1p-Glc7p in response to glucose (Alms *et al.,* 1999; Randez-Gil *et al.,* 1998). Hxk2p appears to regulate Reg1p phosphorylation by the Snf1 kinase complex (Sanz *et al.,* 2000). Moreover, Hxk2p has been found recently to interact directly with Mig1p in vivo in the nucleus (Moreno *et al,* 2005).

2. Glucose as a hormone: extracellular sensing

Not only can glucose be detected after it has been taken up and converted to glucose-6-phosphate in the first step of the glycolysis, it can also be sensed directly at the plasma membrane. The signal results in transcriptional activation of genes encoding hexose transporters. This mechanism is known as glucose induction (reviewed in Kim and Johnston, 2005).

2.1. Glucose sensing occurs at the plasma membrane via two glucose transporter homologues.

Two proteins are responsible for glucose sensing at the plasma membrane: Snf3p and Rgt2p, which have respectively high and low affinities for glucose (Özcan *et al.,* 1996a; Özcan *et al.,* 1998). The C-terminal tails of Snf3p and Rgt2p are necessary for signalling (Özcan *et al.,* 1998). Transfer of the C-terminus of Snf3p to Hxt1p or Hxt2p converts these proteins from being glucose transporters to glucose sensors, and isolation of dominant mutations in *SNF3* and *RGT2* were isolated that confer signalling even in the absence of glucose (Özcan *et al.,* 1998), showing that glucose uptake is not required for glucose induction.

2.2. The Rgt1p transcription factor

The signal generated by Snf3p and Rgt2p is transmitted to target genes and their transcription is then turned on. In the absence of glucose, transcription of the target genes is repressed by the Rgt1 (Restores Glucose Transport) protein (Özcan and Johnston, 1995). Gel shift assays have established that Rgt1p binds to the *HXT1* promoter (Özcan *et al.,* 1996b), while chromatin immunoprecipitation assays have shown binding of Rgt1p to the promoters of *HXT3* and *HXT4* (and *HXT1*) (Flick *et al.,* 2003). Recent work based on DNA arrays and β-galactosidase measurements from the promoters of the potential target genes allowed identification of numerous genes potentially regulated by Rgt1p (Kaniak *et al.,* 2004; also discussed below). The Rgt1p consensus binding site sequence is 5'-

CGGANNA-3', and Rgt1p binds poorly to sequences containing less than five binding sites; moreover, Rgt1p binding to DNA is glucose-regulated (Kim *et al.,* 2003).

2.3. Inactivation of Mth1p and Std1p is required for glucose induction

Mth1p and Std1p are two homologous proteins involved in glucose induction (Özcan *et al.,* 1993; Hubbard *et al.,* 1994; Schulte *et al.,* 2000); two-hybrid screens showed that they interact with the cytoplasmic, C-terminal tails of Snf3p and Rgt2p (Schmidt *et al.,* 1999; Lafuente *et al.,* 2000). Mth1p and Std1p are required for Rgt1p-mediated repression of transcription in the absence of glucose: they actually interact with Rgt1p in a glucose-regulated manner (Tomás-Cobos and Sanz, 2002; Lakshmanan *et al.,* 2003). Inactivation of Mth1p and Std1p is required for glucose induction, and degradation of Mth1p is necessary for dissociation of Rgt1p from the *HXT1, HXT3* and *HXT4* promoters (Flick *et al.,* 2003).

2.4. Phosphorylated Mth1p binds to SCF^{Grr1}, which targets it for degradation by the 26S proteasome

Protein ubiquitination is a major mechanism controlling protein fate by targeting proteins for degradation by the 26S proteasome. Ubiquitination occurs via the action of three enzyme complexes, controlling the three steps of the process (Figure 2-2). A ubiquitin-activating enzyme, E1, is responsible for ubiquitin activation; the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2; a ubiquitin ligase, E3, stands for substrate recognition and facilitates ubiquitin transfer from the E2 enzyme to the substrate. SCF complexes belong to the family of E3 ubiquitin ligases. SCF complexes are composed of Skp1p, a cullin protein and an F-box protein responsible for substrate recognition (Patton *et al.,* 1998).

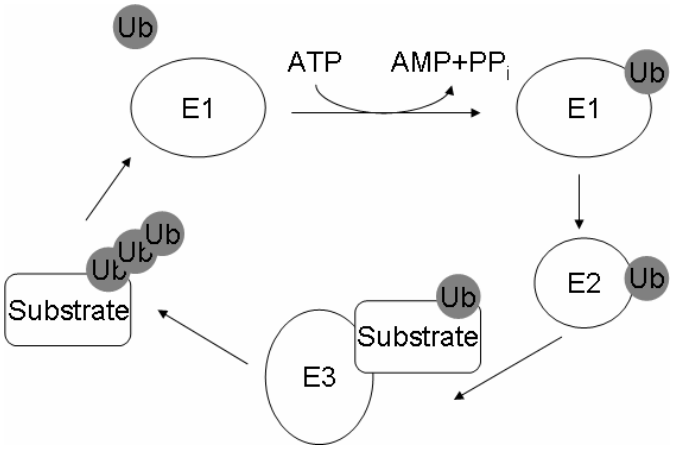

Figure 2-2. Protein ubiquitination by the E1, E2, E3 enzyme complexes.

The F-box protein Grr1 (Glucose Repression Resistant; Bailey and Woodword, 1984) has long been known to be required for glucose induction (Flick and Johnston, 1991; Vallier *et al.,* 1994; Özcan *et al.,* 1994) and it acts in this pathway as part of an SCF complex

(Ning Li and Johnston, 1997). SCF^{Grr1} targets Mth1p, and perhaps Std1p, for degradation (Spielewoy *et al.,* 2004). Recognition of Mth1p requires its prior phosphorylation by the yeast casein kinases Yck1/2p (Spielewoy *et al.,* 2004). This is in agreement with the observation that glucose induction of the *HXT1* promoter is deficient in a *yck1Δ* $yck2^{ts}$ mutant; in addition, this mutant lacks glucose-induced degradation of Mth1p and Std1p (Moriya and Johnston, 2004). The glucose sensors appear to be responsible for recruiting Std1p and Mth1p in the vicinity of the Yck for phosphorylation. This theory is based on the observations that Yck1p interacts with Rgt2p, probably via a transmembrane domain; transferring the C-terminal tail of Rgt2p to Yck1p or overexpressing Yck1p results in constitutive signalling (Moriya and Johnston, 2004).

2.5. Additional factors involved in glucose induction

Besides the proteins mentioned above, a number of additional factors are involved in glucose induction. The co-repressors Ssn6p and Tup1p are necessary for signalling and are recruited via interaction of Ssn6p with Rgt1p (Trumbly, 1986; Tomás-Cobos and Sanz, 2002; Polish *et al.,* 2005). A recent study showed that the proteins Bmh1p and Bmh2p are required for proper glucose induction of the *HXT1* gene (Tomás-Cobos *et al.,* 2005). Σ1278b-derived cells lacking *BMH1* and *BMH2* exhibit no induction of *HXT1* transcription in response to glucose. Bmh1p and Bmh2p are so-called 14-3-3 proteins that are positive regulators of the TOR (Target Of Rapamycin) pathway (Beck and Hall, 1999; Crespo and Hall, 2002). The TOR pathway has diverse regulatory roles in the cell, and is e.g. involved in polarisation of the actin cytoskeleton, autophagy, ribosome biogenesis, and regulation of AAP turnover and nutrient metabolism (Chapter III). The protein kinases Tor1p and Tor2p regulate this pathway, but their action can be inhibited by a complex formed by the immunosuppressive drug rapamycin with the immunophilin FKBP12. Rapamycin inhibits *HXT1* induction in wild-type cells, but not in *tor1* mutants, which are insensitive to rapamycin (Tomás-Cobos *et al.,* 2005). Since TOR regulates the activity of Protein Phosphatase 2A (PP2A), the authors investigated the effect of deletion of *RTS1, CDC55*, both encoding regulatory subunits of PP2A, *TPD3*, encoding its scaffolding subunit, or simultaneous deletion of *PPH21* and *PPH22*, encoding redundant catalytic subunits. Glucose induction from the *HXT1* gene was found to be increased in the *cdc55Δ* mutant as compared to the wild-type, suggesting that PP2A plays a role in down-regulating the pathway.

2.6. Interaction of Mth1p and Std1p with Rgt1p is responsible for transcriptional repression in the absence of glucose

The transcription repressor Rgt1p is phosphorylated both under inducing and non-inducing conditions, but presence of glucose leads, after SCF^{Grr1}-dependent degradation of Mth1p (and Std1p?), to its hyperhosphorylation and subsequent dissociation from the promoters of target genes (Flick *et al.,* 2003). A central region of Rgt1p can interact with its N-terminus, which also contains the DNA-binding domain (Polish *et al.,* 2005). The authors propose the following model for the action of Mth1p and Rgt1p. In the absence of glucose, Rgt1p is hypophosphorylated and interacts with Mth1p, thus the interaction between the central regulatory domain of Rgt1p with its N-terminus is inhibited, and the N-terminal DNA-binding domain is able to bind promoter DNA, thereby repressing transcription. In the presence of glucose, Mth1p is degraded and phosphorylation sites are

unmasked, enabling intramolecular interaction, whereby the DNA-binding domain is masked, which results in dissociation of Rgt1p from promoter DNA.

2.7. The targets of the Snf3/Rgt2-Rgt1 glucose induction pathway

A recent study reported identification of new target genes for the Rgt1p-mediated glucose induction pathway (Kaniak *et al.,* 2004). Genome-wide transcription profiles were determined from galactose-grown *rgt1Δ* cells, *RGT2-1* or *SNF3-1* cells (these mutations confer constitutive signalling independent on extracellular glucose; Özcan *et al.,* 1998), and glucose-grown *snf3Δ rgt2Δ* cells. Genes that were potential targets of Rgt1p were verified by using β-galactosidase reporter assays and chromatin immunoprecipitation. Besides the hexose transporter genes *HXT1, HXT2, HXT3, HXT4, HXT5, HXT8,* of which the transcription levels of *HXT1, HXT2, HXT3* and *HXT4* were found to be down-regulated in glucose-grown *grr1Δ* cells (Eckert-Boulet *et al.,* 2005), new targets were thus identified. Interstingly, *STD1, MTH1, MIG2* and *MIG3* were found to be under Rgt1p-mediated regulation. Mig2p and Mig3p are involved in glucose repression, while Std1p and Mth1p are involved in glucose induction, and the authors conclude that the two pathways are closely intertwined.

2.8. A model for glucose induction

Several questions remain to be answered before the picture of the glucose induction pathway is complete. Which mechanism is responsible for linking the presence of extracellular glucose to the recruitment of Mth1p and Std1p by the glucose sensors? It is likely that binding of glucose to Snf3p or Rgt2p causes a conformational change, and this could lead to uncovering of the binding sites of Mth1p and Std1p present on their cytoplasmic C-terminal tails. How does degradation of Mth1p (and Std1p?) in the cytoplasm affect Rgt1p?

One can assume that Mth1p and Std1p shuttle between nucleus, where they help Rgt1p repress transcription in the absence of glucose, and cytoplasm, where they are targeted for degradation in the presence of glucose. The equilibrium between cytoplasmic and nuclear Mth1p and Std1p could be affected by their phosphorylation and subsequent degradation, so that the amounts of the nuclear forms would decrease, thus inhibiting binding of Rgt1p to the promoters of target genes. The kinase responsible for Rgt1p phosphorylation still remains unidentified. This model is illustrated in Figure 2-3.

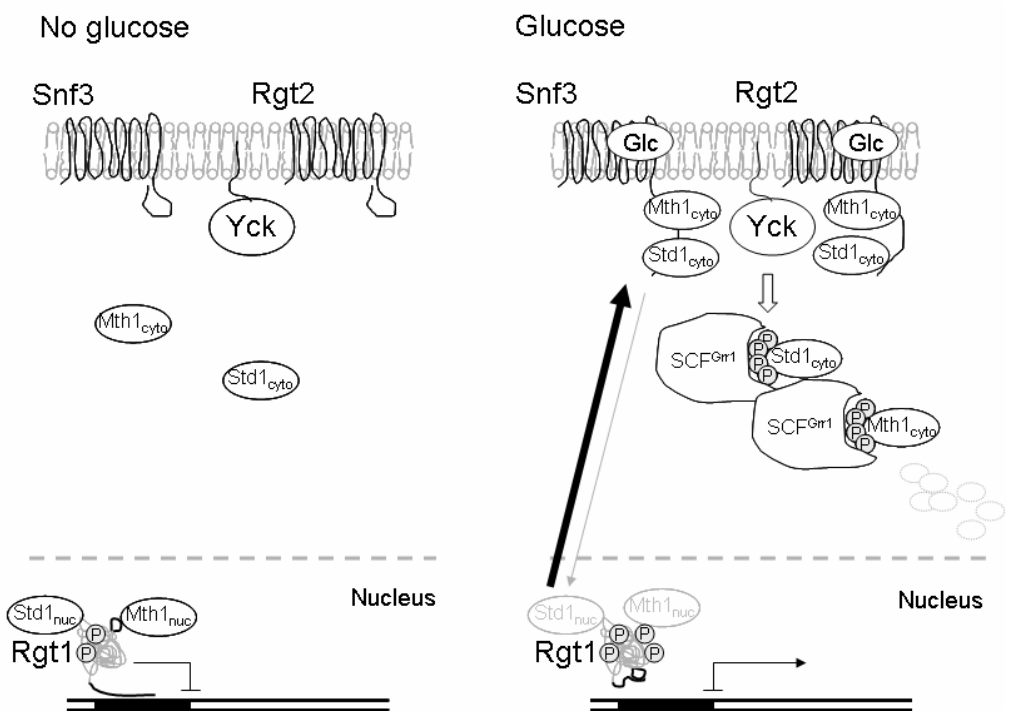

Figure 2-3. Model for Rgt1p-mediated glucose induction of *HXT* genes. When glucose is absent, hypophosphorylated Rgt1p, together with nuclear Mth1p and Std1p, binds promoters of the target genes and represses transcription. When extracellular glucose is available, a conformational change in either of the sensors Snf3p and Rgt2p allows binding of cytoplasmic Mth1p and Std1p to their cytoplasmic tail and brings them close to casein kinase I (Yck). Yck-phosphorylated Std1p and Mth1p are ubiquitylated by SCF^{Grr1} and subsequently degraded by the 26S proteasome (not represented). The equilibrium between the nuclear and cytoplasmic forms of Mth1p and Std1p is pulled towards the cytoplasmic forms, leading to hyperhosphorylation of Rgt1p on the unmasked sites, and its dissociation from the promoters.

3. Conclusions

Glucose is the preferred carbon source of most microorganisms and, as such, is the initiator of complex regulation around its own uptake and metabolism. The Rgt1p-mediated glucose induction pathway leads to increased transcription of hexose transporter genes. Extracellular glucose also affects uptake and metabolism of other, less-favoured carbon sources, by repressing transcription of the genes encoding the necessary proteins. This pathway is mediated by Mig1p and the Snf1 kinase. It is not all too surprising that these two pathways should interact, as highlighted recently (Kaniak *et al.,* 2004). Indeed newly identified targets of Rgt1p are the Mig1p homologue-encoding genes *MIG2* and *MIG3*, but also *MTH1* and *STD1*, indicating that the glucose induction pathway is auto-regulated.

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Chapter III

Nitrogen and amino acid sensing

In order to synthesise proteins, cells need to take up nitrogen sources from the environment. Amino acids can be used as a nitrogen source, and are imported by amino acid permeases (AAPs) (Surdin *et al.,* 1965; Grenson *et al.*, 1966; Grenson, 1966; Gits and Grenson, 1967; Grenson *et al.,* 1970). AAPs exhibit diverse affinities and substrate ranges and are responsible for the uptake of all 20 common amino acids and some other nitrogenous compounds (reviewed in Grenson, 1992). Several pathways are responsible for regulation of the AAPs. Nitrogen Catabolite Repression ensures that AAPs with broad substrate ranges are repressed when cells grow on a good nitrogen source. The Target Of Rapamycin pathway regulates turnover of the General Amino acid Permease Gap1p and the tryptophan permease Tat2p. Finally, the SPS-mediated amino acid induction pathway leads to increased transcription of AAP genes upon the presence of amino acids in the environment.

1. Amino acid permeases

Amino acid permeases belong to the Yeast Amino acid Transporter family (YAT) of the Amino acid/Polyamine/Organocation (APC) superfamily of transporters (Jack *et al.,* 2000). Amino acid transport across the plasma membrane is active and driven by the proton gradient (reviewed in André, 1995; Horak, 1997). Twenty-four *Saccharomyces cerevisiae* proteins have been classified as members of the APC family (Paulsen *et al.,* 1998), but only twenty were found to belong to the YAT family. Table 3-1 presents an overview of the AAP members. They have been grouped in four clusters based on the homology between gene products (Nelissen *et al.,* 1997; Regenberg *et al.,* 1999).

Table 3-1. Overview of the AAP genes members of the APC superfamily (Jack *et al.,* 2000). Adapted and updated from Regenberg *et al.,* 1999; Nelissen *et al.,* 1997. NCR: Nitrogen Catabolite Repression; SPS: Ssy1-Ptr3-Ssy5 sensor of extracellular amino acids, see below; TOR: Target Of Rapamycin.

Cluster	Gene name	ORF name	Substrates	Regulation	References
Cluster I	*AGP1* high-Affinity Glutamine Permease	*YCL025C*	Asn, Gln, neutral amino acids	NCR SPS	1-3
	GNP1 GlutamiNe Permease	*YDR508C*	Gln, Cys, Leu, Met, Ser, Thr, Asn	SPS	4-6
	BAP2 Branched-chain Amino acid Permease	*YBR068C*	Ile, Leu, Val, Cys, Trp, Tyr, Phe, Met	SPS TOR	3, 6-12
	BAP3 Branched-chain Amino acid Permease	*YDR046C*	Val, Cys, Ile, Leu	SPS	3, 5-6, 9, 12-14
	TAT1	*YBR069C*	Tyr, Ile, Leu, Val,	SPS	3, 5-6, 9,

			His, Trp, Cys		12, 15-16
	TAT2	*YOL020W*	Trp, Cys, Tyr, Phe	SPS TOR	3, 5, 15, 17
Cluster II	*HIP1* HIstidine Permease	*YGR191W*	His	TOR	17-19
	GAP1 General Amino acid Permease	*YKR039W*	All 20 common amino acids	NCR	20-22
	MMP1 S-MethylMethionine Permease	*YLL061W*	Met derivatives		23
Cluster III	*ALP1*	*YNL270C*	Arg		3, 24
	CAN1 CANavanine resistance	*YEL063C*	Arg	NCR	25-28
	LYP1 LYsine Permease	*YNL268W*	Lys, Arg		29-30
Unclustered	*DIP5* DIcarboxylic amino acid Permease	*YPL265W*	Asp, Glu, Ala, Gly, Ser, Gln, Asn	SPS	3, 6, 31
	PUT4 Proline UTilisation	*YOR348C*	Pro, GABA, Ala, Gly	NCR	3, 22, 28, 32-34
	AGP2	*YBR132C*	Carnitine, polyamines	SPS, NCR	6, 35-37
	AGP3	*YFL055W*	Leu	NCR	37
	CTR1 Copper TRansport	*YGL077C*	Copper		38
	MUP1 Methionine UPtake	*YGR055W*	Met	SPS	39
	MUP3	*YHL036W*	Met		39
	TPO5 Transport of POlyamines	*YKL174C*	Polyamines		40
	BIO5	*YNR056C*			41
	SAM3 S-AdenosylMethionine metabolism	*YPL274W*	Met derivatives		23
	SSY1 Sulfonylurea Sensitive on YPD	*YDR160W*	Sensor function		42

1, Schreve *et al.,* 1998; 2, Iraqui *et al.,* 1999; 3, Regenberg *et al.,* 1999; 4, Zhu *et al.,* 1996; 5, Düring-Olsen *et al.,* 1999; 6, Eckert-Boulet *et al.,* 2005; 7, Grauslund *et al.,* 1995; 8, Didion *et al.,* 1996; 9, Didion *et al.,* 1998; 10, Nielsen *et al.,* 2001; 11, Omura and Kodama, 2004; 12, Eckert-Boulet *et al,* 2004; 13, de Boer *et al.,* 1998; 14, de Boer *et al.,* 2000; 15, Schmidt *et al.,* 1994; 16, Bajmoczi *et al.,* 1998; 17, Beck *et al.,* 1999; 18, Crabeel and Grenson, 1970; 19, Tanaka and Fink, 1985; 20, Grenson *et al.,* 1970; 21, Jauniaux and Grenson, 1990; 22, Courchesne and Magasanik, 1983; 23, Rouillon *et al.,* 1999; 24, Sychrova and Chevallier, 1994; 25, Grenson *et al.,* 1966; 26, Ahmad and Bussey, 1986; 27, Hoffmann, 1985; 28, Daugherty *et al.,* 1993; 29, Sychrova and Chevallier, 1993; 30, Grenson, 1966; 31, Regenberg *et al.,* 1998; 32, Lasko and Brandriss, 1981; 33, Vandenbol *et al.,* 1989; 34, Jauniaux *et al.,* 1987; 35, Van Roermund *et al.,* 1999; 36, Aouida *et al.* 2005; 37, Schreve and Garrett, 2004; 38, Dancis *et al.,* 1994; 39, Isnard *et al.,* 1996; 40, Tachihara *et al.,* 2005; 41, Entian *et al.,* 1999; 42, Jørgensen *et al.,* 1998.

The amino acids taken up by cells from the environment are metabolised and used for protein synthesis, not only under normal, but also under extreme conditions. Tryptophan uptake, for example, appears to play a determinant role in growth under high-pressure (Abe and Horikoshi, 2000). High pressure causes cell arrest in G_1, but overexpression of

the *TAT2* tryptophan permease gene or high tryptophan concentration in the environment enable high-pressure growth. The authors also report that high pressure leads to reduced tryptophan uptake in wild type cells, and suggest that this is the primary cause of G_1 arrest.

Although uptake of nitrogenous compounds appears to be the primary function of AAPs, two members of the AAP family have been shown to function as sensors that activate two different pathways, independently of their ability to transport amino acids. Ssy1p is an AAP homologue that exhibits a long, cytoplasmic, N-terminal tail that is required for its ability to activate amino acid-induced expression of several AAP genes (detailed below). It has never been possible to show that Ssy1p actually imports amino acids, but it has been shown that transport through Ssy1p is not required for its sensing function (Gaber *et al.,* 2003). The General Amino acid Permease Gap1p has been reported to act not only as an amino acid transporter, but also as a sensor triggering the FGM (Fermentable Growth Medium) pathway (Thevelein, 1994; Donaton *et al.,* 2003). Cells growing on glucose exhibit low stress resistance, low glycogen and trehalose contents and weak cell walls, while glucose-starved cells display the opposite phenotype. Addition of glucose to cells growing on a non-fermentable carbon source results in activation of protein kinase A via a transient spike in cAMP concentrations. The following phosphorylation cascade leads to activation of trehalase and glycogen phosphorylase, thus reducing intracellular trehalose and glycogen contents, but also to repression of STRE-controlled genes, and subsequent loss of stress resistance. The same phenotype is observed when cells starved for nitrogen, phosphate or sulphate are switched to a medium where these nutrients are no longer limiting. Activation of the FGM pathway when a nitrogen source is added to glucose-grown, nitrogen-starved cells is dependent on the presence and activity of Gap1p. Mutants carrying *gap1* alleles deficient in transport have been identified that are still able to induce the FGM pathway (Donaton *et al.,* 2003).

2. Target Of Rapamycin (TOR)

The Target Of Rapamycin (TOR) pathway is dependent on two protein kinases, Tor1p and Tor2p, and is highly conserved from yeast to humans (for review, see Crespo and Hall, 2002). The Tor proteins are inhibited by a complex formed of the immunosuppressive drug rapamycin bound to the immunophilin FKBP12. The TOR pathway has diverse regulatory roles in the cell, and is e.g. involved in polarisation of the actin cytoskeleton, autophagy, ribosome biogenesis, and regulation of AAP turnover and nutrient metabolism.

Three phosphatases have been identified as components of the TOR pathway so far: the type 2A protein phosphatase PP2A, more precisely its catalytic subunits Pph21p and Pph22p, the phosphatase-associated protein Tap42p and the type 2-related phosphatase Sit4p. Tap42p associates with either Pph21/Pph22p or Sit4p depending on nutrient status. Under excess nitrogen conditions, the Tor proteins stimulate interaction of Tap42p with Sit4p, thus inactivating Sit4p; this interaction is disrupted under limiting nitrogen conditions, thus Sit4p is active (Di Como and Arndt, 1996). The Tor proteins can phosphorylate Tap42p, which is then able to compete with Pph21/Pph22p, the catalytic subunits of PP2A, for binding to the Cdc55p-Tpd3p heterodimer, where Cdc55p and

Tpd3p are a regulatory and a scaffold subunit, respectively, of PP2A (Figure 3-1; Jiang and Broach, 1999). The fact that Tap42p is dephosphorylated more slowly than it dissociates from Sit4p indicates that Tap42p phosphorylation status is unlikely to be responsible for regulating Sit4p activity (Di Como and Arndt, 1996).

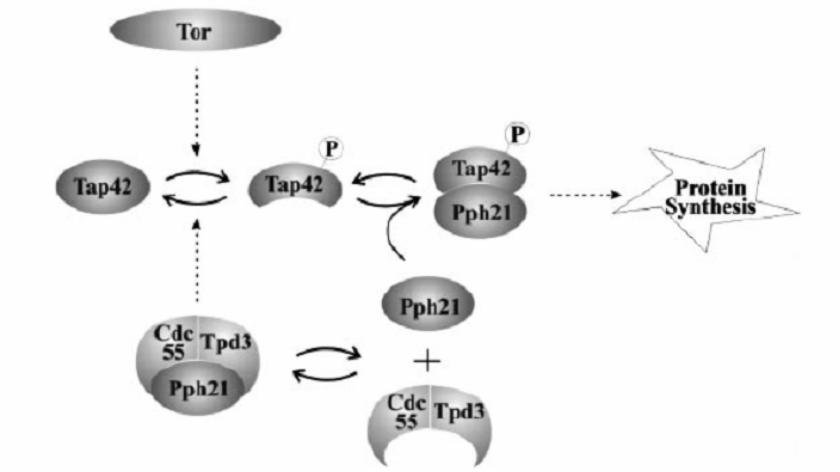


Figure 3-1. **Model for the role of PP2A in TOR regulation of protein synthesis.** Tor phosphorylates Tap42p, while the heterotrimeric PP2A, composed of Cdc55p, Tpd3p and Pph21p, dephosphorylates Tap42p. Phosphorylated Tap42p can associate with the PP2A catalytic subunit Pph21p and regulate protein synthesis; the heterodimer Cdc55p/Tpd3p is then released. Figure adapted from Jiang and Broach, 1999.

The Tor proteins regulate activity and turnover of several AAPs (Crespo and Hall, 2002; Table 3-1). They control phosphorylation of the Ser/Thr nitrogen permease reactivator kinase Npr1p (Schmidt *et al.,* 1998). Npr1p is dephosphorylated and active under limiting nitrogen conditions, where it phosphorylates the general amino acid permease Gap1p, which probably protects it from ubiquitination and degradation (De Craene *et al.,* 2001). Bap2p and Tat2p are regulated inversely to Gap1p, since their degradation is induced by nutrient starvation (Schmidt *et al.,* 1998; Omura *et al.,* 2001). Npr1p targets the tryptophan permease Tat2p for ubiquitination and degradation (Schmidt *et al.,* 1998). Starvation-induced degradation of Bap2p involves Npr1p-dependent phosphorylation (Omura *et al.,* 2004). Thus it appears that nutrient starvation induces degradation of specific, high-affinity permeases, while the low-specificity, broad substrate range permease Gap1p is stabilised.

Besides being involved in the turnover of Gap1p, Bap2p and Tat2p, TOR appears to play a role in down-regulating SPS-mediated induction of AAP genes: rapamycin addition strongly reduces the transcriptional induction observed from the *AGP1* promoter upon the presence of extracellular amino acids (see below).

3. Nitrogen Catabolite Repression (NCR)

Nitrogen sources are qualified of good or preferred nitrogen sources when they are easily converted by the cells into glutamate or glutamine, while poor nitrogen sources necessitate several conversion steps before leading to glutamate or glutamine (Magasanik and Kaiser, 2002, for review). The presence of a preferred nitrogen source results in transcriptional repression of the genes encoding enzymes and transporters required for uptake and metabolism of poor nitrogen sources; this mechanism is known as Nitrogen Catabolite Repression (NCR) (Cooper, 2002, for review).

The GATA family of transcription factors comprises factors able to recognise a nucleotide sequence with GATA at its core. Four GATA factors are involved in NCR: Gln3p and Gat1p are transcription activators, while Dal80p and Deh1p are transcription repressors. Competition for binding to GATA sequences between the activators and repressors is responsible for a tightly regulated transcription of these genes, and for the steady-state that is reached when transcription of NCR-sensitive genes is derepressed, i.e. Dal80p and Deh1p are not involved in repression of NCR-sensitive genes but in regulating their derepression together with Gln3p and Gat1p (Cooper, 2002). Transcription of *GAT1, DEH1* and *DAL80* is regulated by Gat1p, Deh1p, Dal80p and Gln3p (Coffman *et al.,* 1997). *URE2* encodes a protein that negatively regulates Gnl3p and Gat1p (Courchesne and Magasanik, 1988) by regulating their cellular localisation (Figure 3-2; Cox *et al.,* 2000). Ure2p interacts with Gln3p in the cytoplasm (Blinder *et al.,* 1996). The cellular localisation of Gln3p is also related to its phosphorylation status: cytoplasmic Gln3p is hyperphosphorylated but it remains unclear which kinase is involved; the Tap42p- and Pph3p-dependent phosphatases appear to be responsible for dephosphorylation of Gln3p (Bertram *et al.,* 2000). It is not clear whether it is the phosphorylation status of Gln3p or its interaction with Ure2p that sequester it to the nucleus. Rapamycin addition was found to induce expression of many NCR-sensitive genes (Cardenas *et al.,* 1999; Hardwick *et al.,* 1999; Bertram *et al.,* 2000), indicating that the Tor proteins are active under NCR. They have been suggested to regulate the phosphatases involved in Gln3p dephosphorylation (Cooper, 2002). Figure 3-2 presents a possible model for NCR.

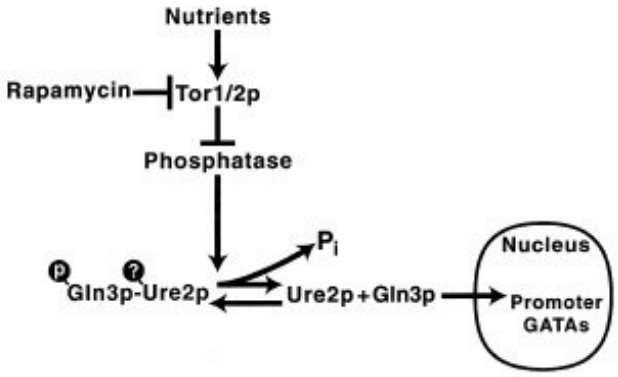


Figure 3-2. Model of the regulatory pathway by which rapamycin induces GATA factor-mediated transcription. Model most closely resembles that proposed by Hall (Beck and Hall, 1999). From Cooper, 2002.

Several AAP genes, encoding transporters with low specificity or transporters of amino acids that are poor nitrogen sources, are under the control of NCR. This is the case for the General Amino acid Permease encoded by *GAP1*, the broad-range AAP-encoding gene *AGP1*, the proline transporter gene *PUT4* and the arginine transporter gene *CAN1* (Table 3-1).

4. Amino acid sensing via the SPS sensor: transcriptional induction

Some AAPs are regulated by the SPS-mediated amino acid sensing pathway. This is the case for *AGP1, BAP2, BAP3, TAT1, TAT2* and *GNP1*, who all belong to Cluster I, and *DIP5* and *AGP2,* who are unclustered members of the AAP family (Regenberg *et al.,* 1999; Table 1); the peptide transporter-encoding gene *PTR2*, which does not belong to the AAP family, is also a target for this pathway (Island *et al.,* 1991). In the absence of amino acids in the environment, transcriptional levels from these genes are low (Iraqui *et al.,* 1999; Gaber *et al.,* 2003; Eckert-Boulet *et al.,* 2004, 2005). Amino acids have different capacities (potencies) to induce transcription: cysteine, proline, arginine, glycine and lysine are unable to induce transcription even when present at the high concentration of 5 mM, while phenylalanine, leucine and isoleucine are very potent and already induce transcription at the concentration of 50 µM (Iraqui *et al.,* 1999; Gaber *et al.,* 2003). Amino acid-induced transcription of the AAP genes depends on the presence of the SPS sensor, comprising Ssy1p, Ptr3p and Ssy5p (Bernard and André, 2001b; Forsberg and Ljungdahl, 2001), of the transcription factors Stp1p and Stp2p (de Boer *et al.,* 2000), of the F-box protein Grr1p (Iraqui *et al.,* 1999) and of the regulatory subunit of PP2A, Rts1p (Eckert-Boulet *et al.,* 2006). The current knowledge is reviewed in the next section and a possible model is suggested.

4.1. The SPS sensor: Ssy1p, Ptr3p and Ssy5p

The Ssy1 protein, encoded by *YDR160W,* is a member of the AAP family (Nelissen *et al.,* 1997). *SSY1* was initially identified in a screen for mutants able to grow on the sulfonylurea herbicide metsulfuron methyl (Jørgensen *et al.,* 1998). Metsulfuron methyl is a sulfonylurea herbicide that inhibits synthesis of branched-chain amino acids; cells are thus dependent on proper amino acid uptake for growth. In other words, mutants deficient in amino acid signalling are growth impaired in the presence of metsulfuron methyl (Tullin *et al.,* 1991; Jørgensen *et al.,* 1997; Jørgensen *et al.,* 1998). Ssy1p is required for proper transcriptional induction of AAP genes, among which *BAP2, BAP3, TAT1*, *AGP1, AGP2* and the peptide transporter gene *PTR2* (Didion *et al.,* 1998; Iraqui *et al.,* 1999; Bernard and André, 2001b; Klasson *et al.,* 1999; Eckert-Boulet *et al.,* 2004; Perry *et al.,* 1994). Ssy1p displays a 276 amino acid long terminal extension as compared to other AAPs (Jørgensen *et al.,* 1998; Iraqui *et al.,* Bernard and André, 2001b). This extension is oriented towards the cytoplasm and is necessary for signalling (Klasson *et al.,* 1999; Bernard and André, 2001b). Ssy1p does not appear to transport amino acids, and the fact that mutations in *SSY1* were identified that confer constitutive activity to Ssy1p supports the theory that its role is to sense amino acids (Gaber *et al.,* 2003). The authors used a strain in which the endogenous potassium transporter genes *TRK1* and *TRK2* were

deleted. The *Arabidopsis thaliana* gene *KAT1*, encoding a potassium transporter, was placed under the control of the amino acid-inducible *AGP1* promoter. Growth of the resulting strain is possible only if the Kat1 channel transporter is expressed. The authors amplified the *SSY1* ORF under mutagenic conditions and introduced the resulting products into an *ssy1Δ trk1Δ trk2Δ* strain carrying the pAGP1-KAT1 construct. Selecting for growth in the absence of amino acids allowed identification of mutations in *SSY1* that result in constitutive signalling.

The other two components of the SPS sensor are encoded by *SSY5* and *PTR3* (Klasson *et al.,* 1999). Ssy5p and Ptr3p are peripherally associated plasma membrane proteins and, like Ssy1p, are essential for amino acid signalling to occur (Barnes *et al.,* 1998; Jørgensen *et al.,* 1998; Klasson *et al.,* 1999; Forsberg and Ljungdahl, 2001; Bernard and André, 2001b). *SSY5* was initially isolated in the same screen as *SSY1* (Jørgensen *et al.,* 1998). *PTR3* was originally isolated in a mutant deficient in amino acid-inducible peptide transport (Island *et al.,* 1991). Further characterisation of Ptr3p showed that it is required for amino acid induction of the peptide transporter gene *PTR2* and of the branched chain amino acid permease *BAP2* (Barnes *et al.,* 1998). Two-hybrid experiments showed that Ptr3p interacts with Ssy5p and with itself (Bernard and André, 2001b). Recent isolation of gain-of-function mutants of *SSY5* and *PTR3* using the pAGP1-KAT1 reporter system described above, together with the fact that constitutive signalling in these mutants still was dependent on each of the other SPS components (Poulsen *et al.,* 2005) supports the notion that Ssy1p, Ptr3p and Ssy5p interact in a complex.

4.2. The Stp transcription factors

Amino acid-induced transcription is also dependent on the transcription factors Stp1p and Stp2p. *STP1* was first identified as encoding a protein involved in pre-tRNA processing and exhibiting zinc-finger motifs (Wang and Hopper, 1988; Wang *et al.,* 1992). Leucine-mediated induction of *BAP2* transcription was found to be dependent on *STP1* (Jørgensen *et al.,* 1997). Deletion of either *STP1* or its homologue *STP2* reduces transcriptional induction, which falls drastically when both genes are deleted (de Boer *et al.,* 1998; Nielsen *et al.,* 2001; Eckert-Boulet *et al.,* 2004); deletion of the *STP3* and *STP4* genes is also required to almost completely abolish induction (Helge A. Andersen, personal communication). It was reported that Stp1p and Stp2p bind to an upstream activating sequence (UAS_{aa}) of the *BAP3* and *BAP2* promoters (de Boer *et al.,* 2000; Nielsen *et al.,* 2001). Initial work suggested that 5'-CGGCTC-3' was the consensus binding sequence (de Boer *et al.,* 2000), while the element (PuCGGC-N_3-PuCGGC) was later proposed to mediate binding (Nielsen *et al.,* 2001). We have found that the sequence element (A/G)(CGGC/GCCG)(C/T)-N_{1-28}-(A/G)(CGGC/GCCG)(C/T) was common in the promoter regions of *GNP1, AGP1, MUP1, BAP2, BAP3, TAT1, TAT2, CAR2* and *DIP5,* and we proposed this sequence is the UAS_{aa} (Eckert-Boulet *et al.,* 2004).

Stp1p was first thought to be a nuclear protein (Wang *et al.,* 1992), but it is now known that Stp1p and Stp2p can also be localised in the cytoplasm (Andréasson and Ljungdahl, 2002; Eckert-Boulet *et al.,* 2004). Stp1p and Stp2p are synthesised as latent cytoplasmic precursors and Stp1p associates with the plasma membrane (Andréasson and Ljungdahl, 2002). In the presence of extracellular amino acids, the proteins relocate to the nucleus

(Andréasson and Ljungdahl, 2002; Eckert-Boulet *et al.,* 2004). Amino acid-induced processing of Stp1p results in cleavage of a 10 kDa fragment of the N-terminus and is dependent on the SPS sensor (Figure 3-3; Andréasson and Ljungdahl, 2002). Stp2p is processed in a similar way. The regulatory domains lie between the amino acid residues 8 and 67 of Stp1p and 2 and 74 of Stp2p (Andréasson and Ljungdahl, 2002). The endoproteolytic processing of Stp1p and Stp2p appears to be responsible for relocation of the Stp factors to the nucleus, and not for their activation: if allowed to enter the nucleus, a mutant, non-cleavable, full-length form of Stp1p can induce transcription (Andréasson and Ljungdahl, 2004).

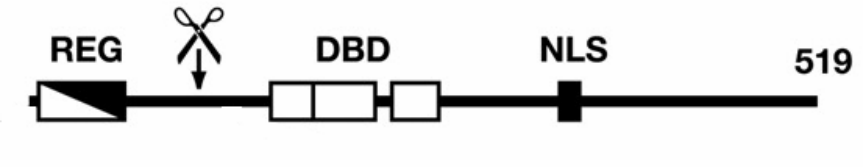


Figure 3-3. Schematic representation of Stp1p. The locations of the inhibitory domain (REG), the DNA-binding domains (DBD), and the putative NLS are depicted in the full-length Stp1p (519 amino acids). From Andréasson and Ljungdahl, 2004.

Recent work showed that proper Stp1p processing requires casein kinase I-mediated phosphorylation of Stp1p and that Ssy5p is the endoprotease responsible for processing (Abdel-Sater *et al.,* 2004b). Stp1p and Stp2p (and their homologues Stp3p and Stp4p?) are not the only transcription factors involved in the pathway. *ABF1* (Autonomously Replicating sequence binding Factor 1), *UGA35/DAL81*, and the co-repressors *SSN6* and *TUP1* are also required for amino acid-induced transcription (de Boer *et al.,* 2000; Iraqui *et al.,* 1999; Bernard and André, 2001b; Abdel-Sater *et al.,* 2004a; Forsberg *et al.,* 2001; Helge A. Andersen, personal communication).

4.3. The SCF^{Grr1} E3 ubiquitin ligase

Another component of the SPS-mediated amino acid sensing pathway is the E3 ubiquitin ligase SCF^{Grr1}. The role of SCF^{Grr1} in amino acid sensing was first highlighted by the finding that *GRR1* required for induction of the *AGP1* gene (Iraqui *et al.,* 1999). Other components of SCF^{Grr1}, namely Skp1p and Hrt1p, as well as ubiquitin and the ubiquitin-conjugating enzyme Cdc34p are also required for induction of *AGP1* and *PTR2* (Bernard and André, 2001a). Whole-genome transcription analysis showed that transcriptional induction of the AAP genes *AGP1, BAP2, BAP3, DIP5, GNP1* and *TAT1* is abolished in a *grr1Δ* mutant (Eckert-Boulet *et al.,* 2005). Stp1p processing does not occur properly in a *grr1Δ* strain (Andréasson and Ljungdahl, 2004; Eckert-Boulet *et al.,* 2006).

4.4. The *RTS1*-encoded regulatory subunit of Protein Phosphatase 2A

The *RTS1* gene encodes one of the two regulatory subunits of PP2A known in *Saccharomyces cerevisiae*. Rts1p is also involved in amino acid-sensing. Deletion of

RTS1 results in constitutive transcription from the *AGP1* and *BAP2* promoters, indicating a role for PP2A in down-regulation of the pathway (Eckert-Boulet *et al.,* 2006). Increased transcription levels in *rts1Δ* strains in the absence of extracellular amino acids are dependent on *SSY1, PTR3, SSY5, GRR1* and the transcription factor-encoding genes *STP1, STP2* and *STP3* (Figure 3-4; Eckert-Boulet *et al.,* 2006).

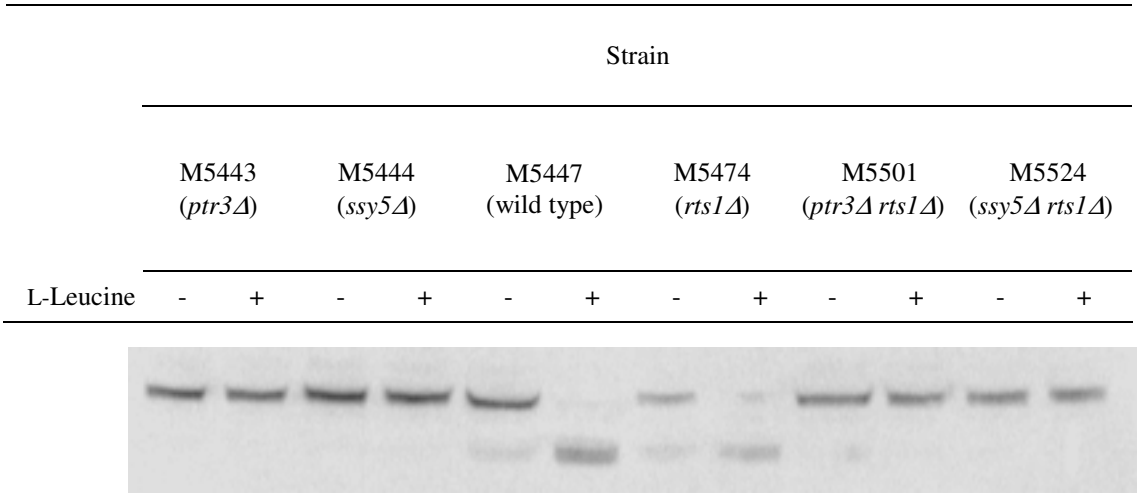


Figure 3-4. Western analysis of Stp1-ZZ processing in *rts1Δ*, *ssy5Δ* and *ptr3Δ* single mutants and in *ssy5Δ rts1Δ* and *ptr3Δ rts1Δ* double mutants, in the absence (-) and 20 minutes after addition (+) of 100 μM L-leucine. Cells were constructed and harvested, and protein extracts were prepared as described (Eckert-Boulet *et al.,* 2006). Equal amounts of proteins were loaded in each lane. Upper band: unprocessed Stp1-ZZ. Lower band: processed Stp1-ZZ.

This observation, taken together with the fact that, as discussed above, TOR regulates PP2A, led to the investigation of the effect of rapamycin on the SPS-mediated pathway. Wild type cells grown on minimal, synthetic medium and incubated for one hour with rapamycin before amino acid addition exhibit a transcriptional induction from the *AGP1* promoter reduced to less than half what is observed without rapamycin incubation (Table 3-2). Quantification of tagged Stp1p showed that Stp1p processing decreased accordingly (Table 3-3). A possible explanation might be provided by the observation that rapamycin inhibits phosphorylation of Tap42p, resulting in increased amounts of free Pph21p able to bind the Cdc55p-Tpd3p dimer (Jiang and broach, 1996). This could limit access to the catalytic subunit of PP2A to Rts1p, which otherwise down-regulates amino acid signalling.

Table 3-2. β-Galactosidase reporter assay on the amino acid-inducible AGP1-promoter in response to amino acids in the absence (A) and presence (B) of rapamycin.

A.

Treatment	Strain			
	M4054 (wild type)		M5397 (*rts1Δ*)	
Rapamycin [a]	-	-	-	-
L-citrulline [b]	-	+	-	+
Activity [c]	0.0 ± 0.1	5.4 ± 0.0	7.9 ± 0.4	8.7 ± 0.6

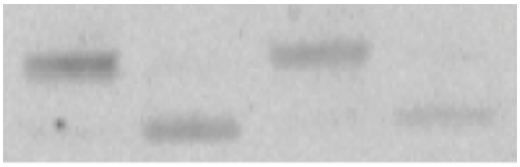
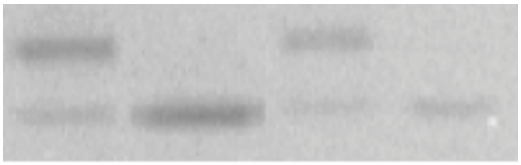
B.

Treatment	Strain			
	M4054 (wild type)		M5397 (*rts1Δ*)	
Rapamycin [a]	+	+	+	+
L-citrulline [b]	-	+	-	+
Activity [c]	0.1 ± 0.1	2.2 ± 0.1	11.2 ± 0.0	13.8 ± 0.4

[a] Rapamycin was added (when indicated) to a final concentration of 200 ng/mL, 1 h 40 min before the samples were harvested.
[b] L-citrulline was added (when indicated) to a final concentration of 5 mM, 40 min before the samples were harvested.
[c] Activity in Miller units ± 1 SEM.

Interestingly, the effect of rapamycin in *rts1Δ* cells is opposite: *AGP1* transcription is activated both in the absence and the presence of extracellular amino acids, and rapamycin addition enhances this phenotype (Table 3-2). Here again, detection and quantification of tagged Stp1p show that increased signalling occurs via increased Stp1p processing (Table 3-3). Thus PP2A appears to down-regulate the SPS-mediated pathway and seems itself to be regulated by TOR in this context.

Table 3-3. Effect of rapamycin on Stp1-ZZ processing. Stp1-ZZ processing was quantified as described (Eckert-Boulet *et al.,* 2006). Cells were constructed and harvested, and protein extracts were prepared as described (Eckert-Boulet *et al.,* 2006). Equal amounts of proteins were loaded in each lane. Upper band: unprocessed Stp1-ZZ. Lower band: processed Stp1-ZZ. Unproc.: unprocessed; Proc.: processed.

Treatment	Strain							
	M5447 (wild type)				M5474 (*rts1Δ*)			
Rapamycin [a]	-	-	+	+	-	-	+	+
L-leucine [b]	-	+	-	+	-	+	-	+
								
Unproc. Stp1-ZZ [c]	89.5 ± 1.7	10.4 ± 0.1	92.2 ± 0.7	17.9 ± 1.6	78.9 ± 0.5	1.8 ± 1.8	71.7 ± 1.7	2.8 ± 1.0
Proc. Stp1-ZZ [c]	10.5 ± 1.7	89.6 ± 0.1	7.8 ± 0.7	82.1 ± 1.6	21.1 ± 0.5	98.2 ± 1.8	28.3 ± 1.7	97.2 ± 1.0

[a] Rapamycin was added (when indicated) to a final concentration of 200 ng/mL, 1 h 40 min before the samples were harvested.

[b] L-leucine was added (when indicated) to a final concentration of 100 μM, 40 min before the samples were harvested.
[c] Relative amounts of unprocessed and processed Stp1p ± 1 SEM.

Interestingly, it has recently been found that the other known regulatory subunit of PP2A in *S. cerevisiae*, Cdc55p, is required for proper glucose induction of the *HXT1* gene (Tomás-Cobos *et al.,* 2005).

While PP2A appears to down-regulate amino acid signaling, a corresponding kinase must be involved in the activation of the pathway. Casein kinase I is a candidate for this kinase activity. The amino acid sensing pathway is inactive in temperature sensitive mutants affected in the casein kinase I genes *YCK1* and *YCK2*, and these strains exhibit loss of Stp1p processing (Abdel-Sater *et al.,* 2004b). Casein kinase I and PP2A are known to act on the same substrate in *Xenopus* embryos (Gao *et al.,* 2002) and it is therefore likely that they may do so in yeast. This hypothesis is substantiated by the finding that the yeast Cdc55p subunit forms a complex with casein kinase I (Ho *et al.,* 2002).

4.5. Possible model for the SPS-mediated amino acid sensing pathway

Still there are questions to be answered: what is the target of SF^{Grr1}? What is the target of PP2A? What is the exact biochemical function of Ptr3p? Targets of SCF^{Grr1} in other pathways are phosphorylated: Cln2p, the target of SCF^{Grr1} in cell cycle regulation, and Mth1p, its target in glucose induction, both need to be phosphorylated before interacting with SCF^{Grr1} (Hsiung *et al.,* 2001; Spielewoy *et al.,* 2005). Thus it is tempting to speculate that this is the case for its target in this pathway as well. The only component of the pathway known to be phosphorylated so far is Stp1p, however it is unlikely that it is the substrate of SCF^{Grr1}. It is also unlikely that Ssy5p be targeted for degradation, as suggested previously (Forsberg and Ljungdahl, 2001), at least not early in the signalling cascade, where it is required to process Stp1p. It was however reported that in response to amino acids Ptr3p is subject to post-translational modifications but not phosphorylation (Forsberg and Ljungdahl, 2001). The slower-migrating electrophoretic band reported by the authors could however be due to ubiquitination of the protein in response to amino acids. With this in mind, one could speculate that Ptr3p is the target of SCF^{Grr1}. This leads to the model proposed in Figure 3-5.

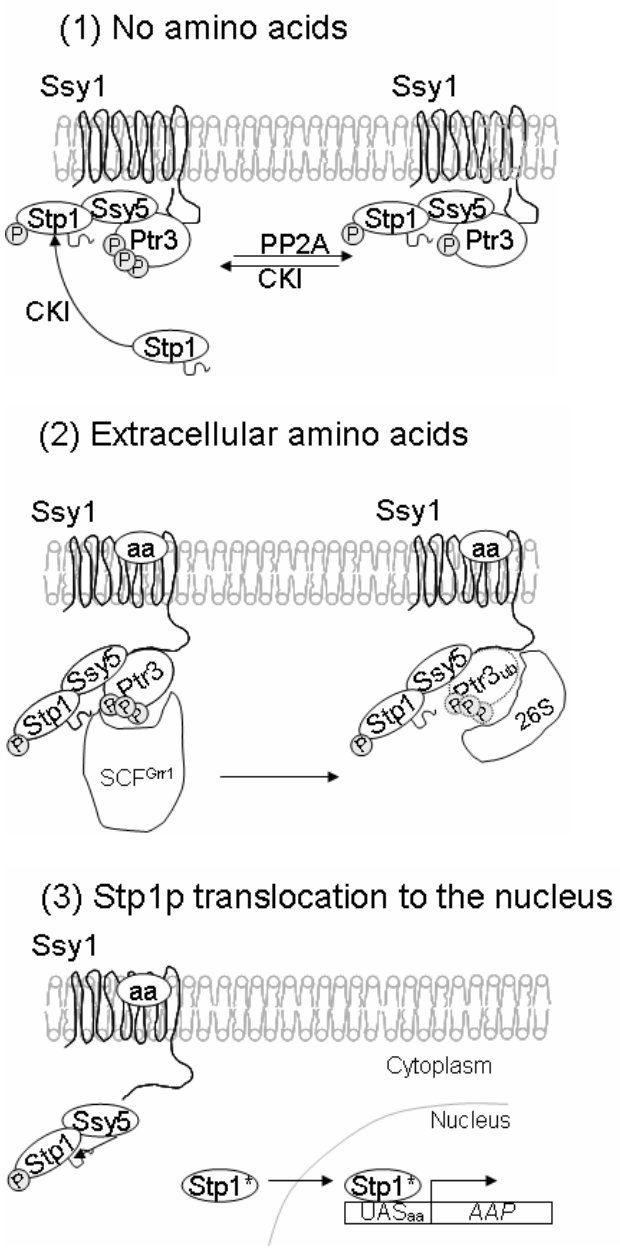


Figure 3-5. Possible model for the SPS-mediated pathway. See text for details.

(1) In the absence of amino acids, Ptr3p is bound to Ssy1p and Ssy5p is bound to Ptr3p, which covers the active site of Ssy5p. Ptr3p exists in two forms: a hyperphosphorylated form, active, and a hypophosphorylated form, inactive. PP2A could be involved in regulating the phosphorylation degree of Ptr3p, together with an unknown kinase, perhaps casein kinase I. Stp1p (we will assume that Stp1p, Stp2p, Stp3p and Stp4p are all involved and have the same fate) is phosphorylated by casein kinase I and binds Ssy5p. (2) Extracellular amino acids bind to Ssy1p and induce a conformational change, limiting access of PP2A to Ptr3p, or opening access of SCF^{Grr1} to the phosphorylated sites of Ptr3p. Ptr3p is ubiquitinated by SCF^{Grr1} and targeted for degradation by the 26S proteasome, liberating the active site of Ssy5p, which cleaves off the inhibitory N-

terminal domain of the Stp factors. (3) The processed Stp factors (Stp1*: active Stp1p) relocalise to the nucleus and bind the UAS_{aa} together with Abf1p and Uga35/Dal81p (not represented). Transcription is induced.

5. Conclusions

The regulation of AAPs is complex. Transcription of the AAP genes is regulated by the SPS pathway via the Stp transcription factors. NCR represses expression of genes encoding AAPs with a broad substrate range on good nitrogen source, while TOR regulates, sometimes differentially, turnover. Post-translational modifications can target AAPs for degradation or can protect them from degradation, as is the case for phosphorylated Gap1p.

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Chapter IV

Similarities and differences between the pathways involved in glucose and amino acid induction

The glucose induction and the amino acid induction pathways share many homologies. They both involve sensor proteins, as well as casein kinase I, the E3 ubiquitin ligase SCF^{Grr1}, transcription factors that bind to target promoters, and PP2A, with different regulatory subunits. Both pathways are dependent on sensor proteins, Snf3p/Rgt2p and Ssy1p respectively, which are members of the transporter family for which genes they induce transcription (Chapter II and Chapter III). Snf3p and Rgt2p are 60% similar and are members of the hexose transporter family (Nelissen *et al.,* 1997), but both exhibit a long C-terminal tail, the amino acid sequences of which are dissimilar, except for a small 17 amino acid-long sequence present twice in Snf3p and once in Rgt2p (Özcan *et al.,* 1996). The amino acid sensor Ssy1p has high similarity to amino acid permeases, but exhibits a long, cytoplasmic N-terminal tail. Mutations have been identified for all three proteins that confer them the ability to signal even in the absence of their substrate; in other words, little change is needed to create a situation where neither glucose uptake nor amino acid uptake is required for signalling (Özcan *et al.,* 1998; Gaber *et al.,* 2003). Interaction of Ssy1p, Ptr3p and Ssy5p within the SPS complex appears to occur independently of the presence of extracellular amino acids, whereas Rgt2p and Sfn3p seem to interact with Mth1p and Std1p only in the presence of glucose.

Glucose induction is dependent on dissociation of a transcription repressor complex from target gene promoters. The transcription repressor Rgt1p, together with the co-repressors Ssn6p and Tup1p, binds promoter DNA in the absence of glucose, inhibiting transcription. Rgt1p appears to be located in the nucleus independently on extracellular glucose availability, while its phosphorylation status is glucose-dependent. The transcription factors Stp1p and Stp2p (and perhaps Stp3p and Stp4p) only bind to promoter DNA of AAP genes when amino acids are present in the environment. In the absence of amino acids, Stp1p and Stp2p are cytoplasmic. Their translocation to the nucleus requires endoproteolytic cleavage of an inhibitory, N-terminal domain, by Ssy5p. The Stp transcription factors interact directly with the sensor complex and are phosphorylated by casein kinase I in response to external amino acids. In the case of glucose induction, there seem to be several intermediate factors between the sensors and the transcription repressor Rgt1p. Post-translational modification of the latter requires prior degradation of Mth1p (and Std1p?), which follows its (their) phosphorylation by casein kinase I.

Both pathways involve the E3 ubiquitin ligase SCF^{Grr1}. Its target in the amino acid sensing pathway is not yet known, but is likely to be a component of the SPS, perhaps Ptr3p, which is probably phosphorylated like other targets of SCF^{Grr1} (Hsiung *et al.,* 2001). SCF^{Grr1} is also known to be involved in cell cycle regulation (Barral *et al.,* 1995), and has recently been found to be required for pheromone sensitivity (Schweitzer *et al.,* 2005) and for actomyosin contraction during cytokinesis (Blondel *et al.,* 2005).

The type 2A protein phosphatase also appears to play a down-regulating role in both pathways, however with different regulatory subunits. Only two regulatory subunits of PP2A, Cdc55p and Rts1p, were identified in yeast so far, but simultaneous disruption of both of them gives rise to viable strains (Shu *et al.,* 1997). As strains lacking functional PP2A are not viable (Ronne *et al.,* 1991), the authors conclude that either PP2A can function without a regulatory subunit in *S. cerevisiae*, or other B subunits exist that remain unidentified. Whichever may be true, the association of Cdc55p or Rts1p with the catalytic and the scaffolding subunits confers distinct functions to PP2A (Shu *et al.,* 1997). The Cdc55p subunit is involved in glucose induction, while it is Rts1p which mediates the role of PP2A in amino acid induction. Taken together with the fact that rapamycin inhibits glucose and amino acid induction, this suggests a connection to the TOR pathway. TOR is believed to promote association of Msn2p and Msn4p with the 14-3-3 proteins Bmh1p and Bmh2p (Beck and Hall, 1999). It would be interesting to check whether the 14-3-3 proteins Bmh1p and Bmh2p, which are required for glucose induction of *HXT1* (Tomás-Cobos *et al.,* 2005), are also required for amino acid induction.

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Chapter V

Transcriptional profiling of extracellular amino acid sensing in *Saccharomyces cerevisiae* and the role of Stp1p and Stp2p

Nadine Eckert-Boulet, Peter Stein Nielsen, Carsten Friis, Margarida Moreira dos Santos, Jens Nielsen, Morten Kielland-Brandt, Birgitte Regenberg

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Research Article

Transcriptional profiling of extracellular amino acid sensing in *Saccharomyces cerevisiae* and the role of Stp1p and Stp2p

Nadine Eckert-Boulet[1,2], Peter Stein Nielsen[2,†], Carsten Friis[3], Margarida Moreira dos Santos[1,#], Jens Nielsen[1], Morten C. Kielland-Brandt[2]* and Birgitte Regenberg[1,‡]

[1]*Centre for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs Lyngby, Denmark*
[2]*Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark*
[3]*Centre for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs Lyngby, Denmark*

*Correspondence to: Morten C. Kielland-Brandt, Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark. E-mail: mkb@crc.dk

†Present Address: Department of Functional Genomics, Exiqon A/S, DK-2950 Vedbæk, Denmark.

#Present Address: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Rua da Quinta Grande 6, Apt 127, 2780-156 Oeiras, Portugal.

‡Present Address: Institut für Mikrobiologie, Goethe-Universität Frankfurt, Marie-Curie-Strasse 9, 60439 Frankfurt am Main, Germany.

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Abstract

S. cerevisiae **responds to the presence of amino acids in the environment through the membrane-bound complex SPS, by altering transcription of several genes. Global transcription analysis shows that 46 genes are induced by L-citrulline. Under the given conditions there appears to be only one pathway for induction with L-citrulline, and this pathway is completely dependent on the SPS component, Ssy1p, and either of the transcription factors, Stp1p and Stp2p. Besides the effects on amino acid permease genes, an *ssy1* and an *stp1 stp2* mutant exhibit a number of other transcriptional phenotypes, such as increased expression of genes subject to nitrogen catabolite repression and genes involved in stress response. A group of genes involved in the upper part of the glycolysis, including those encoding hexose transporters Hxt4p, Hxt5p, Hxt6p, Hxt7p, hexokinase Hxk1p, glyceraldehyde 3-phosphate dehydrogenase Tdh1p and glucokinase (Glk1p), shows increased transcription levels in either or both of the mutants. Also, most of the structural genes involved in trehalose and glycogen synthesis and a few genes in the glyoxylate cycle and the pentose phosphate pathway are derepressed in the *ssy1* and *stp1 stp2* strains. Copyright © 2004 John Wiley & Sons, Ltd.**

Keywords: **amino acid sensing; transcription analysis; NCR; *SSY1*; *STP1*; *STP2***

Introduction

Saccharomyces cerevisiae serves as a model organism for studying adaptation to nutritional changes in the environment and, in a number of biotechnological applications of this yeast, nutrient sensing plays an important role, e.g. beer brewing and baker's yeast production. In *S. cerevisiae* the availability of nitrogen, in the form of ammonium and amino acids, is monitored by systems that affect major parts of the metabolism (Beck and Hall, 1999; Natarajan *et al*., 2001) and more specific systems that only affect genes or proteins specific for a single or few metabolic pathways (e.g. Holmberg and Schjerling, 1996; Iraqui *et al*., 1999a; Wang *et al*., 1999). Several laboratories have recently described a system involved in the transcriptional regulation of amino acid permease genes in response to amino acids in the medium (de Boer *et al*., 1998; Didion *et al*., 1996, 1998; Iraqui *et al*., 1999b; Klasson *et al*., 1999). Seven of these genes are induced by amino acids;

BAP2, BAP3, AGP1, GNP1, DIP5, TAT1 and *TAT2*, besides the arginase gene *CAR1* and the peptide transporter gene *PTR2* (Perry *et al*., 1994; Didion *et al*., 1996, 1998; de Boer *et al*., 1998; Iraqui *et al*., 1999b; Klasson *et al*., 1999; Regenberg *et al*., 1999). L-citrulline and most of the 20 common L-α-amino acids are able to induce the signal, with lle, Leu, Val, Phe, Trp and Tyr being the most potent inducers (Didion *et al*., 1996; de Boer *et al*., 1998; Iraqui *et al*., 1999b; Bernard and André, 2001a). The sensing of amino acids is dependent on the plasma membrane-bound SPS complex (Forsberg and Ljungdahl, 2001), composed of three polypeptides, Ssy1p, Ptr3p and Ssy5p, which is suggested to be responsible for the encounter with the sensed amino acid. *SSY1* encodes a homologue to the amino acid permeases and was first functionally identified in a screen for mutants with decreased uptake of the branched-chain amino acids (Jørgensen *et al*., 1998). Ssy1p itself is not responsible for bulk transport of amino acids, and amino acid uptake is not necessary for signalling (Didion *et al*., 1998; Gaber *et al*., 2003). An N-terminal cytoplasmic domain of Ssy1p appears to be essential for transduction of the signal, since mutations affecting this domain hamper the response (Klasson *et al*., 1999) and expression of the domain alone (276 N-terminal amino acid residues) can partially remedy the non-growth phenotype of *ssy1* on leucine as sole nitrogen source (Bernard and André, 2001a). The three components of the SPS complex are reported to interact physically, Ssy1p to Ptr3p (Uetz *et al*., 2000) and Ptr3p to itself and Ssy5p (Bernard and André, 2001a), and it is believed that they are involved in the same cellular functions, since *ssy1, ptr3* and *ssy5* mutations have identical pleiotropic phenotypes (Jørgensen *et al*., 1998; Klasson *et al*., 1999; Bernard and André, 2001a; Forsberg and Ljungdahl, 2001). The SPS complex alters in response to amino acids; an N-terminally tagged Ssy5p appears to be rapidly degraded, and the two other components, Ssy1p and Ptr3p, are modified and degraded to some extent (Forsberg and Ljungdahl, 2001). One or more of these modifications of the SPS complex may be important for signal transduction, a possibility which is substantiated by the observation that the ubiquitin ligase complex SCF^{Grr1} is required for amino acid sensing (Iraqui *et al*., 1999b; Bernard and André, 2001b).

Several transcription factors have been found to play a role in the regulation of amino acid permease genes, one being Stp1p, which was first isolated as a gene involved in maturation of t-RNAs (Wang and Hopper, 1988), and later in a screen for decreased uptake of neutral amino acids (Tullin *et al*., 1991; Jørgensen *et al*., 1997). *STP1*, and to a lesser extent its homologue *STP2*, are required for amino acid-induced *BAP2* and *BAP3* transcription (de Boer *et al*., 2000; Nielsen *et al*., 2001), and both Stp1p and Stp2p are able to bind the *BAP2* promoter *in vitro* (Nielsen *et al*., 2001), leading to the hypothesis that Stp1p and Stp2p are directly responsible for transcriptional induction of these permease genes in response to amino acid. Stp1p and Stp2p are proteolytically truncated in the sensing process, and genetically truncated forms of Stp1p or Stp2p cause constitutive activity of target promoters (Andréasson and Ljungdahl, 2002). Among other candidates for transcription factors involved in the SPS-mediated response is the general transcription factor Abf1p, which binds the *BAP3* promoter *in vitro*, but binding of Abf1p is not sufficient for induction of *BAP3* (de Boer *et al*., 2000). Also, defects in the transcriptional repressor gene *TUP1* were found to suppress mutants deficient in Ssy1p (Forsberg *et al*., 2001; Nielsen *et al*., 2001) and, at the same time, *TUP1* is required for maximal expression of *BAP2* (Nielsen *et al*., 2001). The transcription factor Dal81p is also suggested to play a role in amino acid sensing, as it is required for *AGP1, PTR2* and *BAP2* expression in response to amino acids (Bernard and André, 2001a).

In this work we investigate the global importance of amino acid sensing using whole-genome expression analysis. To understand the role of the SPS complex in amino acid sensing and to further define the transcription factors involved in this response, we also determined the transcriptional profiles of an *ssy1* mutant and an *stp1 stp2* mutant and compared them to those of the wild-type. During the later stages of this work Forsberg *et al*. (2001) and Kodama *et al*. (2002) also published whole-genome analyses of amino acid sensing comparing transcription profiles of *ssy1* mutants to the corresponding wild-type.

Experimental Procedures

Strains, media and growth conditions

The *gap1 ura3 S. cerevisiae* strain M4054 (Grauslund *et al*., 1995), isogenic to S288C, served as parental control strain for the *ssy1* strain M4238 (Didion *et al*., 1998) and the *stp1 stp2* strain M4272 (Nielsen *et al*., 2001). Cells were grown in liquid SD + uracil, consisting of 1% w/v succinic acid, 0.6% w/v NaOH, 0.67% w/v Yeast Nitrogen Base without amino acids (Difco), 0.002% w/v uracil and 2% w/v glucose monohydrate. For whole-genome expression analysis, cells were grown in a pre-culture until $OD_{600} = 0.25–0.5$ and inoculated into a 4 l batch culture aerated with 4 l air min and an agitation of 700 rpm. pH was kept constant at 5.6 and the temperature at 30 °C. The cultures were grown from $OD_{600} = 0.01$ to $OD_{600} = 2.0$, at which time L-citrulline was added to a final concentration of 2.0 mM. For trehalose and glycogen measurement, cells were grown in 100 ml SD + uracil shake flask cultures until $OD_{600} = 1–2$, while cells for microscopy were grown overnight in SD + uracil at room temperature, and induced with 2.0 mM L-citrulline.

Gas analysis

The mole % of CO_2 in the exhaust gas from the batch cultivations was determined online, every 4 min, using a PC-controlled acoustic gas analyser, model 1308 (Brüel & Kjær, Denmark).

Construction of the *STP1–GFP* fusion gene

Two PCR fragments were generated from *STP1*, one identical to part of the 3′-end and another identical to a part downstream of the stop codon of *STP1*, using the primers 5′-CCGCTGAAGTTATG TTCC, 5′-GTTCTTCTCCTTTACTCATAGCAGC AAATTGTGACCCAAAGTTGT for the first reaction and 5′-GGATGAACTATACAAATAACCAT AAACCAAGTGATTGCAAC, 5′-CCTCTTA CACTCTACTAG for the second reaction. The first fragment was then extended with a red-shifted version of the *GFP* gene, fused to the 5′ two-thirds of the *Kluyveromyces lactis URA3* gene using adaptamer technology, as described (Lisby *et al*., 2001), while the second fragment was extended with *GFP* and the 3′ two-thirds of the *K. lactis URA3*, using 5′-CCGCTGAAGTTATGTTCC and 5′-GAGCAATGAACCCAATAAC for the first extension and 5′-CTTGACGTTCGTTCGACTG and 5′-CCTCTTACACTCTACTAG for the second. The resulting two fragments were co-transformed into M4054, clones containing an *STP1–GFP–K. lactis URA3–GFP* fusion were selected on SD, and their genotype was confirmed by diagnostic PCR. Loss of *URA3* by homologous recombination between the two copies of *GFP* was selected on 5-FOA plates, and the resulting *STP1–GFP* strain was denoted CPB1045.

Fluorescence microscopy

We used a CCD camera mounted on a Nikon Eclipse E400 microscope with a CFI plan fluor 100× objective lens. Illumination was obtained with a 100 W mercury lamp. The filters used to visualize Stp1–GFP (excitation 480 nm and emission 535 nm) and 4′,6-diamidino-2-phenylindole (DAPI, excitation 365 nm and emission 450 nm) were from Nikon and Chroma (Brattleboro, VT), respectively. DNA was stained by adding 10 μg/ml DAPI to the culture 30 min before imaging.

Metabolic flux analysis

Cells were grown in batch cultures in 150 ml SD + uracil with only 0.5% w/v D-glucose, labelled for at least five generations with 0.5% w/v D-[1-^{13}C]glucose, and harvested in mid-logarithmic phase. Derivatisation procedures were performed directly on the crude hydrolysates, as described (Christensen and Nielsen, 2000). Amino acids were analysed as *N*-ethoxycarbonyl amino acid ethylesters and/or *N*-(*N,N*-dimethylaminomethylene) amino acid methylesters, and glucose as glucose pentaacetate, using gas chromatography coupled to mass spectrometry. Labelling patterns of proteogenic amino acids (Ser, Gly, Ala, Val, Leu, Asp, Thr, Ile, Glu, Pro, Lys and Phe) were determined by analysing 26 ions for every biomass sample, as described (Christensen and Nielsen, 1999).

Trehalose and glycogen

These compounds were determined as described (Schulze, 1995).

Whole-genome transcription analysis

Total RNA was purified from cells that had been harvested and cooled below 4 °C within 1 min by mixing 50% culture sample and 50% crushed ice. For total RNA, cells were thawed on ice and RNA isolated using the Fast RNA, RED Kit (BIO101, CA) according to the manufacturer's instructions. cRNA was synthesized and hybridized to the whole-genome oligonucleotide array S98 (Affymetrix, CA) essentially as described (Wodicka *et al*., 1997). After scanning in a GeneArray scanner, the raw data were processed with MicroArray Suite 4.0 software, using a global scaling with all probe sets to a target intensity of 500. The absolute hybridization intensity (average difference) was calculated for each of the probe sets on the array. Many values came out below 20, some of them even negative; they were all increased to 20 in order to eliminate insignificant variations at the low end. The fold changes were then calculated by dividing the average difference values of all transcripts on all eight arrays with the values obtained from the reference array (parental strain M4054, time = 0). Genes that were significantly up- or downregulated were sorted according to two criteria, a fold change value and a comparison analysis. Transcripts that had altered more than threefold (i.e. had changed more than 0.47 in the log 10 value) were considered significantly changed. This cut-off value was determined by repeating fermentations and a second transcription analysis for cells exposed to L-citrulline for 60 min. Differences in expression levels for the two replicates were used as a measure of the biological and analytical variation between any two samples, and the cut-off value was set as ± twice the standard deviation (2 × SD) on log 10-fold changes between the two samples, which corresponds to a 95% confidence level. The comparison analysis was performed with Micro Array Suite 4.0, as described in the user guide. The algorithm was used to determine whether a transcript was increased, decreased or no change (NC) in the *ssy1* mutant M4238 (0 and 30 min), the *stp1 stp2* mutant M4272 (0 and 30 min) and the parental strain M4054 (15, 30 and 60 min), compared to the corresponding one on the reference array (M4054, 0 min induction). All transcript data sets that were NC were discarded in the sorting. We also discarded probe sets that gave no significant signal in any of the eight experiments, as calculated with MicroArray Suite 4.0 software. By this sorting, 501 transcripts were found to be significantly up- or downregulated in at least one of the seven experiments, compared to the level in the reference strain uninduced (M4054, 0 min).

Clustering of expression profiles

Log 10 fold change values of the 501 transcripts found to be differently expressed in one of the seven experiments, as compared to the reference condition, were clustered with XCluster hierarchical clustering (**http://genome-www.stanford.edu/~sherlock/cluster.html**). The algorithm employed by XCluster for hierarchical clustering is Average Linkage, as described (Eisen *et al*., 1998). A centred Pearson correlation was used as distance metric and the resultant dendrogram visualized with Tree View (**http://rana.lbl.gov/EisenSoftware.htm**).

Searching promoter regions for overrepresented patterns

Promoter regions were extracted as 700 bp upstream of the start codon. The positive set (L-citrulline-induced) was searched for patterns overrepresented relative to the negative set (all other genes) by two methods, one a strict word-search algorithm, Saco-Pattern (Jensen and Knudsen, 2000), the other a Gibbs sampling of weight matrix descriptions, ANN-Spec (Workman and Stromo, 2000). Saco-Patterns looks for all possible DNA words up to length 12 bp and is deterministic. ANN-Spec samples the possible weight matrices that describe a degenerate recognition site in DNA and is non-deterministic. For both algorithms, the significance of overrepresentation in the positive set is calculated using the hypergeometric distribution.

Results

L-citrulline induction is predominantly dependent on Stp1p, Stp2p and Ssy1p

To investigate the effect of amino acid induction on whole-genome transcription profiles, *gap1* cells were grown in minimal ammonium medium and exposed to addition of 2 mM L-citrulline. L-citrulline was chosen for induction of the yeast

cells deleted in the general amino acid permease gene, *GAP1*, to avoid interference from intracellular amino acid signals. Gap1p is the main transport system for L-citrulline transport (Grenson *et al*., 1970; Iraqui *et al*., 1999b; Regenberg and Kielland-Brandt, 2001), and gas chromatography mass spectrometry of cell extracts showed that cells grown for eight generations in minimal ammonium medium supplied with 2 mM L-citrulline had assimilated only 10% of the L-citrulline, whereas large amounts of ammonium had been metabolized (results not shown). We therefore conclude that L-citrulline does not significantly contribute to nitrogen metabolism. For transcription profiles, cells were grown in synthetic dextrose medium (SD + uracil) with ammonium as the sole nitrogen source. L-citrulline was added at mid-exponential phase when the glucose concentration was around 15g/L, and samples were taken just before, and 15, 30 and 60 min after, L-citrulline addition. mRNA was purified from the samples, reverse-transcribed into cDNA and *in vitro* transcribed with biotin-labelled nucleotides. The resulting cRNA was hybridized to S98 oligonucleotide arrays from Affymetrix (Wodicka *et al*., 1997). The *gap1* strain without L-citrulline was set as reference, and hybridization intensities were calculated for each of the probe sets on all four arrays. To find the genes that were up- or downregulated in response to L-citrulline, log fold change was calculated for each probe set on the reference chip compared to chips from cells exposed to L-citrulline, and transcripts that had altered more than 0.47 log fold up or down were considered significantly changed. This value was set by repeating one experiment, calculating the fold change of all transcripts in the replicate and considering the 95% confidence level as the level of significance. Forty-six genes and ORFs were found to be significantly upregulated in response to L-citrulline in at least one of the time points (Table 1). Of these, *TAT1, BAP2, BAP3* and *PTR2* are permease genes that are previously reported to be induced by amino acids in an Ssy1p-dependent manner (Perry *et al*., 1994; de Boer *et al*., 1998; Didion *et al*., 1996, 1998). Besides these, another member of the amino acid permease gene family, *AGP2*, was also found to be induced by L-citrulline (Table 1). *TAT1, BAP2* and *BAP3* display maximal induction 30 min after citrulline addition. Only a few other transcripts, the citrate synthase gene *CIT1, YCL058C, YLL032C* and *YKR073C*, had a similar transcription profile, while most of the remaining transcripts showed highest induction after 60 min of exposure to L-citrulline (Table 1). They are *TDH1, SOL4, HXK1, GSY1, GPH1, ARA1, ADR1, AAD10*, all involved in carbon metabolism, *BIO3* and *BIO5*, involved in biotin metabolism, and 30 genes with unknown or other functions.

To investigate the role of the SPS complex and the role of transcription factors responsible for this signal, we grew a *gap1 ssy1* strain, devoid of the membrane-spanning protein Ssy1p in the SPS complex, and a *gap1 stp1 stp2* strain, lacking two of the transcription factors suggested to be involved in amino acid sensing (de Boer *et al*., 2000; Nielsen *et al*., 2001; Andréasson and Ljungdahl, 2002). The cells were treated as described above for the parental strain and taken for transcription profiles before induction with L-citrulline and 30 min after induction. All probe sets were compared to those on the reference chip and the log fold changes calculated. In all eight experiments, 501 transcripts were found to have significantly altered levels (Appendix 1: **http://www3.interscience.wiley.com/cgi-bin/jabout/3895/OtherResources.html**). Of the 46 genes found to be induced in the parental *gap1* strain, only two were also found to be induced in the *ssy1* mutant (*MET28, YKR073C*) and one in the *stp1 stp2* mutant (*EST1*) (Table 1), showing that L-citrulline predominantly induces expression via the SPS component Ssy1p, and the transcription factors Stp1p and Stp2p.

ssy1 and *stp1 stp2* mutants have similar, but not identical, transcription profiles

A large number of genes were affected in the two mutant strains. In the absence and presence of L-citrulline, respectively, 43 and 82 were found to be less expressed in the *ssy1* strain, while 234 and 238 had higher expression in this strain. In the *stp1 stp2* strain, 49 and 62 transcripts were less abundant, while 176 and 173 were more abundant. Importantly, the same transcripts were to a large extent affected in both mutants, so that 80% of the transcripts that were significantly more abundant on the *stp1 stp2* arrays were also found among the significantly more abundant transcripts on the *ssy1* arrays. This pattern is particularly evident when the transcription profiles are clustered. Fold

Table 1. Genes induced more than three-fold by L-citrulline

		gap1			*gap1 ssy1*		*gap1 stp1 stp2*	
Gene	**ORF**	**15 min**	**30 min**	**60 min**	**0 min**	**30 min**	**0 min**	**30 min**
AAD10	*YJR155W*	1.3	2.2	4.0	5.0	4.8	5.4	5.8
ADR1	*YDR216W*	3.2	2.0	3.9	2.3	3.2	1.3	2.2
AGP2	*YBR132C*	2.2	2.3	3.5	2.5	1.3	1.0	1.2
ARA1	*YBR149W*	2.7	2.1	3.6	3.6	4.1	3.5	2.2
BAP2	*YBR068C*	10.2	22.9	13.2	2.8	1.0	1.0	1.0
BAP3	*YDR046C*	22.4	45.7	29.5	1.0	1.1	1.0	1.0
BIO3	*YNR058W*	1.3	2.4	6.0	8.7	8.3	9.3	10.0
BIO5	*YNR056C*	2.2	6.5	23.4	30.9	35.5	38.9	46.8
CIT1	*YNR001C*	4.4	3.5	2.5	3.4	5.2	4.1	4.6
ECM4	*YKR076W*	3.0	2.2	4.1	1.8	3.0	2.3	2.5
EST1	*YLR233C*	6.3	5.0	7.9	11.2	8.1	2.5	11.0
GPH1	*YPR160W*	1.4	1.4	3.5	9.8	13.2	3.0	2.6
GSY1	*YFR015C*	1.1	1.1	3.2	3.2	3.9	1.6	1.5
HSP42	*YDR171W*	1.3	1.1	3.2	6.8	6.6	4.5	3.8
HXK1	*YFR053C*	1.1	0.9	5.5	8.7	9.8	1.9	2.0
INO1	*YJL153C*	2.1	3.7	9.1	30.2	36.3	33.1	28.2
IPK1	*YDR315C*	3.4	2.6	3.2	3.8	3.3	4.7	2.7
MET28	*YIR017C*	1.6	1.8	4.4	0.9	3.5	1.7	3.0
OPT2	*YPR194C*	1.9	1.8	3.5	1.9	3.0	0.4	0.5
PLM2	*YDR501W*	3.1	1.3	1.0	1.1	2.0	1.5	1.0
PTR2	*YKR093W*	3.2	2.5	2.2	1.0	0.8	1.6	1.2
SOL4	*YGR248W*	1.4	1.5	3.8	11.5	17.4	2.8	3.5
TAT1	*YBR069C*	3.9	4.7	1.6	0.4	0.6	0.5	0.8
TDH1	*YJL052W*	1.5	1.7	3.8	11.0	14.8	6.0	5.6
YPS6	*YIR039C*	2.7	2.5	3.5	6.3	9.3	4.5	5.1
YRO2	*YBR054W*	1.5	0.7	3.2	14.5	25.1	1.7	1.3
	YLR031W	1.7	2.6	3.0	12.3	12.0	7.1	6.5
	YER067W	1.4	0.7	3.1	3.1	5.1	1.7	1.7
	YMR135W-A	1.0	1.1	3.1	1.0	1.0	1.0	1.8
	YOL053C	1.2	1.1	3.2	12.0	16.6	4.6	4.7
	SNR6	1.7	1.5	3.2	3.2	1.7	2.1	3.8
	YCR061W	1.7	1.9	3.2	2.0	1.9	2.3	2.3
	YJL152W	2.6	2.9	3.5	4.3	4.8	4.2	6.2
	YJR008W	1.8	2.1	4.0	4.7	6.2	4.1	3.5
	YCL042W	1.4	1.2	4.5	3.8	7.6	4.5	5.0
	YJR154W	1.5	2.3	6.9	1.9	3.9	4.3	4.0
	YLR431C	2.6	3.0	1.9	2.6	3.3	2.8	1.8
	YCL058C	2.6	3.7	1.7	3.6	2.2	1.7	1.3
	SNR45	1.8	3.8	3.9	2.5	3.1	1.5	1.0
	YKR073C	2.8	4.1	2.6	1.0	4.0	1.0	1.2
	TG(GCC)E	2.8	13.2	1.0	25.7	9.3	24.5	18.6
	YLL032C	3.0	3.2	1.8	2.0	1.1	3.4	1.0
	YLR392C	3.1	2.2	1.5	3.5	2.8	2.6	4.3
	YCR106W	3.2	2.1	1.0	1.1	1.0	1.7	3.0
	YER064C	3.3	2.3	1.4	1.3	0.7	0.9	0.9
	TG(GCC)B	5.5	1.0	12.9	26.9	15.5	17.4	23.4

changes of the 501 significantly changed genes from any one of the eight experiments were log transformed and used for hierarchical clustering (Figure 1). The expression profiles of the *ssy1* mutant and the *stp1 stp2* mutant are very similar, but not strictly identical. Deletion of *SSY1* appears to have a somewhat broader effect than deletion of *STP1* and *STP2* (Figure 1). More genes display increased transcription levels in the *ssy1* mutant than in the *stp1 stp2* mutant. This includes several genes involved in glycogen metabolism (*GPH1, GLC3, GSY1, GSY2, TSL1* and *GAC1*) and energy

pathways (*GPM2*), as well as hexose metabolism and transport (*MTH1, HXT6, HXT7, PGM2* and *HXK1*). On the other hand, several genes with decreased expression in the *stp1 stp2* mutant are not affected by *SSY1* deletion. This is true for several tRNA genes and genes of the cell cycle (*MIH1* and *CLB6*).

Besides *TAT1*, the expression of the amino acid permease genes *MUP1, GNP1*, and *AGP1* is decreased in the *ssy1* and the *stp1 stp2* mutants (Appendix 1: **http://www3.interscience.wiley.com/cgi-bin/jabout/3895/OtherResources.html**). On the other hand, expression of several other genes encoding transporters of nitrogenous compounds is increased in the mutants, namely *PUT4* in the *ssy1* mutant, *UGA4* in the *stp1 stp2* mutant, and *BIO5, DAL4, DAL5, THI7, FCY21* and *FCY22* in both mutants. They encode the proline, γ-aminobutyric acid, 7-keto 8-aminopelargonic acid, allantoin, allantoate, thiamine and two putative purine–cytosine transporters, respectively, of which the first three are also members of the

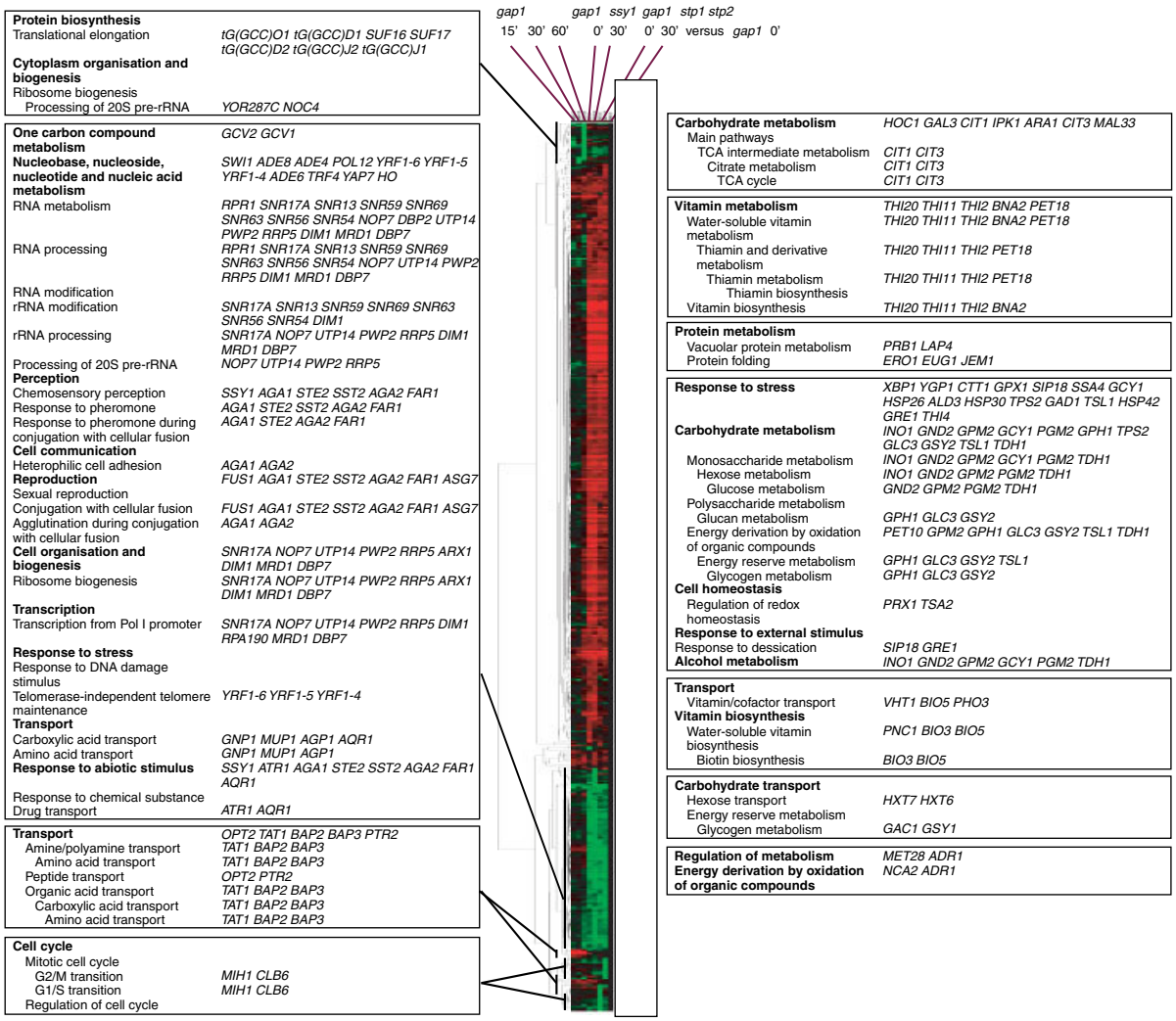

Figure 1. Hierarchical clustering of the 501 genes for which the transcription levels were found significantly changed in at least one experiment. Software from **http://rana.lbl.gov/**. Red refers to higher expression; green refers to lower expression compared to reference. Genes with increased (right part of the figure) and decreased expression (left part of the figure) were grouped in clusters. Over-represented gene ontologies (p value < 0.01) according to the SGD Gene Ontology Term Finder (**http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermFinder**) are listed in the boxes to the right of each tree

amino acid permease family (Nelissen *et al.*, 1997). Besides these transporter genes, we observe a moderate effect on other genes in nitrogen metabolism. Among the genes in amino acid metabolism, only *ARO9, CAR2, GAD1, GDH3* and *PUT1* showed increased expression in both mutants, while *BAT2* showed increased transcription levels in the *stp1 stp2* strain and *GCY1, GDH2* in the *ssy1* mutant. *GCV1, GCV2* and *LYS2* had decreased expression in the *ssy1* strain only, while *MET10* had decreased expression in both mutants. Among the genes involved in nucleotide metabolism, *ADE4* and *ADE8* in the *ssy1* strain and *ADE6* in both strains show decreased transcription levels, while *DAL1, DAL3, DAL80, THI2, THI7, THI11, THI20* and a few others are increased in both mutants. Also in protein synthesis, few genes are affected, with only the expression of *RPP1B* and *YEF3B* being significantly increased in more than one of the experiments, while expression of nine genes involved in protein degradation is increased in at least one of the mutants: *DER1, PAI3, PBI2, YPS3, YPS6* and *AUT7* show increased expression in both mutants, while *LAP4* and *TFS1* are more highly expressed in *ssy1* only and *PRB1* in *stp1 stp2* only.

Surprisingly, the largest functional group of genes affected in the *ssy1* and the *stp1 stp2* mutants are genes involved in carbohydrate metabolism, of which *CDA1, GLC3, GSY1, GSY2, HXK1, HXT4, HXT6, MTH1, PGM2, TPS2* and *TSL1* display higher transcription levels in the *ssy1* mutant only, and *YDL199C* appears to be more transcribed in the *stp1 stp2* strain only, while *ARA1, GPH1, SOL1, SOL4, TDH1, GLK1, GND2, GSC2, AMS1, HXT5, MLS1, NRG1, PDC6, YBR056W* and *YJL045W* showed increased expression in both mutant strains (Appendix 1: **http://www3.interscience.wiley.com/cgi-bin/jabout/3895/OtherResources.html**). Four of these genes, *ARA1, GPH1, SOL4, TDH1*, are also induced by L-citrulline, pointing to a direct effect of extracellular amino acids on carbohydrate metabolism. To test whether the change in gene expression was somehow reflected in the carbon metabolism, we measured the by-product formation of a culture exposed to addition of an L-citrulline solution and one to which a similar volume of water was added (Figure 2). We found that CO_2 production declined in the last part of exponential growth on glucose in the culture exposed to L-citrulline. Experiments with ^{13}C-labelled glucose in the *ssy1* mutant and the *SSY1* strain did not show differences in the flux through the different pathways of the central carbon metabolism (results not shown). Most of the genes that we found to be affected in carbohydrate metabolism encode proteins acting in the upper part of glycolysis, such as hexose transporters (*HXT4, HXT5, HXT6* and *HXT7*), a hexokinase (*HXK1*), glucokinase (*GLK1*), a phosphoglucomutase (*PGM2*), or involved in glycogen synthesis (*GSY1, GSY2* and *GLC3*), trehalose synthesis (*TSL1*) and glycogen degradation (*GPH1*). However, this increased transcription did not lead to any detectable accumulation of glycogen and trehalose in the *ssy1* mutant (results not shown). The effect on carbohydrate metabolism could reflect a stress response in the *ssy1* and *stp1 stp2* mutants, as many of these genes are also found to be upregulated in cells exposed to various types of stress and starvation (Parrou *et al.*, 1997, 1999; Alexandre *et al.*, 2001; Causton *et al.*, 2001). Another large group of genes affected in the *ssy1* and *stp1 stp2* mutants are other genes responding to cell stress, and besides the ones involved in trehalose and glycogen metabolism, transcript levels of 16 genes were found to be increased in at least one of the mutants, viz *CTT1, SIP18, HSP104, HSP30, SSA4, GCY1, PRX1* and *YDR453C* in the *ssy1* mutant and *ICT1, YGP1, CUP2, HSP26, HSP42, PAI3, XBP1* and *YGR043C* in both mutants.

Six genes involved in lipid, fatty-acid, and sterol metabolism are also found to be more expressed

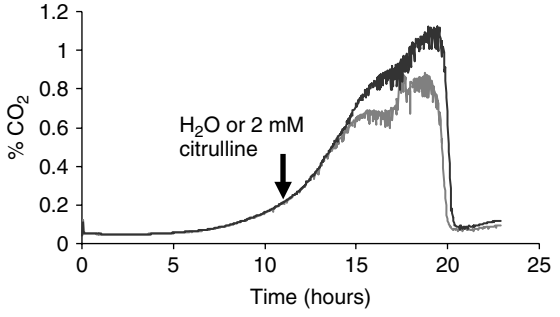

Figure 2. Extracellular L-citrulline affects carbon metabolism. Mole % CO_2 in the exhaust gas from two fermentations of the parental *gap1* strain grown in SD medium with ammonium as sole nitrogen source. To the culture was added either L-citrulline to a final concentration of 2 mM (lower curve) or an equal volume of H_2O (upper curve) in the early exponential phase (time point of addition indicated by the arrow). The figure is representative for four independent replicated fermentations

in both the *ssy1* and the *stp1 stp2* mutants (*CRC1, HES1, IPK1*, *MCD4, INO1* and *PDC6*), and *PHO84, PHO89, SPL2* and *VTC3*, involved in phosphate uptake and accumulation, are also more expressed in both mutants.

A few more functional groups of genes are affected in the *ssy1* and *stp1 stp2* mutants. Some genes involved in mating response are less expressed in the mutants, while a few encoding transcription factors and some involved in energy generation are more expressed. Besides these, a large group of 194 genes have unknown function, while the remaining genes belong to functional classes with only a few genes.

A large number of indirect effects in the *stp1 stp2* and *ssy1* mutants

The large deviation between the number of genes induced by L-citrulline and the number of genes affected in the *ssy1* and *stp1 stp2* mutants could have several causes. One explanation could be that the primary effect of inactivating the amino acid sensing system affects the cell physiology in such a way that a second set of genes are induced. In this view, most of the genes that are affected in the two mutants are not under the direct control of Stp1p, Stp2, and Ssy1p. A second explanation to the broad effects of the mutations could be that most of the affected genes are actually under the direct control of Ssy1p, Stp1p and Stp2p, but that this control is not apparent when the parental strain is induced only with L-citrulline, since these genes are also under the control of other regulatory pathways. The latter explanation may apply to a few of the genes, such as the amino acid permease gene *AGP1*, as this gene is known to be induced by L-citrulline (Iraqui *et al*., 1999b) but also repressed by ammonium (Iraqui *et al*., 1999b; Regenberg *et al*., 1999). To test whether the second hypothesis is generally true, we searched for a common motif in the promoters known to be regulated by amino acids in an *SSY1*-dependent way. Using the consensus sequence proposed by Nielsen *et al*. (2001) and the weight matrix, ANN-Spec (Workman and Stromo, 2000), we found that the promoter regions of *GNP1, AGP1, MUP1, BAP3, BAP2, TAT1, TAT2, CAR2* and *DIP5* have the sequence (A/G)(GGC/GCCG)(C/T)-N_{1-28}-(A/G)(CGGC/GCCG)(C/T) in common (Table 2). Among the 501 genes, the consensus sequence is found in 49 of the promoters (Table 2), of which nine are the amino acid permease genes known to be regulated by Ssy1p. We therefore propose that most of the transcripts that are altered in the mutants are not directly dependent on Stp1p, Stp2p, or Ssy1p, but are rather changed in response to secondary physiological effects that occur in cells missing these factors. The consensus sequence was also found in another four of the genes induced by L-citrulline (Table 1), viz. the citrate synthase gene *CIT1*, the glycogen synthase gene *GSY1*, the heat shock protein gene *HSP42*, and *INO1*, encoding inositol phosphate synthase, suggesting that these genes are directly regulated by the amino acid sensing pathway.

Stp1p re-localizes in response to amino acid induction

Mutational analyses have identified only few components involved in extracellular amino acid signalling in addition to the SPS complex and Stp1p and Stp2p, pointing to the possibility that signalling could involve transport of Stp1p and Stp2p. To investigate this hypothesis, we constructed a strain carrying Stp1p with a C-terminal fusion to a red-shifted version of green fluorescent protein (GFP) called yellow fluorescent protein. This *GFP* was fused to the last codon of the chromosomal copy of *STP1* in the parental *gap1 ura3* strain, using the method and constructs described by Lisby *et al*. (2001). The functionality of the resulting gene product was tested on YPD plus metsulphuron-methyl, which is known to support growth of *STP1* strains but not *stp1* strains (Jørgensen *et al*., 1997). As the *STP1–GFP* strain was found to grow equally as well as the *STP1* parental strain, we conclude that the Stp1p–GFP fusion is functional and supports amino acid induction of the permease genes (results not shown). Stp1p-GFP was visualized by epifluorescence microscopy in cells grown on synthetic dextrose medium and appears to migrate from the cytoplasm to the nucleus upon citrulline addition (data not shown), as reported for leucine addition by Andréasson and Ljungdahl (2001).

Discussion

We have used whole-genome transcription profiling to investigate the effect of extracellular amino

Table 2. Genes containing the consensus [A/G][CGGC/GCCG][C/T]-N_{1-28}-[A/G][CGGC/GCCG][C/T] in the promoter sequence

Consensus				Repeating consensus			Consensus				Repeating consensus		
Gene	**Start**	**Stop**	**Length**	**Start**	**Stop**	**Length**	**Gene**	**Start**	**Stop**	**Length**	**Start**	**Stop**	**Length**
AGP1	−557	−526	31	−696	−660	36	*SPS19*	−173	−153	20			
ARK1	−318	−303	15	−695	−675	20	***TAT1***	−469	−431	38			
ARO9	−232	−217	15	−439	−414	25	*THI7*	−527	−492	35			
BAG7	−521	−491	30				*TOS8*	−290	−255	35			
BAP2	−436	−402	34	−542	−529	13	*USV1*	−410	−399	11			
BAP3	−416	−402	14				*HEF3*	−360	−339	21			
CAR2	−167	−136	31	−211	−177	34	*YBL049W*	−638	−612	26			
CIT1	−353	−342	11				*YBR116C*	−359	−342	17			
DAL3	−394	−363	31				*YCR061W*	−261	−245	16			
DIA1	−509	−473	36				*YEL057C*	−411	−400	11			
EDC2	−154	−122	32	−404	−371	33	*YER064C*	−272	−236	36			
GDH2	−344	−327	17				*YER119C*	−538	−517	21			
GNP1	−380	−345	35	−464	−447	17	*YFR017C*	−550	−528	22			
GSY1	−444	−415	29				*YJR146W*	−582	−560	22			
HSP42	−196	−182	14				*YKL051W*	−339	−301	38			
ICT1	−321	−309	12				*YLR149C*	−232	−196	36			
INO1	−191	−176	15				*YLR312C*	−409	−384	25			
MUP1	−348	−310	38				*YLR346C*	−296	−270	26			
NDT80	−293	−259	34				*YMR085W*	−17	−5	12			
PHO84	−393	−368	25				*YNL194C*	−448	−415	33			
PUT4	−604	−587	17				*FUN34*	−333	−319	14			
RGS2	−594	−576	18				*FIT2*	−409	−380	29			
SML1	−470	−452	18				*YPL033C*	−172	−136	36			
SPL2	−194	−169	25	−391	−376	15	*YPL088W*	−445	−408	37			
							YPR064W	−75	−44	31	−151	−126	25

Genes shown in **bold** were also found to be induced by L-citrulline.

acids on transcription in the entire genome of *S. cerevisiae*. The effect is limited to the genes that were known to be induced by amino acids (e.g. *TAT1, BAP2, BAP3* and *PTR2*) plus a few others (*AGP2, TDH1, SOL4, HXK1, GSY1, GPH1, CIT1, ARA1, ADR1, AAD10, BIO3* and *BIO5*), of which most have a delayed response to amino acids as compared to the amino acid permease genes. With the exception of a few genes, the response is completely dependent on *STP1, STP2* and *SSY1*, suggesting that under the given conditions there is only one pathway for induction with L-citrulline and that this pathway is completely dependent on the integral membrane protein Ssy1p and the transcription factors Stp1p and Stp2p. Obviously the choice of amino acid is important for the result, as the intracellular levels of many amino acids have strong effects on gene expression through regulatory systems other than SPS (Holmberg and Schjerling, 1996; Wang *et al*., 1999; Iraqui *et al*., 1999a). It is complicated to use common L-α-amino acids other than L-citrulline for induction studies, as these amino acids are taken up, which makes it difficult to distinguish between pathways that respond to the extracellular and intracellular levels of amino acids. Two other studies were recently published using DNA arrays to investigate the global effects of an *SSY1* deletion (Forsberg *et al*., 2001; Kodama *et al*., 2002) and the global cellular response to a leucine pulse (Forsberg *et al*., 2001). Kodama and co-workers compared the transcriptional profiles of *ssy1* mutants to wild-type cells on complex medium using Affymetrix oligonucleotide arrays. The use of complex medium implies that the amino acid sensing pathway is turned on in the wild-type cells but not in the *ssy1* strains. As the authors mention, the threshold value for significance was set arbitrarily, implying an increased risk that genes might be overlooked that display lower, yet significant, fold changes. Forsberg *et al*. (2001) investigated the transcriptional responses of a wild-type and of an *ssy1*Δ strain to 0.15 mM L-leucine on

minimal medium. As the authors discuss, their study does not allow differentiation between the effects due to the activation of the amino acid sensing pathway and effects due to leucine uptake. The authors report changes in the transcriptional levels of a number of genes involved in amino acid and nitrogen metabolism in response to leucine addition in the wild-type, some of which appear to be regulated by *SSY1*. The effects of leucine addition observed by Forsberg and co-workers on amino acid permease genes are consistent with the ones we report, with only a few differences. We did not identify *MUP1* or *TAT2*, but *TAT1* and *AGP2* to be induced by amino acid, and we observed different effects of *SSY1* disruption on the transcriptional levels of the AAP genes. Forsberg and co-workers observed decreased expression of the arginine permease gene *CAN1* and the dicarboxylic amino acid permease gene *DIP5*, whereas we observed decreased expression of *AGP1, GNP1* and *MUP1* and increased expression of the proline permease gene *PUT4*. The use of different strain backgrounds or different data analyses might account for these differences. The present study, however, is based on a system that uncouples the effects of amino acid sensing and uptake, since citrulline uptake is inactive in *gap1* Δ cells, and also provides a statistical justification of the data sorting. We therefore propose that by far the majority of the observed effects are significant consequences of the amino acid sensing or the *SSY1/STP1 STP2* deletion.

In agreement with the studies published by Forsberg *et al*. (2001) and Kodama *et al*. (2002), genes encoding amino acid and peptide transporters were found to be induced by amino acids, while genes under nitrogen catabolite repression (NCR) were repressed by amino acids and/or were higher expressed in the *ssy1* mutant. The second group includes *DAL1, DAL4, DAL3, DAL5* and *DAL80*, involved in allantoin metabolism, *PUT1* and *PUT4* involved in proline catabolism, the γ-aminobutyric acid permease gene *UGA4* and the NADH-dependent glutamate dehydrogenase gene *GDH2*. Together, these results indicate that the loss of a functional amino acid sensing pathway leads to derepression of NCR. Forsberg *et al*. (2001) found that several genes involved in amino acid metabolism are repressed in response to amino acids, perhaps reflecting that the added amino acid, in this case L-leucine, may be sensed intracellularly, e.g. via isopropylmalate synthase, α-isopropyl malate and the transcription factor Leu3p. In the study by Kodama *et al*. (2002), a dozen genes involved in methionine metabolism were found to be induced in an *ssy1* mutant grown on complex medium, an effect presumably not caused directly by amino acid sensing but rather by reduced intracellular levels of sulphur-containing amino acids via the dedicated transporters.

Here we also study the role of the transcription factors Stp1p and Stp2p. The transcription profiles of the *ssy1* mutant and the *stp1 stp2* mutant display strong similarities, albeit some genes involved in stress response and energy generation are strongly affected by the *ssy1* mutation but not significantly affected in the *stp1 stp2* mutant. This could mean that Stp1p and Stp2p are not the only transcription factors involved in the amino acid sensing pathway, or that Ssy1p is involved in transcriptional regulation of these genes through another pathway, possibly independently of Stp1/2p. Ssy1p is known to reside in the plasma membrane in concert with Ssy5p and Ptr3p (Klasson *et al*., 1999) and it is suggested to be the first component to act in the amino acid sensing pathway (Bernard and André, 2001a; Didion *et al*., 1998; Gaber *et al*., 2003), while Stp1p can locate to the nucleus (Wang *et al*., 1992; Andréasson and Ljungdahl, 2002) and can bind directly to the promoters of *BAP2* and *BAP3* (de Boer *et al*., 2000; Nielsen *et al*., 2001). There are few candidates for factors acting between Ssy1p, Ptr3p and Ssy5p in the plasma membrane and Stp1p in the nucleus. Most of the mutants that lead to a clear defect in amino acid sensing are affected in *SSY1*, *PTR3, SSY5* and *STP1* (Didion *et al*., 1998; Jørgensen *et al*., 1998; Klasson *et al*., 1999). We therefore considered the possibility that the signal is transmitted from the plasma membrane to the nucleus by relocalization of one of the components in the SPS complex or the transcription factors in response to amino acid sensing. The locations of an Stp1–GFP fusion in the absence and presence of L-citrulline substantiate this hypothesis. Under non-inducing conditions, the fusion protein is to a large extent found in the cytosol, while induction with L-citrulline leads to an enhanced concentration of the fluorescence signal from Stp1p–GFP in the nucleus (data not shown). This observation corresponds to the results of Andréasson and Ljungdahl

(2002), who found that cytosolic Stp1p is proteolytically processed and targeted to the nucleus in response to amino acid induction. It is therefore possible that signalling is mediated by direct interaction between SPS at the plasma membrane and Stp1p in the cytoplasm, resulting in relocalization of Stp1p to the nucleus.

We also used the UAS_{aa} identified by Nielsen *et al*. (2001) as a basis for the identification of [(A/G)(CGGC/GCCG)(C/T)-N_{1-28}-(A/G)(CGGC/GCCG)(C/T)] as a consensus UAS_{aa} in promoters regulated by Stp1p and Stp2p.

The suggested *cis*-acting element is only present in 49 promoters of the 501 genes found to be differentially expressed upon amino acid induction, or in the *stp1 stp2* mutant or the *ssy1* mutant. Hence many of the affected genes do not appear to be under the direct control of Stp1p, Stp2p or Ssy1p, but are still interesting in our understanding of amino acid sensing, as their regulation suggests a cross-talk between amino acid sensing and other regulatory pathways. *DAL1, DAL2, DAL3, DAL4* and *DAL80*, involved in the catabolism of allantoin, the proline-oxidase gene *PUT1*, the proline permease gene *PUT4*, the gene encoding the γ-aminobutyric acid permease *UGA4*, and the glutamate dehydrogenase gene *GDH2* are more highly expressed in the mutants. These genes are known to be controlled by the GATA-binding factors responsible for NCR (Cunningham *et al*., 1996; Soussi-Boudekou *et al*., 1997; Kulkarni *et al*., 2001). Three of these genes, *DAL3, GDH2* and *PUT4*, could be under direct control of Ssy1p, Stp1p and Stp2p, as they contain the consensus sequence described in Table 2, while the remaining effect might be caused by an indirect effect on NCR. Derepression of NCR-controlled genes was reported by Kodama *et al*. (2002) in an *ssy1* mutant. Our data support this finding and show that NCR is also derepressed in an *stp1 stp2* mutant, suggesting that *SSY1, STP1* and *STP2* together have an effect on NCR; however, our data do not allow us to discriminate whether this effect is direct or indirect. NCR uses a more general regulatory network, TOR, which responds to nutritional changes in the cell (Beck and Hall, 1999; Hardwick *et al*., 1999). Although expression of the NCR-regulated genes is increased in the mutants in the current study, we see very little effect on other genes regulated by the TOR pathway, so the mutants seem to have a local effect on NCR. However, we find a big overlap with genes induced by various types of stress, viz. *COX5B, CYC1, ARA1, GLK1, GND2, GPH1, GSY2, HXK1, PGM2, TPS2, TSL1, CTT1, HSP104, HSP26, HSP42, PNC1, SSA4, XBP1, AUT7, ECM4, EDC2, GAD1, GCY1, GDH2, GPM2, GRE1, GYP7, IKS1, OYE3, PB12, PDR15, SOL1, SOL4, SPI1, STF2, TFS1, PHM8, YPS6, YBR056W, YLR345W, YLR327C, YMR090W, YDR453C, YGR043C, YAL061W, YBR047W, YBR053C, YBR116C, YBR137W, YCR061W, YDL110C, YDL204W, YDL222C, YDR070C, YER053C, YER067W, YER175C, YFL030W, YFR017C, YGL121C, YHL021C, YHL087W, YHR138C, YJL017W, YJL048C, YJL185C, YJR008W, YJRO96W, YKL091C, YKL151C, YKR049C, YLR205C, YLR251W, YLR252W, YMR040W, YMR041C, YMR196W, YNR002C, YNR014W, YOR173W, YOR220W, YOR273C* and *YOR289W*, amounting to 38% of the 216 genes found by Causton *et al*. (2001) to be induced under seven different stress conditions. These genes are regulated by the transcription factors Msn2p and Msn4p, which normally reside in the cytoplasm. Upon stress, Msn2p and Msn4p are transported into the nucleus, where they bind to the stress response element (STRE) in promoters of stress-activated genes. The transport of Msn2p and Msn4p to the nucleus can also be induced by the TOR pathway inhibitor rapamycin (Beck and Hall, 1999), and it therefore remains to be seen whether amino acid sensing is acting on stress-induced and NCR genes via the TOR pathway.

Unexpectedly, there is little overlap between the 614 genes regulated by the transcription factor Gcn4p and those identified in this study. Gcn4p is synthesized in amino acid-starved cells (Hinnebusch, 1997) and confers transcriptional induction of amino acid biosynthetic genes in multiple pathways (Arndt and Fink, 1986; Hinnebusch, 1986), as well as genes in purine and vitamin biosynthesis, peroxisomal components, mitochondrial carrier proteins, autophagy and genes in glycogen homeostasis (Natarajan *et al*., 2001). Compared to these results, we only find the citrate synthase genes *CIT1* and *CIT3*, plus the glutamate dehydrogenase *GDH3*, which catalyses the production of glutamate from α-ketoglutarate, *BIO3, BIO4, BIO5*, and *VTH1* involved in biotin metabolism, *GSY1, GSY2*

and *GLC3* involved in the biosynthesis of the storage carbohydrate glycogen, and also *GPH1*, encoding glycogen phosphorylase, responsible for degradation of glycogen, which is expressed less in a *gcn4* mutant and more in the *ssy1* mutant, whereas *BAP2* and *AGP1* are affected in the same way in both mutants. Hence, it seems that the amino acid sensing pathway and general amino acid control, imposed by Gcn4p, work rather independently of one another.

In conclusion, and in accordance with the recent work by Andréasson and Ljungdahl (2002), we propose that extracellular amino acids, such as added L-citrulline, are sensed by the Ssy1p–Ptr3p–Ssy5p complex in the plasma membrane in a process in which the signal is transmitted to the Stp transcription factors, which are released from their cytosolic location to the nucleus. We further propose that the Stps bind to promoters containing the UAS_{aa} and thereby lead to transcriptional induction. Besides these direct effects on permease genes, the current transcription profiles indicate that Stp1p, Stp2p and Ssy1p are involved in the maintenance of NCR and repression of stress-induced genes.

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Appendix 1. Supplementary data

Five hundred and one transcripts were found to be significantly changed in one of the following experiments: parental strain 15 min L-citrulline, 30 min L-citrulline, 60 min L-citrulline, *ssy1* strain 0 min L-citrulline, *ssy1* strain 30 min L-citrulline, *stp1 stp2* strain 0 min L-citrulline, and *stp1 stp2* strain 30 min L-citrulline as compared to the parental strain 0 min L-citrulline. The criteria for significance are described in Materials and Methods.

	ORF	log(gap1 15 min/ gap1 0 min)	log(gap1 30 min/gap1 0 min)	log(gap1 60 min/gap1 0 min)	log(gap1 ssy1 0 min/gap1 0 min)	log(gap1 ssy1 30 min/gap1 0 min)	log(gap1 stp1 stp2 0 min/gap1 0 min)	log(gap1 stp1 stp2 30 min/gap1 0 min)
AAC3	*YBR085C*	-0.01	0	0.18	0.53	0.62	0.57	0.53
AAD10	*YJR155W*	0.13	0.35	0.6	0.7	0.68	0.73	0.76
ABM1	*YJR108W*	0	0	0.29	0.29	0.55	0.22	0
ADE4	*YMR300C*	0.04	-0.12	-0.03	-0.52	-0.63	-0.34	-0.38
ADE6	*YGR061C*	-0.21	-0.02	-0.05	-0.4	-0.5	-0.47	-0.38
ADE8	*YDR408C*	-0.02	-0.06	-0.11	-0.41	-0.5	-0.34	-0.29
ADR1	*YDR216W*	0.51	0.31	0.59	0.37	0.5	0.13	0.34
AGA1	*YNR044W*	0.03	-0.08	-0.01	-0.78	-0.92	-0.54	-0.56
AGA2	*YGL032C*	0.15	-0.03	0.16	-0.39	-0.41	-0.33	-0.5
AGP1	*YCL025C*	0.41	0.41	0.42	-0.93	-1.01	-1.07	-0.92
AGP2	*YBR132C*	0.35	0.36	0.55	0.39	0.12	0	0.09
ALD3	*YMR169C*	0.02	0	0.17	0.6	0.71	0.3	0.26
ALG1	*YBR110W*	0.1	0	-0.01	0.02	-0.04	-0.02	-0.56
AMD2	*YDR242W*	0.23	0.18	0.06	0.34	0.36	0.56	0.4
AMS1	*YGL156W*	0.14	0.17	0.25	1.05	1.03	1.02	0.99
APG17	*YLR423C*	-0.09	-0.06	0.03	1	0.94	1.02	0.99
ARA1	*YBR149W*	0.43	0.32	0.56	0.56	0.61	0.55	0.34
ARK1	*YNL020C*	0	0.2	0.14	0.13	0	0.1	0.63
ARO9	*YHR137W*	0.04	-0.11	-0.11	0.85	0.82	0.93	1.09
ASG7	*YJL170C*	-0.09	-0.1	-0.13	-0.35	-0.4	-0.37	-0.48
ATR1	*YML116W*	-0.11	-0.09	-0.22	-0.38	-0.52	-0.24	-0.24
AUT7	*YBL078C*	0.17	0.15	0.37	0.51	0.64	0.58	0.54
BAG7	*YOR134W*	0	0	0	0.77	0.38	0.27	0
BAP2	*YBR068C*	1.01	1.36	1.12	0.44	0	0	0
BAP3	*YDR046C*	1.35	1.66	1.47	0	0.06	0	0
BAT2	*YJR148W*	0.05	0.05	0.16	0.21	0.25	0.54	0.61
BIO3	*YNR058W*	0.1	0.38	0.78	0.94	0.92	0.97	1
BIO4	*YNR057C*	-0.13	-0.07	0.41	0.73	0.67	0.68	0.72
BIO5	*YNR056C*	0.35	0.81	1.37	1.49	1.55	1.59	1.67
BOP2	*YLR267W*	0	0.06	0	0.64	0.5	0.54	0.42
BTS1	*YPL069C*	0.08	0.05	-0.09	-0.52	0.06	0.18	0.09
CAR2	*YLR438W*	0.2	0.03	0.35	0.45	0.53	0.41	0.53
CDA1	*YLR307W*	0	0	0	0.56	0.47	0.23	0
CIT1	*YNR001C*	0.64	0.55	0.4	0.53	0.72	0.61	0.66
CIT3	*YPR001W*	0	-0.04	0.44	0.44	0.33	-0.15	0.61
CLB6	*YGR109C*	-0.01	0.12	0.11	-0.22	0.03	-0.49	-0.18
CMK2	*YOL016C*	-0.05	-0.14	0	0.5	0.44	0.69	0.55
COS10	*YNR075W*	-0.07	-0.19	-0.01	-0.55	-0.68	-0.13	-0.34
COS12	*YGL263W*	0.06	0.02	0.11	-0.64	-0.64	-0.64	-0.64
COS5	*YJR161C*	0.12	0.04	0.2	-0.76	-0.53	-0.17	-0.48

COS7	*YDL248W*	-0.13	-0.21	0.03	-0.6	-0.63	-0.31	-0.37
COX5B	*YIL111W*	0.01	-0.05	0.17	0.39	0.49	0.39	0.4
CRC1	*YOR100C*	-0.26	-0.12	0.34	0.75	0.76	0.64	0.8
CTR1	*YPR124W*	0.05	0.12	0.28	0.39	0.5	-0.13	0.17
CTT1	*YGR088W*	-0.04	-0.18	0.19	0.81	0.85	0.46	0.44
CUP2	*YGL166W*	-0.03	-0.04	-0.01	0.49	0.46	0.62	0.67
CVT17	*YCR068W*	0.29	0.22	0.2	0.33	0.48	0.45	0.4
CYB2	*YML054C*	-0.05	0.02	0	0.55	0.65	0.44	0.48
CYC3	*YAL039C*	-0.17	-0.11	-0.06	0.49	0.2	0.04	0.04
CYC7	*YEL039C*	-0.22	0	0.32	0.71	0.94	0.19	0.05
DAL1	*YIR027C*	-0.23	-0.3	-0.21	0.57	0.57	0.62	0.59
DAL3	*YIR032C*	-0.07	-0.25	-0.09	0.51	0.56	0.49	0.51
DAL4	*YIR028W*	-0.02	-0.03	-0.24	0.84	0.82	0.88	0.83
DAL5	*YJR152W*	0.02	-0.1	0.05	0.28	0.46	0.7	0.78
DAL80	*YKR034W*	-0.23	-0.36	-0.16	0.27	0.52	0.6	0.61
DBP2	*YNL112W*	-0.05	-0.02	-0.29	-0.25	-0.69	-0.64	-0.68
DBP7	*YKR024C*	-0.08	-0.02	-0.37	-0.15	-0.31	-0.3	-0.51
DER1	*YBR201W*	-0.05	-0.07	0.01	0.49	0.32	0.5	0.44
DIA1	*YMR316W*	-0.19	-0.02	-0.04	0.68	0.44	0.87	0.78
DIM1	*YPL266W*	-0.05	-0.06	-0.21	-0.25	-0.52	-0.28	-0.22
ECM13	*YBL043W*	-0.51	-0.18	-0.54	0	-0.06	-0.36	-0.33
ECM4	*YKR076W*	0.48	0.34	0.61	0.25	0.48	0.36	0.39
EDC2	*YER035W*	0.06	0.05	0.33	0.5	0.53	0.46	0.54
ENA5	*YDR038C*	0.01	0	0	0.34	0.39	0.46	0.48
ENT4	*YLL038C*	-0.09	-0.21	-0.05	-0.35	-0.65	-0.45	-0.22
ERO1	*YML130C*	-0.17	-0.05	0.04	0.54	0.54	0.61	0.44
EST1	*YLR233C*	0.8	0.7	0.9	1.05	0.91	0.4	1.04
EUG1	*YDR518W*	-0.01	-0.07	-0.07	0.46	0.39	0.48	0.42
FAA2	*YER015W*	-0.08	-0.17	-0.23	0	0.02	0.13	0.49
FAD1	*YDL045C*	0.02	-0.1	-0.64	0.22	0.1	0.21	0.19
FAR1	*YJL157C*	0.14	0.16	0	-0.35	-0.51	-0.36	-0.49
FCY21	*YER060W*	-0.28	-0.01	0.03	0.83	0.82	0.77	0.75
FCY22	*YER060W-A*	0	0	0	0.74	0.79	0.38	0.47
FIG2	*YCR089W*	-0.12	-0.19	-0.33	-0.23	-0.4	-0.27	-0.75
FRM2	*YCL026C-A*	-0.03	-0.17	0.28	0.62	0.56	0.67	0.4
FUS1	*YCL027W*	-0.16	-0.2	-0.22	-0.5	-0.59	-0.3	-0.38
GAC1	*YOR178C*	-0.09	0	-0.07	0.37	0.52	-0.09	0.09
GAD1	*YMR250W*	0.12	0.16	0.4	0.96	1.01	0.61	0.56
GAL3	*YDR009W*	0.4	0.21	0.31	0.28	0.56	0.37	0.45
GCV1	*YDR019C*	0.01	0.11	0.08	-0.59	-0.71	-0.38	-0.33
GCV2	*YMR189W*	-0.05	-0.04	-0.03	-0.7	-0.82	-0.44	-0.39
GCY1	*YOR120W*	0.04	-0.04	0.12	0.53	0.51	0.22	0.19
GDH2	*YDL215C*	0.1	0.11	-0.08	0.5	0.52	0.75	0.65
GDH3	*YAL062W*	0.04	-0.15	0.08	0.89	0.96	0.54	0.52
GIP1	*YBR045C*	0	0	0	0.07	0.01	0.25	0.62
GIT1	*YCR098C*	0	0	0	0.57	0.52	0.42	0.34
GLC3	*YEL011W*	0.14	0.07	0.37	0.5	0.61	0.3	0.29
GLK1	*YCL040W*	0.12	0.05	0.44	0.41	0.56	0.42	0.52
GND2	*YGR256W*	0	0	0.27	1.28	1.37	0.27	0.81
GNP1	*YDR508C*	0.17	0.15	0.14	-0.72	-0.79	-0.45	-0.55
GPH1	*YPR160W*	0.15	0.15	0.55	0.99	1.12	0.48	0.42
GPM2	*YDL021W*	0.02	-0.01	0.12	0.57	0.63	0.39	0.37
GPX1	*YKL026C*	-0.02	-0.06	0.01	0.43	0.5	0.28	0.29
GRE1	*YPL223C*	0.09	0.12	0.06	0.4	0.5	0.39	0.29

GSC2	*YGR032W*	0.03	0.08	0.09	1.01	0.96	1	1.06
GSP2	*YOR185C*	0.11	0.04	0.42	0.72	0.78	0.44	0.59
GSY1	*YFR015C*	0.05	0.03	0.5	0.51	0.59	0.21	0.19
GSY2	*YLR258W*	-0.02	-0.02	0.25	0.43	0.51	0.2	0.23
GUP2	*YPL189W*	0.1	-0.06	0.09	0.13	0.48	0.37	0.28
GYP7	*YDL234C*	0	-0.03	0	0.39	0.32	0.5	0.33
HEM3	*YDL205C*	-0.07	-0.08	-0.02	-0.43	-0.3	-0.58	-0.3
HES1	*YOR237W*	0	0	0.04	0.52	0.34	0.48	0
HMS2	*YJR147W*	-0.02	0.01	-0.27	-0.78	-0.97	-0.6	-0.47
HNT2	*YDR305C*	0	0.22	0	0.5	0	0	0.26
HO	*YDL227C*	0.09	0.08	0.05	-0.27	-0.16	-0.57	-0.44
HOC1	*YJR075W*	0.17	0.1	0.45	0.3	0.24	0.57	0.28
HPA3	*YEL066W*	0.24	0.12	0.18	0.51	0.51	0.36	0.51
HSP104	*YLL026W*	0.02	0	0.47	0.59	0.53	0.43	0.27
HSP26	*YBR072W*	0.19	0.09	0.34	1.72	1.7	0.85	0.87
HSP30	*YCR021C*	-0.35	-0.14	-0.05	0.97	1.28	0.25	0.45
HSP42	*YDR171W*	0.1	0.05	0.51	0.83	0.82	0.65	0.58
HXK1	*YFR053C*	0.04	-0.03	0.74	0.94	0.99	0.27	0.31
HXT4	*YHR092C*	0.13	0.3	0.28	0.51	0.57	-0.16	0.12
HXT5	*YHR096C*	0.17	0.1	0.28	1.89	1.97	1.65	1.6
HXT6	*YDR343C*	0.06	0.05	0.17	0.49	0.59	-0.04	-0.05
HXT7	*YDR342C*	0.04	-0.01	0.19	0.42	0.52	-0.06	-0.11
ICT1	*YLR099C;*	0.03	0.02	0.17	0.88	0.83	0.85	0.81
IKS1	*YJL057C*	-0.01	0.03	-0.04	0.72	0.73	0.51	0.6
INO1	*YJL153C*	0.32	0.57	0.96	1.48	1.56	1.52	1.45
IPK1	*YDR315C*	0.53	0.41	0.51	0.58	0.52	0.67	0.43
JA2	*YKL078W*	0.02	-0.06	-0.3	-0.33	-0.48	-0.46	-0.47
JEM1	*YJL073W*	0.06	-0.01	-0.06	0.47	0.31	0.48	0.32
KEL3	*YPL263C*	-0.22	-0.03	-0.38	-0.37	-0.5	-0.27	-0.26
KTR2	*YKR061W*	-0.17	-0.01	-0.21	0.62	0.26	0.57	0.64
LAP4	*YKL103C*	0.1	-0.04	0.29	0.46	0.52	0.41	0.43
LEE1	*YPL054W*	0.05	-0.13	0.1	0.59	0.72	0.63	0.38
LEU9	*YOR108W*	0.03	-0.02	-0.15	-0.43	-0.53	-0.39	-0.45
LHS1	*YKL073W*	-0.01	-0.03	-0.08	0.37	0.32	0.49	0.41
LOC1	*YFR001W*	-0.08	0.04	-0.15	-0.12	-0.19	-0.03	-0.5
LRG1	*YDL240W*	-0.11	-0.16	0.01	-0.38	-0.51	-0.32	-0.37
LYS14	*YDR034C*	0.07	0.09	0.09	1.47	1.5	1.37	1.33
LYS2	*YBR115C*	0.03	0.06	-0.06	-0.53	-0.51	-0.11	-0.24
MAE1	*YKL029C*	0.12	0.01	-0.05	-0.47	-0.49	-0.35	-0.31
MAL33	*YBR297W*	0.09	-0.05	0.21	0.02	0.01	0.51	0.44
MBR1	*YKL093W*	-0.04	-0.16	0.14	0.56	0.59	0.24	0.07
MCD4	*YKL165C*	0.13	0.14	0.02	0.51	0.48	0.55	0.5
MET10	*YFR030W*	-0.05	-0.23	-0.29	-0.5	-0.56	-0.53	-0.51
MET28	*YIR017C*	0.21	0.26	0.64	-0.03	0.55	0.22	0.48
MIH1	*YMR036C*	0.06	0.02	0.02	0.16	0.01	0.13	-0.69
MLS1	*YNL117W*	0.1	0.2	0.14	1.28	1.37	1.34	1.46
MPD1	*YOR288C*	0.1	0	-0.14	0.54	0.61	0.66	0.6
MRD1	*YPR112C*	-0.15	-0.06	-0.58	-0.24	-0.48	-0.28	-0.47
MSC2	*YDR205W*	0.27	0.18	0.05	0.38	0.28	0.33	0.48
MTH1	*YDR277C*	-0.16	-0.1	0.17	0.38	0.48	0.08	0.23
MTL1	*YGR023W*	0.24	0.2	0.47	0.56	0.47	0.24	0.41
MUP1	*YGR055W*	0.39	0.41	0.31	-0.76	-0.89	-0.81	-0.78
NAM2	*YLR382C*	0.02	-0.09	0.02	-0.09	-0.13	-1.08	-0.27
NCA2	*YPR155C*	-0.05	-0.08	-0.15	-0.98	-0.13	-0.09	-0.12

NCA3	*YJL116C*	0.09	0.09	0.06	0.51	0.34	0.03	0.19
NDT80	*YHR124W*	0.08	-0.14	0.31	0.48	0.23	0.51	0.52
NOP13	*YNL175C*	-0.09	-0.08	-0.4	-0.23	-0.51	-0.35	-0.43
NRG1	*YDR043C*	0.14	0.17	0.09	0.42	0.47	0.52	0.49
NRP1	*YDL167C*	-0.09	-0.04	-0.29	-0.29	-0.51	-0.31	-0.36
NVJ1	*YHR195W*	-0.03	-0.03	0.08	0.15	0.14	-0.98	0.03
OM45	*YIL136W*	0.05	0.08	0.27	0.56	0.62	0.28	0.27
OPT2	*YPR194C*	0.28	0.26	0.54	0.29	0.48	-0.41	-0.3
OYE3	*YPL171C*	0.18	0.09	0.24	0.56	0.32	0.43	0.22
PAI3	*YMR174C*	0.18	0.21	0.31	0.7	0.76	0.54	0.62
PAU1	*YJL223C*	0.22	0.1	0.2	0.37	-0.65	0.23	0.72
PBI2	*YNL015W*	0.03	-0.04	0.18	0.61	0.7	0.6	0.61
PDC6	*YGR087C*	0	0	0	0.63	0.6	0.47	0.7
PDR15	*YDR406W*	-0.11	-0.07	-0.05	0.49	0.53	0.01	0.24
PET18	*YCR020C*	0.09	0.01	0.05	0.8	0.91	0.8	0.86
PGM2	*YMR105C*	0.07	0.1	0.46	0.79	0.84	0.36	0.31
PHM6	*YDR281C*	-0.1	-0.13	-0.57	0.66	0.32	0.27	0.28
PHM7	*YOL084W*	-0.04	0.03	0.35	0.47	0.62	0.45	0.35
PHM8	*YER037W*	0.12	-0.06	0.39	0.84	0.73	0.52	0.4
PHO11	*YAR071W*	0.09	0.19	0.26	0.45	0.44	0.62	0.71
PHO3	*YBR092C*	0.1	0.25	-0.05	0.43	0.51	0.13	0.23
PHO84	*YML123C*	-0.01	-0.08	0.3	2.21	2.19	1.93	2
PHO89	*YBR296C*	0.08	0	0.08	0.78	0.59	0.74	0.81
PIG1	*YLR273C*	0.08	0.16	0.35	0.42	0.51	0.19	0.33
PLB1	*YMR008C*	0.01	0.06	0.09	0.38	0.38	0.46	0.49
PLM2	*YDR501W*	0.49	0.11	0	0.05	0.31	0.17	0
PNC1	*YGL037C*	0.02	-0.02	0.35	0.37	0.48	0.28	0.28
POL12	*YBL035C*	-0.1	-0.13	-0.06	-0.31	-0.48	-0.35	-0.29
POT1	*YIL160C*	0.2	0	0.31	0	0.5	0.16	0.07
PPM2	*YOL141W*	-0.21	-0.09	-0.54	-0.47	-0.54	-0.54	-0.54
PPT1	*YGR123C*	0.03	-0.03	-0.29	-0.34	-0.47	-0.6	-0.45
PRB1	*YEL060C*	0.1	0.01	0.28	0.43	0.44	0.5	0.48
PRM1	*YNL279W*	-0.04	-0.07	-0.2	-0.76	-0.83	-0.4	-0.46
PRM4	*YPL156C*	-0.06	-0.15	-0.19	0.43	0.4	0.58	0.5
PRM7	*YDL039C*	-0.02	-0.11	-0.07	0.51	0.19	0.49	0.48
PRX1	*YBL064C*	-0.14	0	0.17	0.52	0.54	0.32	0.33
PRY3	*YJL078C*	0.08	-0.03	-0.17	-0.33	-0.32	-0.53	-0.47
PST1	*YDR055W*	-0.09	-0.18	-0.03	0.52	0.49	0.19	0.2
PTP2	*YOR208W*	0.12	0.05	0.12	0.49	0.42	0.45	0.4
PTR2	*YKR093W*	0.5	0.4	0.34	-0.02	-0.1	0.21	0.07
PUT1	*YLR142W*	-0.09	-0.27	0.08	0.66	0.84	0.92	0.95
PUT4	*YOR348C*	0.22	0.11	0.16	0.47	0.64	0.4	0.42
PWP2	*YCR057C*	-0.04	-0.05	-0.26	-0.26	-0.56	-0.26	-0.47
RGS2	*YOR107W*	0	0	0	0.64	0	0	0.09
RIM4	*YHL024W*	-0.16	-0.49	0.24	0.34	0.32	-0.08	-0.46
RNR3	*YIL066C*	0.32	0	0	0.64	0	0.4	0.17
ROM1	*YGR070W*	0.03	0.01	0.07	0.71	0.66	0.29	0.33
RPA190	*YOR341W*	-0.02	-0.06	-0.28	-0.34	-0.51	-0.39	-0.4
RPL16A	*YIL133C*	-0.04	0.03	0.03	-0.08	-0.02	-0.64	-0.01
RPL7B	*YPL198W*	-0.01	-0.02	0.02	-0.35	-0.49	-0.47	-0.32
RPM2	*YML091C*	0.08	0.16	0.05	0.05	-0.09	-0.61	-0.27
RPP1B	*YDL130W*	0.08	0.03	0.21	0.54	0.5	0.31	0.29
RRP5	*YMR229C*	-0.09	-0.05	-0.29	-0.29	-0.49	-0.36	-0.39
RRS1	*YOR294W*	-0.13	-0.07	-0.55	-0.06	-0.16	-0.14	-0.16

RTA1	*YGR213C*	0	0	0.07	1.13	1.1	1.43	1.41
SDC25	*YLL016W*	0.14	0.08	0	0.4	0.34	0.54	0.25
SFB3	*YHR098C*	0	0.01	-0.01	0.03	0.04	-0.48	0.08
SGA1	*YIL099W*	-0.08	0.13	0.32	0.96	0.87	0.63	0.6
SHE4	*YOR035C*	0	0.12	0.23	0.5	0.44	0.47	0.47
SIP18	*YMR175W*	0	0	0	0.75	0.83	0.35	0.39
SML1	*YML058W*	0	-0.28	-0.28	1.35	1.2	1.08	1.1
SOL1	*YNR034W*	-0.09	-0.02	0.25	1.2	1.35	0.55	0.4
SOL4	*YGR248W*	0.15	0.19	0.58	1.06	1.24	0.44	0.55
SPI1	*YER150W*	0.06	-0.07	0.26	0.79	0.82	0.5	0.55
SPL2	*YHR136C*	0	0	0.16	1.58	1.62	1.24	1.36
SPR1	*YOR190W*	0.09	0	0.16	0.59	0.5	0	0.35
SPS19	*YNL202W*	0.04	0.09	0.09	0.48	0.5	0.4	0.4
SPS4	*YOR313C*	-0.38	-0.45	-0.45	0.52	0.46	0.53	0.46
SPT5	*YML010W*	0.2	0.13	0.09	-0.08	-0.05	0.07	-0.64
SSA4	*YER103W*	-0.02	-0.06	0.12	0.59	0.54	0.26	0.25
SSF1	*YHR066W*	-0.04	0.01	-0.33	-0.01	-0.17	-1.08	-0.19
SST2	*YLR452C*	-0.01	0	-0.08	-0.68	-0.69	-0.43	-0.44
SSY1	*YDR160W*	-0.11	-0.15	-0.05	-0.87	-0.87	0.1	-0.11
STE2	*YFL026W*	-0.01	-0.01	-0.01	-0.51	-0.45	-0.27	-0.32
STE6	*YKL209C*	-0.09	0.02	-0.11	-0.55	-0.39	-0.29	-0.29
STF2	*YGR008C*	0.02	0.01	0.36	0.73	0.72	0.71	0.62
SUV3	*YPL029W*	0.05	0.05	-0.58	0.1	-0.01	0.03	-0.11
SWI1	*YPL016W*	-0.07	-0.14	-0.15	-0.2	-0.56	-0.03	-0.2
TAD2	*YJL035C*	-0.08	-0.04	-0.08	0.57	0.43	0.46	0.4
TAT1	*YBR069C*	0.59	0.67	0.21	-0.36	-0.22	-0.26	-0.11
TCI1	*YDR161W*	-0.09	-0.03	-0.25	-0.46	-0.49	-0.32	-0.37
TDH1	*YJL052W*	0.19	0.22	0.58	1.04	1.17	0.78	0.75
TFS1	*YLR178C*	0.03	-0.03	0.31	0.68	0.77	0.32	0.31
THI11	*YJR156C*	0.06	-0.1	-0.26	1.02	1.1	0.87	0.89
THI2	*YBR240C*	-0.17	-0.08	-0.18	0.64	0.72	0.57	0.51
THI20	*YOL055C*	-0.01	-0.05	-0.29	1.2	1.26	1.05	1.14
THI4	*YGR144W*	0	-0.13	-0.15	0.47	0.73	0.33	0.38
THI7	*YLR237W*	0.09	0.07	-0.07	0.85	1.02	0.68	0.84
TIF4631	*YGR162W*	-0.01	-0.01	-0.11	-0.4	-0.58	-0.31	-0.46
TIS11	*YLR136C*	-0.05	-0.05	-0.02	1.07	0.9	0.96	0.92
TOS2	*YGR221C*	0.04	0.04	0	-0.53	-0.61	-0.69	-0.33
TOS3	*YGL179C*	0	-0.13	0	0.65	0.28	0.38	0.44
TOS8	*YGL096W*	-0.11	0.03	0.2	0.5	0.55	0.23	0.26
TPS2	*YDR074W*	0.07	0.08	0.19	0.5	0.47	0.25	0.2
TRF4	*YOL115W*	-0.13	-0.06	-0.09	-0.33	-0.49	-0.48	-0.47
TSL1	*YML100W*	0.1	0.06	0.28	0.54	0.56	0.31	0.33
UGA4	*YDL210W*	-0.23	-0.39	-0.17	0.33	0.2	0.51	0.39
USV1	*YPL230W*	0	0	0	0.68	0.83	0.43	0.04
VHT1	*YGR065C*	0.22	0.36	0.46	0.42	0.44	0.41	0.5
VPS8	*YAL002W*	-0.01	-1.12	-0.02	0.19	0.16	0.1	0.06
VTC3	*YPL019C*	-0.03	0	0.1	0.57	0.49	0.57	0.5
XBP1	*YIL101C*	0	0	0.29	1.12	1.06	0.71	0.62
YAP1801	*YHR161C*	0.03	0.01	0.15	0.18	0.17	-0.75	0.26
YAP7	*YOL028C*	-0.04	-0.02	0.01	-0.46	-0.36	-0.24	-0.54
YEF3B	*YNL014W*	-0.3	0.18	-0.3	0.55	0.55	0.44	0.66
YGP1	*YNL160W*	-0.01	-0.04	0.3	1.17	1.27	0.85	0.8
YIP3	*YNL044W*	0.21	0.09	0.13	0.5	0.5	0.55	0.48
YPS3	*YLR121C*	0	0	0.33	0.98	0.88	0.86	0.9

YPS6	*YIR039C*	0.43	0.4	0.54	0.8	0.97	0.65	0.71
YRF1-4	*YLR466W*	0.02	0.05	0.26	-0.66	-0.51	-0.54	-0.64
YRF1-5	*YLR467W*	0.23	0.2	0.32	-0.38	-0.5	-0.42	-0.5
YRF1-6	*YNL339C*	0.15	0.11	0.23	-0.62	-0.62	-0.57	-0.41
YRO2	*YBR054W*	0.19	-0.15	0.51	1.16	1.4	0.23	0.1
YVH1	*YIR026C*	-0.08	-0.07	-0.33	-0.3	-0.49	-0.31	-0.27
	YFL021C	0	0	0	0.41	0.52	0.1	0
	RDN25-1	0.24	-0.1	0.41	-0.56	-0.38	-0.4	-0.28
	RDN37-1	0.38	0.06	0.47	-0.5	-0.2	-0.29	-0.23
	RDN37-1	0.02	-0.01	0.21	-0.49	-0.45	-0.31	-0.47
	RPR1	0.06	-0.02	0.1	-0.26	-0.87	0.42	0.33
	SNR13	0.14	0.13	0.21	-0.44	-0.69	-0.64	-0.52
	snr17a	-0.15	-0.18	-0.33	-0.36	-0.61	-0.1	-0.12
	SNR31	-0.25	-0.16	-0.55	0.09	-0.1	-0.12	-0.55
	SNR44	-0.04	0.04	-0.02	-0.36	-0.54	-0.55	-0.54
	SNR45	0.25	0.58	0.59	0.4	0.49	0.17	0
	SNR54	-0.3	-0.06	-0.28	-0.44	-0.49	-0.49	-0.49
	SNR56	-0.06	0.05	-0.21	-0.4	-0.37	-0.51	-0.51
	SNR59	-0.08	-0.05	0	-0.21	-0.51	-0.46	-0.46
	SNR6	0.24	0.17	0.5	0.51	0.23	0.32	0.58
	SNR63	0.12	0.09	-0.06	-0.43	-0.39	-0.7	-0.8
	SNR66	-0.04	-0.04	0.05	0.32	0.59	-0.04	-0.04
	SNR69	-0.1	0.02	0.13	-0.36	-0.51	-0.51	-0.47
	SNR75	0.14	0.07	0.09	0.55	0.48	0.1	0.11
	TA(AGC)P	-0.07	0.1	-0.1	-0.12	-0.36	-0.19	-0.5
	TC(GCA)P1	0.06	0.11	-0.23	0.47	0.41	0.05	-0.64
	TG(GCC)B	0.74	0	1.11	1.43	1.19	1.24	1.37
	TG(GCC)C	-0.22	0.13	-1.03	0.4	0.03	0.15	0.31
	TG(GCC)D1	0.28	0.05	-0.96	0.52	0.21	0.36	0.35
	TG(GCC)D2	-0.57	-0.01	-1.05	0.34	0.26	0.11	0.13
	TG(GCC)E	0.45	1.12	0	1.41	0.97	1.39	1.27
	TG(GCC)J1	0.04	-1.05	-0.82	0.29	0.21	0.16	0.19
	TG(GCC)J2	-1.33	-0.38	-1.33	0.02	0.14	-0.04	0.05
	TG(GCC)O1	-0.25	-0.52	-1.31	0.22	0	-0.01	0.05
	TG(GCC)O2	-0.82	0.04	-1.32	0.08	-0.21	-0.22	-0.07
	TG(UCC)N	0.02	0.29	0.27	0.5	0.27	0.18	0.27
	TLC1	-0.01	0.04	-0.12	-0.6	-0.57	-0.64	-0.59
	TN(GUU)K	0.05	0.08	-0.06	0.57	0.54	0.6	0.55
	TQ(UUG)C	0.01	0.13	0.15	-0.1	-0.18	-0.2	-0.71
	TQ(UUG)D1	-0.05	-0.07	0.22	0.13	-0.22	-0.19	-0.58
	TQ(UUG)E1	0.15	0.24	0.22	0.1	-0.14	-0.03	-0.56
	TR(ACG)D	-0.31	0.14	-0.02	0.52	0.38	0.19	0.38
	TR(ACG)K	-0.44	0.07	0.06	0.61	0.35	0.3	0.24
	TR(ACG)L	-0.1	0.13	-0.07	0.58	0.37	0.29	0.31
	TR(ACG)O	-0.02	0.13	0.02	0.72	0.51	0.34	0.31
	TS(AGA)D2	-0.1	0.18	-0.05	0.51	0.26	-0.06	0.14
	TT(UGU)P	-0.01	0.07	-0.31	0.91	0.72	0.53	0.45
	TV(UAC)B	-0.23	-0.23	-0.23	0.52	0.44	0.05	0.28
	YAL061W	0.13	-0.03	0.47	0.74	0.9	0.47	0.31
	YAR029W	0.16	0.07	0	0.68	0.49	0.52	0.48
	YAR069C	-0.06	-0.49	-0.49	0.27	-0.19	0.2	0.25
	YBL048W	0.13	0.02	-0.15	1.2	1.23	0.99	0.97
	YBL049W	-0.01	0.07	0.32	0.63	0.72	0.52	0.54
	YBL054W	-0.06	-0.06	-0.75	-0.12	-0.42	-0.32	-0.29

YBL083C	-0.11	-0.08	-0.07	-0.26	-0.48	-0.16	-0.21
YBR005W	-0.02	0.05	-0.1	0.6	0.33	0.5	0.42
YBR032W	-0.79	-0.3	-0.48	-0.41	-0.9	-0.76	-0.9
YBR033W	0.02	0	0	0.54	0.6	0.45	0
YBR047W	-0.01	-0.26	-0.36	0.06	0.08	0.53	0.53
YBR053C	0.18	0.11	0.35	0.68	0.74	0.68	0.61
YBR056W	-0.09	-0.02	-0.2	1.21	1.05	1.55	1.45
YBR116C	-0.16	-0.16	-0.1	0.37	0.6	-0.06	-0.23
YBR137W	-0.02	0.02	0.08	0.4	0.37	0.54	0.41
YBR271W	0.04	-0.09	-0.48	-0.07	-0.2	-0.32	-0.16
YBR292C	-0.01	-0.09	-0.29	-0.04	-0.54	-0.2	-0.19
YCL042W	0.15	0.08	0.65	0.58	0.88	0.65	0.7
YCL058C	0.41	0.57	0.22	0.56	0.35	0.23	0.1
YCR061W	0.24	0.27	0.5	0.31	0.29	0.36	0.37
YCR072C	-0.02	-0.01	-0.54	-0.21	-0.55	-0.36	-0.27
YCR106W	0.5	0.33	0	0.05	0.02	0.23	0.48
YDL038C	0	0	0	0.4	0.38	0.51	0.44
YDL110C	-0.05	-0.04	0.21	0.53	0.55	0.43	0.42
YDL123W	0.18	0.15	0.03	0.49	0.35	0.36	0.26
YDL124W	0.08	0.1	0.24	0.64	0.65	0.66	0.55
YDL172C	0.45	0.31	0.22	0.17	0.6	0.12	0.4
YDL199C	-0.03	0.08	0.31	0.45	0.21	0.51	0.43
YDL204W	0.06	0.19	0	0.44	0.53	0.23	0.2
YDL222C	0.23	0.14	0.2	0.91	1.02	0.67	0.63
YDL241W	-0.03	-0.03	-0.07	-0.68	-0.69	-0.63	-0.37
YDR042C	0	0	0.1	0.85	0.79	0.36	0.52
YDR070C	-0.25	0.03	0.12	1.06	1.24	0.51	0.49
YDR093W	-0.01	-0.84	-0.14	-0.1	-0.18	-0.17	-0.16
YDR101C	-0.02	0	-0.37	-0.16	-0.53	-0.24	-0.32
YDR119W	0.07	-0.04	-0.13	-0.36	-0.49	-0.18	-0.24
YDR209C	0.18	0.14	0.12	0.41	0.49	0.43	0.42
YDR210W	0.1	0.07	0.06	0.45	0.49	0.51	0.56
YDR366C	0.28	0.43	0.01	0.71	0.47	0.63	0.47
YDR380W	-0.19	-0.19	-0.19	0.53	0.37	0.89	0.85
YDR384C	0.04	-0.01	-0.18	-0.62	-0.72	-0.55	-0.58
YDR453C	0.08	0.09	-0.03	0.63	0.61	0.34	0.31
YDR540C	0.03	0.07	0.12	0.48	0.54	0.61	0.66
YEL057C	-0.18	-0.18	-0.18	0.53	0.46	0.46	0.16
YEL059W	0	0	0	0.4	0.64	0.31	0.32
YER053C	-0.03	0.06	0.2	0.43	0.52	0.16	0.16
YER064C	0.52	0.37	0.14	0.1	-0.16	-0.03	-0.03
YER067W	0.15	-0.13	0.49	0.49	0.71	0.23	0.24
YER119C	0.06	-0.03	0.14	0.27	0.53	0.18	0.18
YER121W	0.22	0.25	0.37	0.66	0.74	0.62	0.62
YER158C	-0.04	0.12	0.28	0.62	0.53	0.26	0.26
YER175C	0.08	-0.14	-0.28	0.24	0.19	0.5	0.47
YER185W	-0.09	0.03	-0.1	0.61	0.24	0.6	0.54
YFL030W	-0.09	-0.02	0.13	0.48	0.46	0.41	0.43
YFR017C	0	-0.03	0.34	0.65	0.73	0.38	0.34
YFR026C	-0.01	0.09	0.07	1.09	0.88	1.02	0.87
YFR032C	-0.55	-0.5	-0.32	-0.21	-0.84	-0.9	-0.8
YGL121C	-0.08	0	0.1	1.18	1.33	0.63	0.77
YGR039W	0.08	0	0	1.16	1.09	1.13	1.02
YGR043C	-0.04	-0.17	-0.06	1	1.03	1.03	0.92

YGR103W	-0.08	-0.01	-0.23	-0.28	-0.54	-0.35	-0.58
YGR110W	0.1	-0.04	0.1	0.58	0.55	0.69	0.61
YGR138C	0.05	0.05	-0.03	0.87	0.85	0.31	0.24
YGR145W	-0.03	-0.03	-0.32	-0.25	-0.54	-0.31	-0.38
YGR146C	0	-0.22	0	0.78	0.7	0.53	0.59
YGR160W	-0.52	-0.09	-0.52	-0.52	-0.4	-0.52	-0.52
YGR161C	-0.09	-0.11	0.28	0.56	0.55	0.61	0.62
YHL021C	-0.02	0.03	0.33	0.36	0.44	0.67	0.55
YHL050C	0.13	0.06	0.17	-0.53	-0.33	-0.37	-0.5
YHR032W	0	0.04	-0.1	-0.13	-0.22	-1.23	-0.36
YHR052W	-0.2	-0.15	-0.22	-0.23	-0.51	-0.38	-0.33
YHR063C	0.06	0.02	-0.55	-0.07	-0.05	-0.04	0.06
YHR087W	0.1	0.06	0.46	0.68	0.78	0.56	0.52
YHR126C	0.04	0.11	0.21	0.49	0.31	0.29	0
YHR138C	-0.02	-0.01	0.13	0.62	0.6	0.57	0.55
YHR140W	0.02	-0.1	0.18	0.54	0.67	0.62	0.52
YHR162W	-0.06	0	0.02	-0.05	0.06	-1.44	0.1
YHR196W	0.01	0.03	-0.12	-0.13	-0.19	-0.54	-0.19
YHR209W	-0.11	-0.12	-0.13	0.48	0.55	0.32	0.56
YIL059C	-0.02	0.05	-0.13	0.33	0.68	0.24	0.46
YIL079C	0.03	0	-0.43	-0.09	-0.28	-0.15	-0.92
YIL121W	0.17	0.11	0.17	-0.69	-0.79	-0.32	-0.34
YIR042C	-0.08	-0.1	0.01	-0.74	-0.75	-0.75	-0.75
YIR043C	0.03	0.12	-0.03	-0.56	-0.56	-0.56	-0.56
YJL017W	0.11	0.19	0.19	0.51	0.6	0.45	0.39
YJL037W	0.12	0.34	0.11	1.09	0.96	0.76	0.82
YJL045W	0.11	0.1	0.03	0.48	0.55	0.57	0.5
YJL048C	-0.03	0.05	0.2	0.39	0.48	0.5	0.41
YJL067W	-0.18	-0.15	-0.13	-0.03	-0.53	-0.11	-0.19
YJL119C	0	0	0.36	0.67	0.61	0.43	0.45
YJL144W	0.15	0.03	0.2	1.19	1.26	1.27	1.16
YJL152W	0.41	0.46	0.55	0.63	0.68	0.62	0.79
YJL160C	0.07	-0.28	-0.02	-0.04	-0.42	-0.42	-0.51
YJL161W	0.33	0.36	0.36	1.08	1.19	0.76	0.82
YJL200C	-0.04	-0.18	-0.03	-0.44	-0.5	-0.37	-0.32
YJL213W	-0.03	0	0.19	0.11	0.25	0.51	0.39
YJR008W	0.26	0.33	0.6	0.67	0.79	0.61	0.55
YJR078W	0.09	0.14	0.31	0.96	0.88	0.9	0.87
YJR079W	0.26	0.17	0.2	0.92	0.78	0.86	0.8
YJR096W	-0.01	-0.03	0.07	0.54	0.51	0.38	0.39
YJR146W	-0.01	-0.08	-0.05	-0.55	-0.22	-0.4	-0.58
YJR149W	0.04	0.14	0.26	0.16	0.17	0.57	0.52
YJR154W	0.17	0.37	0.84	0.29	0.59	0.63	0.6
YKL031W	0	0	0	0.77	0.69	0.58	0.66
YKL051W	0.05	0.02	0.08	-0.26	-0.21	-0.3	-0.57
YKL086W	-0.34	0.13	-0.14	0.7	0.72	0.2	0.48
YKL151C	-0.03	-0.05	0.06	0.55	0.57	0.51	0.44
YKL161C	0.14	0	0	0.45	0.34	0.5	0.54
YKL162C	0	0	0.46	0.32	0.59	0.25	0.48
YKL177W	0.27	0.16	0.07	0.5	0.5	0.58	0.69
YKR046C	0.16	0.18	0.4	0.92	0.93	0.79	0.82
YKR049C	0	0.02	0.07	0.6	0.52	0.5	0.48
YKR073C	0.45	0.61	0.41	0	0.6	0	0.08
YLL032C	0.48	0.5	0.26	0.3	0.04	0.53	0

YLL057C	-0.02	0.11	-0.06	0.46	0.5	0.49	0.51
YLL067C	0.06	0.02	0.13	-0.5	-0.42	-0.46	-0.47
YLR004C	0.1	0.16	0.08	1.25	1.56	1.28	1.37
YLR031W	0.24	0.42	0.48	1.09	1.08	0.85	0.81
YLR042C	-0.08	0	-0.03	-0.44	-0.53	-0.24	-0.62
YLR053C	-0.31	-0.39	-0.12	1.1	1.15	1.12	1.2
YLR054C	-0.02	-0.27	0.1	0.62	0.4	0.69	0.58
YLR149C	0.13	0	0.47	0.64	0.81	0.71	0.62
YLR194C	-0.09	-0.14	-0.06	0.85	0.52	0.84	0.81
YLR205C	-0.07	-0.14	0.06	0.58	0.43	0.57	0.54
YLR251W	0.06	-0.07	0.08	0.65	0.65	0.58	0.39
YLR252W	-0.01	-0.1	0.1	0.52	0.52	0.48	0.39
YLR271W	0.22	0.33	-0.01	0.58	0.6	0.44	0.36
YLR312C	-0.08	-0.21	0.09	0.53	0.59	0.45	0.25
YLR327C	-0.22	-0.27	0.3	0.9	1.19	0.77	0.79
YLR346C	-0.13	-0.11	-0.33	1.01	1.1	1.16	1.12
YLR392C	0.49	0.34	0.19	0.55	0.44	0.41	0.63
YLR413W	0.02	0.05	-0.18	-0.69	-0.79	-0.72	-0.76
YLR414C	-0.07	-0.08	-0.05	0.47	0.4	0.56	0.58
YLR415C	0	0	0	0.03	0.59	0.53	0.44
YLR428C	0.11	0.17	0	0.25	0.43	0.41	0.58
YLR431C	0.41	0.48	0.28	0.42	0.52	0.44	0.26
YLR464W	0.02	0.18	0.22	-0.62	-0.67	-0.63	-0.67
YML093W	-0.04	-0.14	-0.25	-0.27	-0.59	-0.25	-0.38
YML128C	0	-0.13	0.25	0.52	0.72	0.19	0.12
YMR007W	-0.04	0.11	0.2	0.54	0.55	0.53	0.26
YMR025W	0.15	0.2	0.22	0.36	0.53	0.39	0.46
YMR034C	0.11	0.14	0	0.99	0.7	0.88	0.95
YMR040W	-0.09	0.02	-0.01	1.14	1.04	1.14	1.03
YMR041C	0.05	0.04	-0.21	0.49	0.4	0.45	0.38
YMR085W	-0.13	-0.01	0.23	0.46	0.36	0.51	0.41
YMR090W	-0.01	0.01	0.22	0.59	0.61	0.33	0.37
YMR101C	0	0.03	0.19	0.17	0.21	0.08	0.73
YMR107W	0	0	0	1.32	1.34	1.26	1.17
YMR135W-A	0.01	0.04	0.49	0	0	0	0.26
YMR145C	0.1	0.07	0.01	-0.25	-0.31	-0.5	-0.49
YMR184W	-0.04	-0.06	-0.06	0.57	0.51	0.6	0.6
YMR191W	0.13	-0.07	0.19	0.41	0.3	0.57	0.33
YMR194C-A	0.23	0.29	-0.03	0.36	0.22	0.41	0.49
YMR196W	0.06	-0.05	0.3	0.61	0.72	0.3	0.35
YMR278W	0.06	0.19	0.22	0.38	0.57	0.3	0.43
YMR316C	0.13	0.1	-0.04	0.55	0.37	0.61	0.54
YNL065W	-0.03	0.02	-0.18	-0.26	-0.62	-0.4	-0.7
YNL140C	0.08	0.05	0	0.44	0	0	0.59
YNL179C	-0.26	-0.09	0.01	-0.75	-0.58	-0.44	-0.24
YNL194C	0	0	0.21	0.67	1.01	0.5	0.5
YNL254C	-0.03	-0.05	-0.06	-0.26	-0.57	-0.07	-0.06
YNR002C	-0.12	0.02	-0.17	0.74	0.96	0.47	0.63
YNR014W	0.09	-0.16	0.22	0.39	0.49	0.12	0.15
YOL015W	-0.22	-0.22	0.07	0.08	0.27	0.09	0.55
YOL024W	0	0	0	0.54	0.1	0.27	0
YOL031C	-0.07	-0.13	-0.04	0.63	0.62	0.74	0.68
YOL047C	-0.04	-0.18	-0.39	0.56	0.42	0.42	0.57
YOL053C	0.07	0.04	0.5	1.08	1.22	0.66	0.67

YOL153C	0.08	0	0.33	0.37	0.66	0.14	0.44
YOL155C	0.02	-0.15	-0.08	-0.29	-0.03	-0.66	-0.52
YOL155C	0.02	-0.07	-0.03	-0.08	0.01	-0.6	-0.47
YOR049C	0	0	0	0.94	1.08	0.71	0.64
YOR102W	0	0	0.12	0.77	0.14	0.01	0
YOR152C	0.23	0.14	0.18	0.52	0.34	0.43	0.53
YOR173W	-0.01	-0.06	0.3	0.65	0.78	0.42	0.39
YOR192C	0.11	0.05	-0.4	0.61	0.67	0.44	0.65
YOR220W	0.09	-0.05	0.06	0.57	0.47	0.67	0.52
YOR252W	-0.13	-0.07	-0.55	-0.15	-0.41	-0.14	-0.14
YOR273C	0.01	-0.13	0.24	0.47	0.39	0.52	0.5
YOR284W	-0.03	-0.05	-0.98	-0.07	-0.03	0.02	0.06
YOR287C	-0.34	-0.09	-0.59	-0.19	-0.23	-0.13	-0.09
YOR289W	0.05	0.02	0.05	0.68	0.67	0.69	0.65
YOR338W	-0.26	-0.18	-0.26	0.62	0.01	0.12	0.16
YOR343C	-0.18	-0.22	-0.25	-0.21	-0.28	-0.61	-0.19
YOR359W	-0.14	-0.05	-0.28	-0.2	-0.42	-0.46	-0.51
YOR382W	0.18	-0.03	-0.11	0.81	0.89	0.67	0.68
YPL017C	0	0	0	0.39	0.61	0.15	0.06
YPL033C	0	0	0	0.28	0.25	0.48	0.4
YPL088W	-0.2	-0.15	-0.16	0.63	0.4	0.52	0.45
YPL095C	0.04	0.16	-0.03	0.18	0.13	0.59	0.49
YPL186C	-0.04	-0.03	0.22	0.21	0.57	0.16	0.04
YPR064W	0.16	-0.14	-0.14	0.15	-0.05	0.62	-0.14
YPR077C	0	0	0	0.62	0.43	0	0.65
YPR142C	-0.27	-0.25	-0.52	0.01	-0.28	-0.07	-0.27
YPR144C	0.09	0	-0.57	-0.15	-0.37	-0.38	-0.27
YPR151C	-0.14	-0.17	-0.1	0.65	0.57	0.25	0.41
YPR195C	0.27	0.03	0.42	0.04	0.51	0	0.32

Chapter VI

Grr1p is required for transcriptional induction of amino acid permease genes and proper transcriptional regulation of genes in carbon metabolism of *Saccharomyces cerevisiae*

Nadine Eckert-Boulet, Birgitte Regenberg, Jens Nielsen

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RESEARCH ARTICLE

Nadine Eckert-Boulet · Birgitte Regenberg · Jens Nielsen

Grr1p is required for transcriptional induction of amino acid permease genes and proper transcriptional regulation of genes in carbon metabolism of *Saccharomyces cerevisiae*

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Abstract The F-box protein Grr1p is involved in cell cycle regulation, glucose repression and transcriptional induction of the amino acid permease (AAP) gene *AGP1*. We investigated the role of Grr1p in amino acid-mediated induction of AAP genes by performing batch cultivations with a wild-type strain and a *grr1*Δ strain and adding citrulline in the exponential phase. Whole-genome transcription analyses were performed on samples from each cultivation, both immediately before and 30 min after citrulline addition. Transcriptional induction of the AAP genes *AGP1*, *BAP2*, *BAP3*, *DIP5*, *GNP1* and *TAT1* is fully dependent on Grr1p. Comparison of the *grr1*Δ strain with the reference strain in the absence of citrulline revealed that *GRR1* disruption leads to increased transcription of numerous genes. These encode enzymes in the tricarboxylic acid cycle, the pentose-phosphate pathway and both glucose and starch metabolism. Promoter analysis showed that many of the genes with increased transcription display Mig1p- and/or Msn2p/Msn4p-binding sites. Increased expression of glucose-repressed genes in the *grr1*Δ strain may be explained by the reduced expression of the hexose transporter genes *HXT1*, *HXT2*, *HXT3* and *HXT4* and a subsequent lowering of the glucose uptake; and the effect of *GRR1* deletion on general carbon metabolism may therefore be indirect. Finally, none of the genes known to be primarily involved in cell cycle regulation displayed different expression levels in the *grr1*Δ cells as compared with the reference strain, suggesting that the role of Grr1p in cell cycle regulation does not include any transcriptional component.

Keywords Amino acid-sensing · Glucose-sensing · Glucose repression · Transcription analysis · *GRR1*

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N. Eckert-Boulet · B. Regenberg · J. Nielsen (✉)
Center for Microbial Biotechnology, BioCentrum–DTU,
Technical University of Denmark, Building 223,
BioCentrum–DTU, 2800 Lyngby, Denmark
E-mail: jn@biocentrum.dtu.dk
Tel.: +45-45252696
Fax: +45-45884148

N. Eckert-Boulet
Department of Physiology, Carlsberg Research Center,
Gamle Carlsberg vej 4–6, 2500 Valby, Denmark

Present address: B. Regenberg
Institut für Mikrobiologie, Johann Wolfgang
Goethe-Universität Frankfurt am Main,
Marie-Curie-Str. 9, 60439 Frankfurt am Main, Germany

Introduction

In its natural habitat, the yeast *Saccharomyces cerevisiae* is exposed to highly varying environments; and this yeast has therefore developed a complex regulatory network to be able to rapidly adapt to environmental changes (Horak 1997; Forsberg and Ljungdahl 2001b; Rolland et al. 2001). A well known example is the transcriptional regulation of the hexose transporter genes in response to extracellular glucose. In the absence of glucose, the transcriptional repressor Rgt1p binds to the promoters of the glucose transporter genes *HXT1*, *HXT2*, *HXT3* and *HXT4* (Özcan et al. 1996b; Flick et al. 2003; Mosley et al. 2003). Transcription of these genes is induced in the presence of glucose, which is detected by either of two hexose transporter-like sensors, Snf3p and Rgt2p, displaying high- and low-glucose affinities, respectively (Özcan et al. 1996a, 1998; Özcan and Johnston 1999 for a review). Two closely related proteins, Mth1p and Std1p (Hubbard et al. 1994) are known to mediate the signal from the membrane-bound receptors to the *HXT* genes (Schmidt et al. 1999; Schulte et al. 2000), by affecting the phosphorylation state of Rgt1p (Mosley et al. 2003; Flick et al. 2003). Two different models have been proposed to explain how these genes are reactivated in the presence of glucose: (1) phosphorylation of Rgt1p results in its inability to bind the *HXT* promoters and thereby its inactivation (Flick et al. 2003), (2) at high levels of glucose, phosphorylated

Rgt1p indirectly activates transcription of the *HXT1* gene (Özcan et al. 1996b; Mosley et al. 2003). Besides these components, the F-box protein Grr1p (Glucose Repression Resistant) is also essential for glucose-mediated induction of transcription of the *HXT* genes (Özcan et al. 1994). Grr1p is part of the E3 ubiquitin ligase SCF^{Grr1} (Skp1-Cullin-F-box) complex that targets proteins for degradation by ubiquitination (Skowyra et al. 1997, 1999). Inactivation of *GRR1* prevents phosphorylation of Rgt1p and its dissociation from the *HXT* promoters, although the mechanism has not been completely elucidated (Mosley et al. 2003; Flick et al. 2003). Flick and co-workers (2003) suggest that Mth1p and Std1p sterically prevent phosphorylation of Rgt1p in the absence of glucose and might be targeted for degradation by SCF^{Grr1}-mediated ubiquitination in response to glucose. SCF^{Grr1} is also needed for the function of another nutrient-sensing pathway, which results in induced transcription of the amino acid permease (AAP) gene *AGP1* upon the presence of amino acids in the environment (Iraqui et al. 1999). Little is known about the role of Grr1p in the amino acid-sensing pathway, although deletion of each of the genes encoding subunits of SCF^{Grr1} suggests that all of them are important for induction (Iraqui et al. 1999; Bernard and André 2001). The AAP genes *AGP1*, *BAP2*, *BAP3*, *GNP1*, *TAT1* and *TAT2* are subject to transcriptional induction by extracellular amino acids (Schmidt et al. 1994; Grauslund et al. 1995; Zhu et al. 1996; Didion et al. 1998; Schreve et al. 1998; Iraqui et al. 1999). The amino acids are first recognised at the plasma membrane by the permease homologue Ssy1p (Didion et al. 1998; Jørgensen et al. 1998; Iraqui et al. 1999; Klasson et al. 1999). Ssy1p forms the SPS complex together with the peripherally associated proteins Ssy5p and Ptr3p. These proteins (Barnes et al. 1998; Jørgensen et al. 1998) are essential for transmission of the signal and are degraded in response to amino acids (Forsberg and Ljungdahl 2001a). The resulting signal, the nature of which remains unclear, is followed by recruitment of the homologous transcription factors Stp1p and Stp2p (Jørgensen et al. 1997; de Boer et al. 1998, 2000), which appear to be responsible for the transcription of all the genes induced by this pathway (Eckert-Boulet et al. 2004). Stp1p and Stp2p are potential targets for Grr1p, as they undergo an endoproteolytic process in response to amino acids in the medium, resulting in their targeting to the nucleus (Andréasson and Ljungdahl 2002). However, there is no positive proof that this proteolytic process is dependent on Grr1p. SCF^{Grr1} is also known for its role in cell cycle control (Barral et al. 1995). The SCF^{Grr1} complex targets the G_1 cylins Cln1p and Cln2p for degradation by the 26S proteasome, thereby delaying entry into the S-phase of the cell cycle by preventing cyclin-mediated activation of the Cdc28p kinase. Grr1p was shown to be responsible for recognition of Cln1p and Cln2p by the ubiquitin-ligase complex via its leucine-rich repeats and carboxy terminus (Kishi and Yamao 1998; Hsiung et al. 2001). Cells lacking Grr1p initiate entry into the S-phase too early, resulting in elongated cellular shapes caused by the accumulation of cyclins (Barral et al. 1995).

The involvement of Grr1p in glucose-induced expression of hexose transporter genes and in the amino acid-sensing pathway, together with its role in cell cycle regulation, indicates that Grr1p is of primordial importance in cellular processes by coupling nutrient availability to cell growth. We performed the present study to get a better understanding of the role of SCF^{Grr1} in nutrient sensing and to investigate whether Grr1p has an impact on cell cycle regulation at the transcriptional level. This was done by whole genome transcription analysis of a *grr1*Δ strain and its parental strain in the absence and presence of the inducing amino acid L-citrulline.

Materials and methods

Strains and media

The wild-type strain CEN.PK-113-7D (*MAT*a) and the otherwise isogenic *grr1* deletion strain CEN.PK-513-3A (*MAT*a *grr1::loxP-KanMX-loxP*), in which the *GRR1* ORF was replaced with the loxP-KanMX-loxP cassette over nucleotides 4–2,883 (kindly provided by Dr. P. Kötter, Frankfurt, Germany) were used for this study. Cells cultured for expression analysis were grown in a defined, ammonium sulphate-based minimal medium modified from Verduyn et al. (1992). The medium contained (per litre): 7.5 g $(NH_4)_2SO_4$, 14.4 g KH_2PO_4, 0.5 g $MgSO_4$·dodo7H_2O, 10.0 g D-glucose, 0.05 ml antifoam 289 (A-5551, Sigma-Aldrich), 2.0 ml trace metal solution and 1.0 ml vitamin solution. The trace metal solution contained (per litre): 15.0 mg EDTA (Titriplex III), 4.5 mg $ZnSO_4$·7H_2O, 0.82 mg $MnCl_2$·2H_2O, 0.3 mg $CoCl_2$·6H_2O, 0.3 mg $CuSO_4$·5-H_2O, 0.4 mg Na_2MoO_4·2H_2O, 4.5 mg $CaCl_2$·2H_2O, 3.0 mg $FeSO_4$·7H_2O, 1.0 mg H_3BO_3 and 0.1 mg KI. The vitamin solution contained (per litre): 0.05 mg biotin, 0.2 mg *p*-benzoic acid, 1.0 mg nicotinic acid, 1.0 mg Ca-pantothenate, 1.0 mg pyridoxin HCl, 1.0 mg thiamin HCl and 25.0 mg myo-inositol.

Batch cultivations

Single colonies were transferred from yeast/peptone/dextrose plates to 500-ml shake-flasks containing 100 ml of defined minimal medium and incubated for 24 h at 30°C in an orbital shaker agitated at 150 rpm. Aerobic batch cultivations were then carried out in 5-l bioreactors manufactured in-house, mounted with four metal baffles and with a working volume of 4 l. Fermentors were inoculated at an initial optical density at 600 nm (OD_{600}) of 0.001–0.002. Aeration was set at 1 vvm of atmospheric air and agitation at 750 rpm. The temperature was kept constant at 30°C and pH was maintained at 5.00 ± 0.05 by addition of KOH. OD_{600} measurements, dry weight

and carbon dioxide measurements were determined throughout the fermentations, to ensure that conditions were identical for all fermentations. Dry weight and carbon dioxide measurements were determined as described by Bro et al. (2003), while the concentration of glucose in the growth medium was quantified by filtration through a 0.45-μm pore-size acetate filter (Cameo 25Gas 0.45; Osmonics, USA) and HPLC on a HPX-87H column (Aminex; Biorad) according to Zaldivar et al. (2002). L-Citrulline (©-7629, Sigma-Aldrich, St. Louis, Mo.,USA) was added to a final concentration of 2.0 mM when the glucose concentration was approximately 15 g/l.

Whole-genome transcription analyses

Samples for whole-genome transcription analysis were harvested and treated as described by Bro et al. (2003). Microarrays were scanned in an Gene array scanner (Agilent; Affymetrix) and the resulting images were processed with Microarray Suite ver. 4.0 software, using global scaling and normalisation of the four arrays to each other. Average difference values representing the absolute hybridisation intensities were then calculated for each probe set. To enable further data processing, all average difference values below 20 were set to 20.

We performed three comparisons of expression levels: (1) condition j = reference strain, 30 min after citrulline addition and condition k = reference strain, immediately before citrulline addition, (2) condition j = *grr1*Δ, 30 min after citrulline addition and condition k = *grr1*Δ, immediately before citrulline addition, (3) condition j = *grr1*Δ before the pulse and condition k = reference strain before the pulse. In any comparison, genes called absent or marginal by the Affymetrix software were discarded from the analysis. x-Fold change values were calculated as the average difference of condition j (probe set I) divided by the average difference of condition k (probe set I). Genes displaying x-fold changes with absolute values greater than 3.0 in any comparison were considered significantly affected and were further analysed.

Function assignment

Genes that had changed expression levels more than 3.0-fold in the *grr1*Δ strain compared with the parental strain were listed with assigned functional categories in January 2004 according to the Comprehensive yeast genome database (http://mips.gsf.de/genre/proj/yeast/index.jsp). Genes affected more than 3.0-fold were grouped in genes with increased and decreased transcription levels and each of the groups was used as input for the Gene Ontology (GO) term finder tool (http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermFinder) available on the *Saccharomyces* Genome Database webpage. We considered gene ontologies to be over-represented if the P-value was lower than 0.01 according to the GO term finder tool.

Promoter analysis

Promoter sequences of the genes of interest were retrieved on the Regulatory sequence analysis tools homepage (http://rsat.ulb.ac.be/rsat/) from −800 bp to −1 bp relative to the start codon (van Helden 2003). They were queried for the Mig1p consensus-binding site (G/C C/T GG G/A G) and for stress response elements (STREs; AGGGG). This was performed using the DNA pattern tool, searching both strands, preventing overlapping matches and forbidding substitutions.

Results

GRR1 is required for citrulline induction of *BAP2*, *BAP3*, *TAT1*, *DIP5*, *AGP1* and *GNP1*

To explore the effect of a *GRR1* deletion on amino acid-sensing in *S. cerevisiae*, the genome-wide transcriptional responses to 2.0 mM L-citrulline of a wild-type strain (CEN.PK-113-7D) and a *grr1*Δ strain (CEN.PK-513-3A) were analysed immediately before and 30 min after the pulse. L-Citrulline is known to induce the amino acid-sensing pathway (Iraqui et al. 1999; Eckert-Boulet et al. 2004) and is reported to be taken up exclusively by the general amino acid permease Gap1p (Grenson et al. 1970; Regenberg and Hansen 2000). Gap1p is under the control of nitrogen catabolite repression and is nitrogen catabolite inactivated; and as such it is only active when cells are grown on a poor nitrogen source (Grenson et al. 1970; Stanbrough and Magasanik 1995). In the present study, cells were grown on the repressing nitrogen source ammonium, meaning that L-citrulline was not imported; and it could therefore be assumed that the effects reported here were the effects of amino acid-sensing and not those of the catabolism of L-citrulline. Cultivations of the reference strain and the *grr1*Δ strain were performed in defined minimal medium, with ammonium as the sole nitrogen source. The cells were grown aerobically to mid-exponential phase before they were exposed to 2.0 mM L-citrulline. Samples for transcription profile analyses were harvested immediately before the addition of L-citrulline (when the glucose concentration was approximately 15 g/l) and 30 min thereafter (when the transcriptional response of amino acid-induced genes was maximal; de Boer et al. 1998; Forsberg and Ljungdahl 2001a; Nielsen et al. 2001; Eckert-Boulet et al. 2004). mRNA was purified from all four samples and processed into labelled cRNA, which was then hybridised onto oligonucleotide arrays. The resulting images were analysed to calculate expression levels from all probe sets. Arrays were compared pairwise and transcripts that were present under at least one of the conditions and had changed more than 3.0-fold were

considered significantly changed (Table 1). Sixty-one genes were significantly affected by the L-citrulline pulse in the reference strain, of which 45 were repressed and 16 induced (Table 1). The functional group with most genes induced by L-citrulline were AAP genes, among which six genes had altered expression. These genes were the

Table 1 Genes displaying transcription levels changed more than 3.0-fold in the reference strain after L-citrulline addition. Classifications were retrieved from the MIPS database (http://mips.gsf.de/genre/proj/yeast/index.jsp). Gene *YOR146W* is also affected by the citrulline pulse in a *grr1*Δ mutant. *N.S.* Genes displaying *x*-fold changes with absolute values lower than 3.0

Gene/ORF name	MIPS classification	*x*-Fold change in reference strain upon citrulline addition	*x*-Fold change in *grr1*Δ upon citrulline addition
AGP1	Amino acid transport	3.7	N.S.
AIR2	RNA transport	3.2	N.S.
BAP2	Amino acid transport	7.8	N.S.
BAP3	Amino acid transport	17.1	N.S.
DIP5	Amino acid transport	3.5	N.S.
DPM1	Carbohydrate metabolism	3.2	N.S.
FYV5	Unclassified	−3.1	N.S.
GNP1	Amino acid transport	3.1	N.S.
HEM3	Metabolism of vitamins, cofactors, and prosthetic groups	−3.8	N.S.
HXT1	Carbohydrate transport	−3.6	N.S.
LAG2	Cell wall	−3.2	N.S.
MDS3	Sporulation and germination	4.2	N.S.
MRP20	Ribosome genesis	3.1	N.S.
SPO21	Meiosis	3.1	N.S.
SPT15	rRNA synthesis	−3.0	N.S.
TAT1	Amino acid transport	5.6	N.S.
TEF4	Translation	3.3	N.S.
VPS69	Unclassified	−5.0	N.S.
YBL053W	Unclassified	−3.3	N.S.
YBL062W	Unclassified	−3.4	N.S.
YBL112C	Unclassified	−3.3	N.S.
YBR089W	Unclassified	−3.2	N.S.
YCR013C	Unclassified	−3.8	N.S.
YDL172C	Unclassified	−4.9	N.S.
YDL228C	Unclassified	−3.7	N.S.
YDR008C	Unclassified	−5.5	N.S.
YDR157W	Unclassified	−3.2	N.S.
YDR327W	Unclassified	−3.1	N.S.
YDR445C	Unclassified	−4.4	N.S.
YER064C	Unclassified	5.7	N.S.
YFL068W	Unclassified	−3.0	N.S.
YFR035C	Unclassified	−3.2	N.S.
YGL041C	Unclassified	−3.5	N.S.
YGR018C	Unclassified	−3.1	N.S.
YGR228W	Unclassified	−4.8	N.S.
YJL009W	Unclassified	−3.0	N.S.
YJL075C	Unclassified	−5.0	N.S.
YKL111C	Unclassified	−3.4	N.S.
YLR053C	Unclassified	−4.9	N.S.
YLR076C	Unclassified	−3.3	N.S.
YLR162W	Unclassified	−3.5	N.S.
YLR230W	Unclassified	−3.0	N.S.
YLR281C	Classification not yet clear-cut	3.0	N.S.
YNL150W	Unclassified	−3.5	N.S.
YNL235C	Unclassified	−3.0	N.S.
YNL276C	Unclassified	−4.4	N.S.
YOL035C	Unclassified	−4.0	N.S.
YOR135C	Unclassified	−3.2	N.S.
YOR146W	Unclassified	−3.9	−3.5
YOR200W	Unclassified	−3.5	N.S.
YOR331C	Unclassified	−4.2	N.S.
YPL014W	Unclassified	6.0	N.S.
YPL142C	Unclassified	−3.1	N.S.
YPR013C	Transcription	4.1	N.S.
YPR038W	Unclassified	−3.6	N.S.
YPR044C	Unclassified	−3.6	N.S.
YPR050C	Unclassified	−3.0	N.S.
YPR059C	Unclassified	−3.7	N.S.
YPR130C	Unclassified	−3.6	N.S.
YPR142C	Unclassified	−3.1	N.S.

branched-chain amino acid permeases *BAP2* and *BAP3*, the tyrosine transporter *TAT1*, the broad-spectrum amino acid permease *AGP1*, the dicarboxylic amino acid permease *DIP5* and the high-affinity glutamine permease *GNP1*. The branched-chain AAP genes *BAP2* and *BAP3* displayed the greatest *x*-fold changes of all the genes affected by the citrulline pulse, while *AGP1*, *TAT1*, *DIP5* and *GNP1* were induced more modestly. None of the six AAP genes induced by L-citrulline in the reference strain displayed a significant *x*-fold change in the *grr1*Δ strain upon citrulline addition. As a matter of fact, no hybridisation could be detected using the probe sets representing *DIP5* and *BAP3*, reflecting very low transcription levels. Our data therefore indicate that L-citrulline-induced transcription of the AAP genes *AGP1*, *BAP2*, *BAP3*, *DIP5*, *GNP1* and *TAT1* is completely dependent on the presence of *GRR1*.

Expression of the glucose transporter gene *HXT1* was slightly down-regulated 30 min after the addition of citrulline. This can to some extent be accounted for by the decreasing glucose concentration during this time. *HXT1* is a low-affinity glucose transporter (Reifenberger et al. 1997) and its expression is sensitive to changes in the glucose concentrations in the range used in the current study (Özcan and Johnston 1995). Besides the genes involved in amino acid and glucose transport, there was little functional relationship between genes affected by addition of the amino acid in the reference strain.

GRR1 disruption affects transcription levels of genes involved in nitrogen metabolism

The whole-genome transcription profiles of a wild-type strain and a *grr1*Δ strain were also compared in the absence of citrulline in order to investigate the global effects of the *GRR1* deletion. This analysis revealed that 434 genes had changed expression more than 3.0-fold in the *grr1*Δ strain. Using the GO term finder tool (http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermFinder), these genes were grouped according to the process in which the gene product is involved. Over-represented ontologies and the corresponding gene names are listed in Table 2. Comparison of the transcription profiles from the *grr1*Δ strain and the parental strain before citrulline addition showed that the AAP genes *BAP3*, *DIP5*, *GNP1* and *TAT2* displayed reduced transcription in *grr1*Δ cells (Table 2). Hence, *GRR1* appears not only to be required for the citrulline induction of AAP genes: it is also found to have an effect on the basal level of transcription of AAP genes. One of the many phenotypes of a *grr1*Δ mutant is increased sensitivity to nitrogen starvation (Flick and Johnston 1991). This effect may be due to up-regulation of the $NADP^+$-dependent glutamate dehydrogenase gene (*GDH3*) and the glutamate decarboxylase gene (*GAD1*) and a reduced expression of the NAD^+-dependent glutamate synthase gene (*GLT1*), which together possibly result in altered levels of glutamate in the *grr1*Δ strain (Table 2).

GRR1 disruption affects transcriptional regulation of numerous genes involved in the central carbon metabolism

Another phenotype of *grr1*Δ mutations is reduced glucose uptake (Özcan et al. 1993; Gamo et al. 1994) as a consequence of reduced induction of the glucose transporter-encoding genes *HXT1*, *HXT2*, *HXT3* and *HXT4* (Özcan et al. 1994; Özcan and Johnston 1995). Indeed, we found that transcription of these four well characterised hexose transporter genes was reduced in the *grr1*Δ strain as compared with the reference strain (Table 2). Expression of the hexose transporter genes *HXT5*, *HXT6*, *HXT16* and *HXT17* was increased in *grr1*Δ cells as compared with wild-type cells. The *HXT5* and *HXT6* genes encode medium- and high-affinity transporters known to be transcribed at low levels when glucose levels are high (Liang and Gaber 1996; Buziol et al. 2002). Our data therefore suggested that glucose repression was affected in the *grr1*Δ strain. This hypothesis was substantiated by the observation that transcription of many of the genes known to be under glucose repression was increased in the *grr1*Δ mutant (Table 2). This included genes involved in maltose transport and metabolism (*MAL11*, *MAL32*, *MAL31*) and genes encoding glycolytic/gluconeogenic enzymes (*HXK1*, *GLK1*, *FBP1*, *PGM2*), suggesting that gluconeogenesis was active in the *grr1*Δ strain and that carbon could be led in the direction of starch and sucrose metabolism as well as towards the pentose phosphate pathway. The fact that the invertase gene *SUC2* was also more transcribed in *grr1*Δ cells, like the genes involved in glycogen and trehalose metabolism, supports this hypothesis. Two major glycogen-synthetic enzymes, the starch phosphorylase *GPH1* and the branching enzyme *GLC3*, displayed higher expression levels in *grr1*Δ cells, which may lead to glycogen accumulation in *grr1*Δ cells. However, the effect of *GRR1* disruption on trehalose metabolism was not so clear. The neutral trehalase gene *NTH1* and the *TPS2* gene, coding for a subunit of the trehalose-6-phosphate phosphatase, both showed increased transcription, while the rest of the genes involved in trehalose synthesis were not affected in the *grr1*Δ strain. Expression of nine tricarboxylic acid cycle genes (*IDP2*, *KGD1*, *KGD2*, *LSC2*, *MDH1*, *PCK1*, *SDH1*, *SDH2*, *SDH4*) was higher in the *grr1*Δ strain. Of these, four (*SDH1*, *SDH2*, *SDH3*, *SDH4*) are also involved in oxidative phosphorylation; and the expression of genes in the pentose-phosphate pathway (*TKL1*, *TKL2*, *SOL1*, *SOL4*) was also higher in the *grr1*Δ strain, suggesting an increased capacity in each of these pathways. Finally, several ATP synthase subunit-encoding genes (*ATP5*, *ATP7*, *ATP16*, *ATP20*) displayed higher transcription in the *grr1*Δ strain, reflecting a partial derepressing of respiration in this strain.

Table 2 Over-represented gene ontologies among the genes displaying transcription levels affected more than 3.0-fold by *GRR1* disruption. The genes with higher and lower transcription in *grr1*Δ cells were queried for over-represented ($P < 0.01$) gene ontologies using the GO term finder tool (http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermFinder). The hierarchy is illustrated: the main groups are indicated in italics, while subgroups are indented

GO process	Up-regulated genes ($P < 0.01$)	Down-regulated genes ($P < 0.01$)
Cell organisation biogenesis		
Cytoplasm organisation and biogenesis		
Ribosome biogenesis and assembly		*RPS0B*
Ribosome biogenesis		
rRNA processing		*NSR1 FAL1 RPF2 KRR1*
Processing of 20S pre-rRNA		*IMP3 NOC4/UTP19 NOP7 UTP8 ENP1*
Ribosome–nucleus export		*LSG1 RIX1*
Ribosome large subunit export		*ECM1 NOG2*
Metabolism		
Alcohol metabolism	*GRE3 GAL4 PCK1 XKS1 GPM2 TKL2 MCR1 ALD4 FBP1 CAT8 GCY1 GUT1 PGM2 INO1 HXK1*	
Aldehyde metabolism	*AAD16 ICL1 AAD4 MLS1*	
Amino acid metabolism		
Aspartate family amino acid metabolism		*SAM1*
Aspartate family amino acid synthesis		*AAT1*
Lysine biosynthesis		
Lysine biosynthesis, aminoadipic pathway		*LYS4 LYS20 LYS1*
Amino acid and derivative metabolism		*DYS1*
Amino acid metabolism		*MAE1 GLT1 GCV1 LYS4 AAT1 FMT1 LYS20 SAM1 LYS1*
Glutamine family amino acid metabolism		
Glutamate metabolism	*CIT1 GDH3 IDP2 GAD1*	
Carbohydrate metabolism	*TPS1 GRE3 TPS2 GAL4 ADR1 XKS1 HAP4 AMS1 GLK1 GCY1 PGM2 GUT2 INO1 HXK1*	
Carbohydrate biosynthesis	*PIG1 GLG2 PCK1 GPM2 GLG1 FBP1 CAT8 TSL1*	
Carbohydrate catabolism	*NTH1 SUC4 XKS1 SUC2 GPM2 TKL2 GDB1 GPH1 MAL32*	
Main pathways of carbohydrate metabolism		
Tricarboxylic acid intermediate metabolism	*CIT1 FUM1 LSC2 MDH1 IDP2*	
2-oxoglutarate metabolism	*KGD1 KGD2*	
Tricarboxylic acid pathway	*CIT1 SDH3 FUM1 KGD1 KGD2 PCK1 GPM2 ICL1 LSC2 MDH1 TKL2 SDH2 SDH4 FBP1 SDH1 CAT8 IDP2 MLS1*	
Disaccharide metabolism	*NTH1 SUC4 SUC2 TSL1 MAL32*	
Monosaccharide metabolism		
Hexose metabolism	*GAL4 PCK1 GPM2 TKL2 FBP1 CAT8 PGM2 INO1 HXK1*	
Pentose metabolism	*GRE3 XKS1 GCY1*	
Polysaccharide metabolism		
Glucan metabolism	*GSY2 GDB1 GAC1 GSY1 GLC3 GPH1*	
Glycogen metabolism	*PIG1 GLG2 GLG1*	
Catabolism		
Regulation of catabolism		
Regulation of proteolysis and peptidolysis	*TFS1 PBI2*	
Coenzyme and prosthetic group metabolism		
Coenzyme metabolism	*LSC2 TKL2 PNC1 ACH1 GUT2*	
Group transfer coenzyme metabolism	*ATP20 ATP16 ATP5 ATP7 INH1 STF2*	
Electron transport	*MCR1 CYC7 CYB2*	
Protein-disulfide reduction	*SDH3 SDH2 SDH4 NDI1 SDH1*	
Energy pathways		
Energy derivation by oxidation of organic compounds	*MBA1 COX20 ATF1 ADR1 HAP4 AAC1 MBR1 ISF1*	
Energy reserve metabolism	*NTH1 TSL1*	
Glycogen metabolism	*PIG1 GLG2 GLG1 GSY2 GDB1 GAC1 GSY1 GLC3 GPH1*	
Tricarboxylic acid pathway	*CIT1 SDH3 FUM1 KGD1 KGD2 PCK1 GPM2 ICL1 LSC2 MDH1 TKL2 SDH2 SDH4 FBP1 SDH1 CAT8 IDP2 MLS1*	

Table 2 (Contd.)

GO process	Up-regulated genes ($P < 0.01$)	Down-regulated genes ($P < 0.01$)
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism		
Nucleotide metabolism		
Nucleoside triphosphate metabolism		
Nucleoside triphosphate biosynthesis		
Purine nucleoside triphosphate biosynthesis		
Purine ribonucleoside triphosphate biosynthesis	*ATP20 ATP16 ATP5 ATP7 INH1 STF2*	
Nucleotide biosynthesis		
Purine nucleotide biosynthesis	*YNK1*	
Purine ribonucleotide biosynthesis	*ATP20 ATP16 ATP5 ATP7 INH1 STF2*	
RNA metabolism		*DBP2 TRL1 LHP1 FMT1*
rRNA metabolism		*NSR1 IMP3 NOC4/UTP19 SNR128 FAL1 NOP7 UTP8 RPF2 SNR76 KRR1 ENP1*
snoRNA metabolism		*SHQ1 NAF1*
Nucleoside phosphate metabolism		
ATP metabolism		
ATP biosynthesis	*ATP20 ATP16 ATP5 ATP7 INH1 STF2*	
Oxidative phosphorylation	*ATP20 ATP16 SDH3 ATP5 ATP7 INH1 SDH2 SDH4 NDI1 SDH1 STF2*	
ATP synthesis coupled electron transport	*NDI1*	
Mitochondrial electron transport, succinate to ubiquinone	*SDH3 SDH2 SDH4 SDH1*	
Oxygen and reactive oxygen species metabolism	*TTR1 MCR1 SOD2 GPX1 GAD1 HSP12*	
One carbon compound metabolism		*GCV2 GCV1 SHM2*
Phosphorus metabolism		
Phosphate metabolism	*GIP2 PHO5*	
Phosphorylation	*YAK1 ATP20 ATP16 SDH3 ATP5 ATP7 INH1 SDH2 SDH4 NDI1 SDH1 STF2 MRK1*	
Protein metabolism		
Protein folding	*SSA1 HSP78 SSE2 HSP104 SSA4 HSP26 HSP30*	
Response to stress	*DDR48 TPS1 GRE3 MRE11 MYO3 HAL1 NTH1 TTR1 TPS2 HSP78 HSP104 UBI4 MCR1 SSA4 XBP1 YJL144W PHO5 GPX1 GAD1 GAC1 HSP42 TSL1 STF2 GCY1 HSP12 YGP1 HSP26 SIP18 HSP30 MRK1*	
Response to stimulus	*DDR48 TPS1 GRE3 MRE11 MYO3 HAL1 NTH1 TTR1 TPS2 MF(ALPHA) 1 AFR1 HSP78 HSP104 SNF3 UBI4 MCR1 SSA4 XBP1 CRS5 PHO5 GPX1 GAD1 GAC1 HSP42 TSL1 GCY1 YGP1 HSP26 HSP30 MRK1*	
Response to external stimulus		
Response to abiotic stimulus		
Response to water		
Response to water deprivation		
Response to desiccation	*YJL144W STF2 HSP12 SIP18*	
Transport		
Carbohydrate transport		
Monosaccharide transport		
Hexose transport	*HXT7 HXT5 HXT6 MAL31 HXT17 MAL11 HXT16*	*HXT1 HXT4 HXT3 HXT2*
Hydrogen transport		
Proton transport		
Energy coupled proton transport, down electrochemical gradient		
ATP synthesis coupled proton transport	*ATP20 ATP16 ATP5 ATP7 INH1 STF2*	

Table 2 (Contd.)

GO process	Up-regulated genes ($P < 0.01$)	Down-regulated genes ($P < 0.01$)
Intracellular transport		
Nucleocytoplasmic transport		*AIR1 LSG1 RIX1 ECM1 THP2 SRP40 NOG2*
Intracellular protein transport		
Protein targeting		
Protein-nucleus export		*LSG1 RIX1 ECM1 NOG2*
Ion transport	*SUL1 SFC1 JEN1*	
Cation transport	*COX17 ATP20 ATP16 ATP5 ATP7 ALR2 INH1 CTR3 STF2*	
Monovalent inorganic cation transport	*ATP20 ATP16 ATP5 ATP7 INH1 STF2*	
Organic acid transport		*PDR12*
Carboxylic acid transport		*AQR1*
Amino acid transport		*GNP1 DIP5 BAP3 TAT2*

Hence, the general picture of the *grr1*Δ transcription analysis was an increased expression of many genes involved in carbon metabolism. Many of these genes are also known to be under glucose repression. We therefore speculated that loss of *GRR1* relieves genes under glucose repression. Several pathways are known to impose glucose repression in yeast, such as the main glucose repression pathway, which involves the transcriptional repressor Mig1p (for a review, see Rolland et al. 2001). To further investigate whether this pathway is affected in the *grr1*Δ mutant, we queried promoter sequences of up-regulated genes (Table 2) in the *grr1*Δ strain for Mig1p-binding sites (G/C C/T GG G/A G). Of the genes that were significantly up-regulated in the *grr1*Δ strain, 40% contained at least one Mig1p consensus-binding site, with an average of 2.5 sites per promoter, compared with 42% of all genes in the genome, with an average of 1.6 binding site per promoter.

Growth on glucose also coincides with loss of stress resistance. This mechanism is mediated via the protein kinase A (PKA), which can be activated by cAMP and by the Sch9p-dependent fermentable growth medium (FGM) pathway (Thevelein et al. 2000; Rolland et al. 2001). Inactivation of PKA results in migration of the transcription factors Msn2p and Msn4p to the nucleus (Görner et al. 1998, 2002), where they bind to STRE motifs and induce transcription (Ruis and Schuller 1995). Promoters of the genes displaying higher expression in the *grr1*Δ strain were also queried for STRE elements (AGGGG); and 23% of these promoters displayed at least one STRE element, with an average of 4.3 STRE elements per promoter, against 32% of all genes in the genome with an average of 1.4 STRE elements per promoter. As discussed below, this observation suggested that the effect of *GRR1* deletion on the transcription of tricarboxylic acid cycle genes, genes involved in gluconeogenesis, glycogen synthesis, electron transport, *MAL* genes and the invertase gene *SUC2* was a result of Msn2p/Msn4p activation and Mig1p inactivation.

Discussion

We studied the whole-genome transcriptional response of a wild-type strain to citrulline addition under conditions where citrulline uptake is prevented. The transcriptional response to citrulline in the wild type shows induced transcription of the AAP genes *AGP1*, *BAP2*, *BAP3*, *TAT1*, *GNP1* and *DIP5*. All but *DIP5* were previously reported to be amino acid-inducible via the membrane-bound Ssy1p sensor (Didion et al. 1996, 1998; de Boer et al. 1998; Iraqui et al. 1999) and the transcription factors Stp1p and Stp2p (Eckert-Boulet et al. 2004). This discrepancy could be due to differences in promoter sequence, depending on the genetic background, since other strain types (such as S288C) were used in earlier studies while CEN.PK was chosen for the current analysis. These two strain types are indeed reported to display significant differences in the sequence of certain genes (Daran-Lapujade et al. 2003) and this may also explain why other genes that were previously reported to be induced by amino acids such as *CAR1* and *PTR2* were not found to be induced in the current analysis. We also found that the transcription levels of another 56 genes are affected more than 3.0-fold in response to citrulline (Table 1) in a manner that is almost completely dependent on the presence of *GRR1*. This observation shows that Grr1p is involved in amino acid-induced transcription of AAP genes, as already reported for *AGP1* (Iraqui et al. 1999). Besides its role in amino acid-sensing, Grr1p is involved as part of the SCF^{Grr1} complex in the ubiquitin-dependent degradation of the G_1 cyclin Cln2p. However, mutations in *GRR1* that inactivate binding of Grr1p to Cln2p do not have any effect on *AGP1* or *HXT1* induction, suggesting that different residues recognise the cyclins and the targets of the nutrient-sensing pathways (Hsiung et al. 2001). The target of Grr1p in the amino acid-sensing pathway is unknown, although the dependency on ubiquitin for amino acid induction of *AGP1* (Bernard and André 2001) suggests that one of the components in the amino

acid-sensing pathway may be degraded as part of the activation. Likely candidates for such a degradation could be the transcription factors Stp1p/Stp2p that are known to be activated via an endoproteolytic process (Andréasson and Ljungdahl 2002) or Ssy5p, which is degraded in response to extracellular amino acids (Forsberg and Ljungdahl 2001a). Besides the requirement for *GRR1* for amino acid-induced transcription of the AAP genes, our data show that *BAP3*, *DIP5*, *GNP1* and *TAT2* display a reduced basal transcription in *grr1*Δ cells. Previous work reported that transcription levels of several AAP genes were decreased in *stp1 stp2* and /or in *ssy1* cells (Eckert-Boulet et al. 2004). Together with our present data, this suggests that the factors involved in the amino acid-sensing pathway are required, at least to some extent, for basal expression of the AAP genes, perhaps for a basal activity of the pathway.

In the current work, we observe that transcription of the hexose transporter genes *HXT1–HXT4* is decreased in the *grr1*Δ mutant, which is in agreement with previous results showing the involvement of Grr1p in glucose induction of *HXT* genes (Özcan and Johnston 1995; Westergaard, personal communication). Down-regulation of *HXT1–HXT4* does not abolish glucose uptake in *grr1*Δ cells but reduces it substantially (Wieczorke et al. 1999), thereby also reducing intracellular amounts of glucose, which is likely to result in the relief of glucose repression. For the main glucose repression pathway, this would mean that the Snf1p kinase is activated, resulting in the accumulation of the transcriptional repressor Mig1p in the cytoplasm and derepression of the genes under its control. The fact that a large fraction of the genes that have higher transcription levels in *grr1*Δ cells is also found to display Mig1p consensus-binding sites in their promoters is in agreement with this model. Moreover, 23% of the genes with increased transcription in *grr1*Δ cells display one or more Msn2p/Msn4p-binding sites (STREs) in their promoters. This observation suggests that another glucose response pathway, the cAMP-dependent PKA pathway, is inactivated in the *grr1*Δ strain. The PKA pathway appears to regulate processes such as glycogen accumulation and stress response by negatively regulating STRE-dependent gene expression (Marchler et al. 1993; Smith et al. 1998) by sequestering the transcription activators Msn2p and Msn4p in the cytoplasm (Görner et al. 1998; Beck and Hall 1999; Görner et al. 2002). PKA is activated by intracellular and extracellular glucose via cAMP (for a review, see Thevelein and de Winde 1999), but can also be activated by extracellular glucose via the FGM-induced pathway (Thevelein et al. 2000). In *grr1*Δ cells, where intracellular glucose is likely to be present in reduced amounts, PKA can be expected to be less active than in wild-type cells, thereby allowing increased migration of Msn2p and Msn4p to the nucleus. Hence, the observed up-regulation of genes involved in energy metabolism, glycogen synthesis and the tricarboxylic acid cycle could be a consequence of reduced Mig1p and PKA activity. This implies that the transcriptional induction of genes in carbon and energy metabolism in the *grr1*Δ mutant may be an indirect effect of reduced glucose uptake in a mutant that has lost its ability to induce hexose transporter genes via the Snf3p/Rgt2p-dependent pathway. Although this hypothesis is currently the most plausible explanation for the increased transcription of many genes in the *grr1*Δ strain, it is also possible that SCF^{Grr1} also has a direct role in glucose repression.

Besides its role in nutrient sensing, SCF^{Grr1} is also involved in cell cycle control (Barral et al. 1995). Binding of Grr1p to the G_1 cyclins Cln1p and Cln2p via its leucine-rich repeats was shown to be the first step in the ubiquitin-mediated degradation of these unstable proteins (Skowyra et al. 1997). Subsequent entry into the S-phase is delayed, as Cln1p/Cln2p-mediated activation of the cyclin kinase Cdc28 is required for the cell to progress through the cell cycle. *grr1*Δ mutants display a morphological defect shown to be the consequence of cyclin accumulation and anticipated entry into the S-phase (Barral et al. 1995). In our study, only a single cell cycle gene is affected by *GRR1* disruption, namely *SCM4*, which is a suppressor of temperature-sensitive *cdc4* alleles. Hence, Grr1p does not appear to have a transcriptional impact on cell cycle regulation. Consistent with the proposed model of cyclins being regulated at the protein level (for a review, see Clarke 2002), we did not observe any significant up- or down-regulation of the transcriptional levels of any cyclin genes.

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Appendix 2. Supplementary data

Gene name	MIPS functional category	Fold change in *grr1* vs wild type	Number of STRE elements in promoter	Number of Mig1 binding sites in promoter
ACH1	Lipid, fatty-acid and isoprenoid metabolism	11,6	4	1
ADE5,7	Nucleotide metabolism	-3,2	1	2
ADR1	Transcriptional control	4,2	0	0
AFR1	Cell differentiation	4	1	1
AIR1	Cell growth/morphogenesis	-10,9	0	0
ALD4	Respiration	6,4	2	1
ALR2	Cellular import	4,1	0	0
AMS1	C-compound and carbohydrate metabolism	5,9	0	1
AQR1	Detoxification	-3,3	1	1
ATF1	C-compound and carbohydrate metabolism	3,8	1	0
ATO3	Nitrogen and sulfur metabolism	-5,4	4	2
ATP16	Respiration	3,2	1	0
ATP20	Respiration	3,1	0	1
ATP5	Respiration	3,4	0	0
ATP7	Respiration	3,6	0	1
ATR1	Detoxification	-8,8	0	0
BAP3	amino acid transport	-9,5	1	2
BLM3	Stress response	-3,3	0	1
BOP3	Unclassified	-3,7	n.a.	n.a.
BTN2	Regulation of/Interaction with extracellular environment	9,5	0	1
CAT2	Lipid, fatty-acid and isoprenoid metabolism	5,1	0	2
CAT8	Glycolysis and gluconeogenesis	8	1	2
CBP4	Respiration	3,6	0	2
CIT1	C-compound and carbohydrate metabolism	3,1	2	0
COX17	Respiration	3,0	0	0
COX20	Energy	3,3	0	1
CRC1	Amino acid transport	12,7	1	3
CRS5	Detoxification	7,1	2	1
CSR2	Unclassified	9	0	5
CST9	Cell cycle and DNA processing	3,1	0	0
CTR3	Regulation of/Interaction with extracellular environment	10,2	0	0
CYB2	Respiration	13,6	2	2
CYC7	Respiration	6,9	3	1
DAN4	n.a.	4,2	0	2
DBP2	Transcription	-3,9	0	0
DDR48	Stress response	3,0	2	0
DIP5	amino acid transport	-14,8	1	0
DYS1	Amino acid biosynthesis	-3,0	0	0
ECM1	Cell wall	-3,2	1	0
ECM13	Control of Cellular Organisation	4,2	0	1
EDS1	Transcription	22,4	n.a.	n.a.
EMI2	C-compound and carbohydrate metabolism	10,4	n.a.	n.a.
EMI5	Unclassified proteins	3,0	n.a.	n.a.
ENP1	Protein modification	-3,0	1	0
ERG1	lipid, fatty-acid and isoprenoid metabolism	-4,1	0	0
FAL1	Transcription	-3,2	0	0
FBP1	Glycolysis and gluconeogenesis	7,6	2	4

FCY2	Nucleotide transport	-3,1	0	2
FIS1	Control of cellular organisation	5,7	1	0
FIT2	Unclassified	17,5	1	2
FIT3	Classification not yet clear-cut	13,1	0	1
FMT1	Transcription	-3,1	1	0
FOX2	Lipid, fatty-acid and isoprenoid metabolism	3,0	0	0
FRE3	Ionic homeostasis	3,4	0	1
FUI1	Nucleotide metabolism	-3,3	1	2
FUM1	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	3,4	2	0
FUN19	Unclassified	7,5	0	2
FUN34	C-compound and carbohydrate metabolism	12,8	1	2
FYV13	Unclassified	-5,9	n.a.	n.a.
FYV4	Unclassified proteins	3,4	0	0
GAC1	Metabolism of energy reserves	12,3	1	2
GAD1	Amino acid metabolism	10,9	1	0
GAL4	Transcriptional control	3,9	0	2
GCV1	Amino acid metabolism	-3,7	0	0
GCV2	Amino acid metabolism	-4	0	0
GCY1	C-compound and carbohydrate metabolism	15,2	1	2
GDB1	Metabolism of energy reserves	6,5	1	3
GDH2	Amino acid degradation	3,1	0	1
GDH3	Amino acid metabolism	5,4	0	1
GIP2	C-compound and carbohydrate metabolism	3,0	3	3
GLC3	Metabolism of energy reserves	29,1	3	2
GLG1	Metabolism of energy reserves	5,6	0	2
GLG2	C-compound and carbohydrate metabolism	3,4	2	0
GLK1	Glycolysis and gluconeogenesis	10,5	3	0
GLT1	Amino acid metabolism	-6,5	1	1
GNP1	amino acid transport	-19,6	0	2
GPG1	Intracellular signalling	36,8	0	0
GPH1	Metabolism of energy reserves	80,6	3	3
GPM2	Glycolysis and gluconeogenesis	4,5	1	1
GPX1	Detoxification	10	2	3
GPX2	Detoxification	-3,6	1	1
GRE3	C-compound and carbohydrate metabolism	3,1	1	0
GRR1	C-compound and carbohydrate metabolism	-5,8	0	0
GSP2	Nuclear transport	4,3	2	3
GSY1	Metabolism of energy reserves	12,6	2	3
GSY2	Metabolism of energy reserves	6,4	2	3
GUT1	C-compound and carbohydrate metabolism	25,1	1	2
GUT2	C-compound and carbohydrate metabolism	37,7	1	1
HAL1	Stress response	3,4	1	1
HAP4	Transcriptional control	4,4	1	5
HGH1	Unclassified proteins	-3,3	0	2
HLR1	Unclassified proteins	-3,1	1	0
HMS2	Transcription	-5,5	0	1
HPA2	Cell cycle and DNA processing	10,4	1	1
HSP104	Stress response	4,4	3	0
HSP12	Stress response	26,3	2	0
HSP26	Stress response	34,7	4	1
HSP30	Stress response	73,5	0	0
HSP42	Stress response	12,3	3	3
HSP78	Stress response	4,2	3	0
HXK1	C-compound and carbohydrate metabolism	86,1	5	5
HXT1	C-compound, carbohydrate transport	-97	1	3

HXT16	C-compound, carbohydrate transport	215,6	1	3
HXT17	C-compound, carbohydrate transport	46	1	4
HXT2	C-compound, carbohydrate transport	-5,7	0	8
HXT3	C-compound, carbohydrate transport	-26,3	1	7
HXT4	C-compound, carbohydrate transport	-62,7	0	2
HXT5	C-compound, carbohydrate transport	3,6	2	2
HXT6	C-compound, carbohydrate transport	6,4	2	3
HXT7	C-compound and carbohydrate transport	3,3	4	3
ICL1	Glycolysis and gluconeogenesis	4,6	0	1
IDP2	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	10,8	0	4
IKS1	Unclassified proteins	3,3	n.a.	n.a.
IMD4	Nucleotide metabolism	-5,2	0	0
IMP2	DNA repair	3,2	0	0
IMP3	Transcription	-4,5	0	0
INH1	Energy generation	4,4	2	0
INO1	C-compound and carbohydrate metabolism	56,8	0	0
ISF1	Nucleotide metabolism	103,1	1	1
JEN1	C-compound, carbohydrate transport	83,2	1	4
KGD1	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	3,7	2	1
KGD2	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	4,1	1	1
KIC1	Mitotic cell cycle and cell cycle control	-3,2	0	1
KRE30	Transport facilitation	-3,2	n.a.	n.a.
KRE33	Intracellular signalling	-3,8	0	1
KRR1	n.a.	-3,0	0	0
LHP1	Transcription	-3,1	1	0
LSC2	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	4,6	0	2
LSG1	Unclassified	-4,1	0	1
LYS1	Amino acid biosynthesis	-3,0	1	0
LYS20	Amino acid biosynthesis	-3,1	0	1
LYS4	Amino acid biosynthesis	-3,3	2	1
MAE1	C-compound, carbohydrate transport	-6,9	1	0
MAL11	C-compound, carbohydrate transport	133	0	0
MAL31	C-compound, carbohydrate transport	14,7	1	4
MAL32	Metabolism of energy reserves	213,6	1	4
MBA1	Respiration	3,0	1	0
MBR1	Transcriptional control	6,3	1	2
MCR1	Respiration	5,7	2	1
MDH1	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	4,7	1	2
MED1	Transcription	-3,1	0	0
MF(ALPHA)1	Pheromone response, mating-type determination, sex-specific proteins	3,8	0	0
MGA1	Stress response	3,0	4	0
MIG2	Transcriptional control	-7,4	2	6
MKC7	Protein fate (folding, modification, degradation)	-3,3	0	0
MLS1	C-compound and carbohydrate metabolism	17,5	1	1
MPM1	Unclassified proteins	6	1	0
MRE11	DNA recombination and DNA repair	3,2	0	0
MRF1	Translation	9,9	0	0
MRK1	Classification not yet clear-cut	10,45	2	5
MRP20	Ribosome biogenesis	3,1	0	1
MSC1	Meiosis	8,2	2	1

MSS2	Transcription	3,2	1	0
MSY1	Aminoacyl-tRNA synthesis	3,2	0	1
MUC1	C-compound and carbohydrate metabolism	5,3	0	0
MYO3	Cell fate	3,3	0	0
NAF1	Unclassified proteins	-3,2	0	2
NDI1	Respiration	6,2	1	1
NOG2	Unclassified proteins	-3,0	1	0
NOP7	Ribosome biogenesis	-3,1	0	0
NRP1	Unclassified proteins	-3,0	0	0
NSR1	Transcription	-4,9	0	2
NTH1	Metabolism of energy reserves	3,6	3	0
ODC1	C-compound, carbohydrate transport	18,4	0	1
OM45	n.a.	17,8	3	0
OYE3	Other energy generation activities	60,3	0	2
PAB1	Transcription	-3,3	0	1
PAI3	Stress response	6,7	3	1
PAU1	Stress response	3,7	3	1
PBI2	Protein fate (folding, modification, degradation)	9,3	1	0
PBP2	Transcription	-3,1	0	0
PCK1	Glycolysis and gluconeogenesis	4,3	0	4
PDR12	Transport facilitation	-4,9	0	0
PDR15	Transport facilitation	3,1	5	4
PGM2	Metabolism of energy reserves	28,7	6	2
PHO5	Phosphate utilisation	7,6	0	2
PIG1	C-compound and carbohydrate metabolism	3,2	0	2
PIR3	Stress response	3,8	1	2
PNC1	Unclassified	4,9	4	1
POR2	Mitochondrial transport	-4,5	2	0
PRM10	Unclassified proteins	-3,4	2	3
PRM4	Unclassified	4,8	0	1
PRX1	Detoxification	7,8	1	5
PRY1	Cell differentiation	4,1	1	1
PRY3	Cell differentiation	-3,7	1	1
PUF6	Unclassified proteins	-3,4	0	0
PUT4	Amino acid transport	11,8	3	2
PXA2	Lipid and fatty-acid transport	4,6	0	1
RAS1	Nucleotide metabolism	-3,3	1	2
REG2	C-compound and carbohydrate metabolism	14,6	1	2
RGS2	Intracellular signalling	3,6	0	2
RHR2	C-compound and carbohydrate metabolism	-7,4	0	0
RIM4	Cell cycle and DNA processing	5,9	1	4
RIX1	Unclassified proteins	-3,2	0	0
ROM1	C-compound and carbohydrate metabolism	7,5	1	1
RPF2	Unclassified proteins	-3,1	0	0
RPL7B	Ribosome biogenesis	-4	0	0
RPM2	Transcription	3,3	0	0
RPP1B	Ribosome biogenesis	6,4	1	0
RPS0B	Ribosome biogenesis	-3,0	0	1
RPS7B	Ribosome biogenesis	-3,3	1	1
RSM23	Ribosome biogenesis	3,1	0	1
RTN2	Unclassified proteins	7,3	1	2
SAM1	Amino acid metabolism	-3,0	0	0
SCM4	Cell cycle and DNA processing	-4,3	0	0
SDH1	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	7,8	1	2

SDH2	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	5,2	1	3
SDH3	Respiration	3,3	0	1
SDH4	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	6	1	1
SDS24	Protein fate (folding, modification, degradation)	5,3	2	0
SFC1	C-compound and carbohydrate metabolism	5,2	3	1
SGD1	Osmosensing	-3,2	0	0
SHM2	Nucleotide metabolism	-3,7	0	0
SHQ1	Nucleotide metabolism	-5,8	0	0
SIP18	Stress response	42	3	1
SNF3	C-compound and carbohydrate metabolism	4,4	0	3
SNR128	Transcription	-3,2	n.a.	n.a.
SNR76	n.a.	-3,0	n.a.	n.a.
SNZ1	Stress response	-3,1	0	0
SNZ2	Stress response	3,7	0	3
SOD2	Detoxification	5,9	1	0
SOL1	Transcription	151,3	0	0
SOL4	Transcription	18,9	n.a.	n.a.
SPI1	Cell wall	17,7	3	2
SPT15	Transcription	-3,0	1	0
SRP40	Transcription	-3,1	2	1
SSA1	Protein fate (folding, modification, destination)	3,0	2	1
SSA4	Stress response	6,1	3	1
SSE2	Stress response	4,3	1	0
SST2	Cell differentiation	-5,3	0	0
STD1	Transcriptional control	-7,7	1	0
STF2	Respiration	14,3	2	3
STL1	C-compound, carbohydrate transport	31,7	1	3
STP4	Transcription	4,2	2	4
SUC2	C-compound and carbohydrate metabolism	4,4	0	0
SUC4	n.a.	4,3	n.a.	n.a.
SUL1	Cellular import	3,1	0	3
SUN4	C-compound and carbohydrate metabolism	-4,1	0	0
SYM1	n.a.	3,0	n.a.	n.a.
TA(AGC)J	n.a.	-3,0	n.a.	n.a.
TA(AGC)K2	n.a.	-3,1	n.a.	n.a.
TAT2	amino acid transport	-3,7	1	2
TEF4	Translation	-3,2	0	1
TFS1	Mitotic cell cycle and cell cycle control	6,5	2	0
THP2	Classification not yet clear-cut	-3,1	0	0
TIP1	Stress response	8,9	0	0
TKL2	Pentose-phosphate pathway	4,7	2	1
TL(UAA)J	n.a.	-3,0	n.a.	n.a.
TOS8	Ionic homeostasis	3,1	3	2
TPS1	Metabolism of energy reserves	3,0	6	2
TPS2	Metabolism of energy reserves	3,7	5	0
TRL1	Transcription	-3,2	0	0
TSL1	Metabolism of energy reserves	12,5	5	3
TT(UGU)P	n.a.	-3,2	n.a.	n.a.
TTR1	Stress response	3,6	2	1
UBI4	Stress response	4,6	1	3
UGX2	Unclassified proteins	3,7	1	1
UIP4	Unclassified proteins	9,4	3	1
URA10	Nucleotide metabolism	3,6	2	1

UTP19	Unclassified proteins	-3,3	n.a.	n.a.
UTP8	Transcription	-3,1	1	1
XBP1	Transcription	6,3	0	1
XKS1	C-compound and carbohydrate metabolism	4,3	2	1
YAK1	Mitotic cell cycle and cell cycle control	3,1	3	1
YAL061W	C-compound and carbohydrate metabolism	4,3	2	1
YAR075W	Nucleotide metabolism	-3,1	0	1
YBL048W	Unclassified proteins	7,7	0	0
YBL049W	Unclassified proteins	6,3	1	2
YBL059W	Unclassified proteins	3,0	0	0
YBL071C	Unclassified proteins	-3,6	0	0
YBR116C	Unclassified proteins	9,3	0	2
YBR139W	Protein fate (folding, modification, degradation)	3,2	2	0
YBR141C	Unclassified proteins	-5,1	0	0
YBR224W	Unclassified proteins	-5,7	1	0
YBR230C	Unclassified proteins	6,8	2	3
YBR238C	DNA processing	-3,8	0	0
YBR262C	Unclassified proteins	3,0	1	3
YCL042W	Unclassified proteins	13,2	0	0
YCR061W	Unclassified proteins	-4,3	1	0
YCR072C	n.a.	-3,2	0	0
YCR087W	Unclassified proteins	-4,1	0	0
YDL027C	Unclassified proteins	3,1	1	0
YDL071C	Unclassified proteins	3,3	0	4
YDL110C	Unclassified proteins	3,3	2	0
YDL124W	C-compound and carbohydrate metabolism	4,2	2	2
YDL228C	Unclassified proteins	-3,7	0	1
YDR018C	Unclassified proteins	3,9	1	2
YDR031W	Unclassified proteins	4	0	0
YDR070C	Unclassified proteins	20,5	1	0
YDR089W	Unclassified proteins	-3,2	2	0
YDR222W	Unclassified proteins	-4,5	0	2
YDR413C	Unclassified proteins	-4,5	0	1
YDR527W	Unclassified proteins	-4,4	0	1
YEL041W	Unclassified proteins	3,1	1	1
YER049W	Unclassified proteins	-3,5	0	0
YER053C	Vacuolar transport	14,3	1	0
YER067C	n.a.	35,0	n.a.	n.a.
YER067W	Unclassified proteins	118,5	4	3
YER079W	Unclassified proteins	3,4	1	1
YER121W	Unclassified proteins	4,8	2	1
YER188W	Unclassified proteins	5,1	1	1
YFL030W	Amino acid biosynthesis	53,1	1	3
YFL049W	Unclassified proteins	-5,8	0	0
YFL067W	Unclassified proteins	-4,5	0	0
YFR011C	Classification not yet clear-cut	3,2	0	1
YFR017C	Unclassified proteins	29,1	2	2
YGL068W	Ribosome biogenesis	3,4	1	0
YGL081W	Unclassified proteins	3,4	0	1
YGL146C	Unclassified proteins	4,3	0	1
YGL157W	Metabolism of vitamins, cofactors and prosthetic group	-8,3	2	0
YGP1	Stress response	31,1	2	0
YGR021W	Unclassified proteins	3,0	1	0
YGR052W	Unclassified proteins	3,8	1	0

YGR053C	Unclassified proteins	6,2	1	0
YGR064W	Unclassified proteins	-3,1	0	1
YGR067C	Transcription	5	3	4
YGR127W	Unclassified proteins	3,1	1	1
YGR146C	Unclassified proteins	4,2	0	2
YGR228W	Unclassified proteins	-3,3	0	0
YGR243W	Unclassified proteins	22,6	1	2
YGR287C	C-compound and carbohydrate metabolism	3,8	0	6
YGR290W	Unclassified proteins	8	0	0
YHL021C	Unclassified proteins	7,1	3	2
YHR033W	Amino acid biosynthesis	4,3	1	2
YHR063W	n.a.	-3,1	n.a.	n.a.
YHR087W	Unclassified proteins	25,5	4	0
YHR138C	Unclassified proteins	3,0	1	1
YIL057C	Unclassified proteins	8,4	0	2
YIL059C	Unclassified proteins	4,7	1	1
YIL090W	Unclassified proteins	-4,3	1	1
YIL096C	Unclassified proteins	-4,4	0	2
YIL176C	Unclassified proteins	3,6	3	1
YIR016W	Unclassified proteins	3,1	2	2
YIR043C	Unclassified proteins	-4,2	n.a.	n.a.
YJL037W	Unclassified proteins	4,3	1	0
YJL052C-A	Unclassified proteins	8,5	1	1
YJL103C	Transcription	3,2	3	1
YJL119C	Unclassified proteins	3,5	0	1
YJL142C	Unclassified proteins	3,7	1	2
YJL144W	Unclassified proteins	6,9	1	0
YJL161W	Unclassified proteins	8,1	1	0
YJL200C	Tricarboxylic acid pathway (citrate cycle, Krebs cycle, TCA cycle)	-3,3	0	2
YJR008W	Unclassified proteins	12,6	2	1
YJR018W	Unclassified proteins	-3,2	0	2
YJR061W	Unclassified proteins	3,0	0	1
YJR115W	Unclassified proteins	10,4	3	3
YKL030W	Unclassified proteins	-8,4	0	0
YKL044W	Unclassified proteins	-6,5	2	1
YKL066W	Unclassified proteins	5	0	0
YKL084W	Unclassified proteins	-3,0	0	0
YKL086W	Unclassified proteins	6,5	0	0
YKL091C	Unclassified proteins	3,7	3	2
YKL111C	Unclassified proteins	-3,8	0	0
YKL151C	Unclassified proteins	4,6	2	1
YKR016W	Respiration	3,0	0	1
YKR049C	Unclassified proteins	5,5	2	1
YLL020C	Unclassified proteins	3,8	3	4
YLL067C	Unclassified proteins	-3,9	0	2
YLR031W	Unclassified proteins	4	0	0
YLR108C	Unclassified proteins	-3,0	3	1
YLR149C	Unclassified proteins	6,3	2	1
YLR252W	Unclassified proteins	4,9	0	0
YLR280C	Unclassified proteins	4,3	1	0
YLR281C	Classification not yet clear-cut	6,3	0	0
YLR312C	Unclassified proteins	12,3	2	5
YLR327C	Protein fate (folding, modification, destination)	123,4	2	3
YLR339C	Unclassified proteins	-3,9	0	3

YLR346C	Unclassified proteins	3,6	2	0
YLRCDELTA9	n.a.	3,0	n.a.	n.a.
YLRWTY2-1	n.a.	-3,5	n.a.	n.a.
YMC2	Mitochondrial transport	-7,6	1	0
YML081C	n.a.	3,1		
YML087C	Respiration	15,5	0	0
YML089C	Unclassified proteins	3,5	1	0
YMR040W	Unclassified proteins	3,6	0	0
YMR090W	Unclassified proteins	8,4	1	0
YMR103C	Unclassified proteins	5,3	0	0
YMR114C	Unclassified proteins	4,4	0	1
YMR153C	n.a.	-3,3	n.a.	n.a.
YMR196W	Unclassified proteins	6,5	2	1
YMR206W	Unclassified proteins	28,4	2	3
YMR245W	Unclassified proteins	5,5	0	0
YMR278W	C-compound and carbohydrate metabolism	3,1	1	0
YMR31	Ribosome biogenesis	3,9	1	1
YMR322C	Unclassified proteins	3,1	4	2
YMRCDELTA11	n.a.	3,0	n.a.	n.a.
YNK1	Nucleotide metabolism	4,9	0	0
YNL057W	Unclassified proteins	4,3	1	0
YNL100W	Unclassified proteins	3,7	1	1
YNL109W	Unclassified proteins	-3,5	0	1
YNL114C	Unclassified proteins	-3,9	0	0
YNL144C	Unclassified proteins	19	3	3
YNL174W	Unclassified proteins	-3,1	0	0
YNL200C	Unclassified proteins	4,3	1	1
YNL274C	C-compound and carbohydrate metabolism	12,2	1	4
YOL014W	Unclassified proteins	-3,8	2	1
YOL032W	Unclassified proteins	5,8	0	0
YOL053C	n.a.	38,4	n.a.	n.a.
YOL053W	Unclassified proteins	4,2	0	0
YOL101C	Unclassified proteins	4,6	0	3
YOL114C	Unclassified proteins	3,0	1	2
YOR012W	Unclassified proteins	-3,9	0	2
YOR051C	Unclassified proteins	-3,8	0	1
YOR146W	Unclassified proteins	-6,5	2	1
YOR173W	Unclassified proteins	12	2	0
YOR203W	Unclassified proteins	-3,3	2	2
YOR215C	Unclassified proteins	3,8	0	1
YOR289W	Unclassified proteins	4,2	0	0
YOR331C	Unclassified proteins	-4,5	0	0
YOR343C	Unclassified proteins	13,9	0	2
YPL014W	Unclassified proteins	15,9	3	1
YPL113C	C-compound and carbohydrate metabolism	9,5	0	2
YPL201C	Unclassified proteins	22,1	0	2
YPL222W	Unclassified proteins	3,0	0	3
YPL230W	Transcription	8,9	6	5
YPL247C	Unclassified proteins	4,3	2	0
YPR003C	Ionic homeostasis	6,3	1	1
YPR013C	Transcription	5,9	0	0
YPR061C	Unclassified proteins	3,7	1	1
YPR071W	Unclassified proteins	-3,7	0	1
YPR150W	Unclassified proteins	5,5	0	1

YPR151C	Unclassified proteins	6,8	1	1
YPRWDELTA14	n.a.	3,2	n.a.	n.a.
YPS3	Protein fate (folding, modification, destination)	4,0	2	3
YPS6	Protein fate (folding, modification, destination)	3,8	1	1
YRF1-4	DNA processing	-3,5	0	1
YRO2	Stress response	6,6	1	1
YTP1	Respiration	4,1	1	0
ZTA1	Classification not yet clear-cut	4,4	1	0
AAC1	Nucleotide transport	4,4	0	0
AAC3	Nucleotide transport	-3,7	1	1
AAD16	C-compound and carbohydrate metabolism	3,2	0	0
AAD4	C-compound and carbohydrate metabolism	7,3	0	0
AAT1	Amino acid biosynthesis	-3,1	0	0

Chapter VII

Deletion of *RTS1*, encoding a regulatory subunit of Protein Phosphatase 2A, results in constitutive amino acid signaling via increased Stp1p processing

Nadine Eckert-Boulet, Katrin Larsson, Boqian Wu, Peter Poulsen, Birgitte Regenberg, Jens Nielsen, Morten Kielland-Brandt

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Deletion of *RTS1*, Encoding a Regulatory Subunit of Protein Phosphatase 2A, Results in Constitutive Amino Acid Signaling via Increased Stp1p Processing

Nadine Eckert-Boulet,[1,2] Katrin Larsson,[1]‡ Boqian Wu,[1] Peter Poulsen,[1] Birgitte Regenberg,[2]† Jens Nielsen,[2] and Morten C. Kielland-Brandt[1]*

Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark,[1] and Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs Lyngby, Denmark[2]

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In *Saccharomyces cerevisiae*, extracellular amino acids are sensed at the plasma membrane by the SPS sensor, consisting of the transporter homologue Ssy1p, Ptr3p, and the endoprotease Ssy5p. Amino acid sensing results in proteolytic truncation of the transcription factors Stp1p and Stp2p, followed by their relocation from the cytoplasm to the nucleus, where they activate transcription of amino acid permease genes. We screened a transposon mutant library for constitutively signaling mutants, with the aim of identifying down-regulating components of the SPS-mediated pathway. Three isolated mutants were carrying a transposon in the *RTS1* gene, which encodes a regulatory subunit of protein phosphatase 2A. We investigated the basal activity of the *AGP1* and *BAP2* promoters in *rts1*Δ cells and found increased transcription from these promoters, as well as increased Stp1p processing, even in the absence of amino acids. Based on our findings we propose that the phosphatase complex containing Rts1p keeps the SPS-mediated pathway down-regulated in the absence of extracellular amino acids by dephosphorylating a component of the pathway.

The yeast *Saccharomyces cerevisiae* has developed a complex regulatory network enabling it to control the production of nutrient transporters depending on substrate availability in the environment. Hexose transporters and amino acid transporters are examples of nutrient transporters that are transcriptionally induced by their substrates (14, 24, 44). Amino acids are imported into the cells through amino acid permeases (AAPs) (20). The yeast AAPs belong to the amino acid/polyamine/organocation (APC) superfamily of transporters (28). They exhibit a range of affinities and specificities for all 20 common amino acids and a number of other compounds (42). The presence of extracellular amino acids results in increased transcription of about a third of the AAP genes (14). Amino acids induce transcription of the AAPs with different efficiencies, the most potent amino acid being leucine, while signaling by, e.g., arginine and proline is undetectable (16).

Ssy1p is an AAP homologue with 12 membrane-spanning domains, but compared to other members of the *S. cerevisiae* AAP family its cytoplasmic N-terminal tail is unusually long (30). Ssy1p has been shown to act as an amino acid sensor able to detect extracellular amino acids at the plasma membrane (8, 26, 32). Ssy1p is part of the so-called SPS sensor complex that includes Ssy1p, Ptr3p, and Ssy5p (13, 41). Ssy5p (30) and Ptr3p (27) are peripherally associated proteins, which, like Ssy1p, are essential for amino acid induction (14).

* Corresponding author. Mailing address: Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark Phone: 45 3327 5331. Fax: 45 3327 4765. E-mail: mkb@crc.dk.

† Present address: Institut für Mikrobiologie, Goethe-Universität Frankfurt, Marie Curie Strasse 9, D-60439, Frankfurt-am-Main, Germany.

‡ Present address: Bioinvent Int. AB, Sölvegatan 41, SE-22370 Lund, Sweden.

Transcriptional induction is mediated by the homologous transcription factors Stp1p and Stp2p, which bind to the promoters of the AAP genes at the UAS_{aa} (upstream activating sequence) first identified in the *BAP3* promoter (6) and then in the *BAP2* promoter (37), but later also found in the promoter sequences of the AAP genes *GNP1*, *AGP1*, *MUP1*, *TAT1*, *TAT2*, and *DIP5* (10). In the absence of amino acids, Stp1p and Stp2p are present mainly in the cytoplasm (3, 10). When amino acids are detected in the environment, 10 kDa of the N terminus are endoproteolytically cleaved off, resulting in relocation of the transcription factors to the nucleus (3). Ssy5p is the endoprotease responsible for processing of Stp1p (1, 2). Signaling and processing are moreover dependent on the F-box protein Grr1p (1, 5, 11, 26), which is part of the E3 ubiquitin ligase SCF (Skp1-Cullin-F-box) complex SCF^{Grr1} (38). Though SCF^{Grr1} normally targets proteins for degradation by the 26S proteasome, activation of Stp1p is not dependent on the 26S proteasome (1). Other factors involved in amino induction include casein kinase I (1). Furthermore, Ptr3p is found to interact with Yfr021wp in a two-hybrid screen (18). The same work reports that *yfr021w*Δ and *ypl100w*Δ strains exhibit *ptr3*Δ-like phenotypes, i.e., they are unable to grow at a high concentration of histidine.

Mutants that exhibit constitutive expression of the target AAP genes independently of the components of the SPS sensor have been identified (15). Here we report a screen likewise aimed at identifying factors down-regulating SPS-mediated signaling, but allowing for factors that, when mutated, cause (constitutive) signaling only if the sensor is present. Using a strain deficient in histidine synthesis, we selected for growth on minimal ammonium medium supplemented with the dipeptide Gly-His. Dipeptides, including Gly-His, are taken up by the permease Ptr2p (39), which is under transcriptional control of

TABLE 1. Strains used in this study

Strain	Relevant genotype	Reference
M4054	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ	19
M4605	*MAT***a** *SUC2 mal gal2 CUP1 ura3 his3 leu2 gap1*Δ	This work
M5397	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ	This work
M4871	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *ssy1*Δ	P. S. Nielsen
M4723	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *ptr3*Δ	P. S. Nielsen
M4724	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *ssy5*Δ	P. S. Nielsen
M5470	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *grr1*Δ	This work
M4600	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *ssy1*Δ	This work
M5437	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *ptr3*Δ	This work
M5439	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *ssy5*Δ	This work
M4608	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *stp1*Δ *stp2*Δ *stp3*Δ	This work
M5471	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *grr1*Δ *rts1*Δ	This work
M5447	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *STP1-ZZ*	41
M5445	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *ssy1*Δ *STP1-ZZ*	41
M5443	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *ptr3*Δ *STP1-ZZ*	41
M5444	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *ssy5*Δ *STP1-ZZ*	41
M5559	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *grr1*Δ *STP1-ZZ*	This work
M5474	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *STP1-ZZ*	This work
M5501	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *ptr3*Δ *STP1-ZZ*	This work
M5524	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *ssy5*Δ *STP1-ZZ*	This work
M5629	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *ssy1*Δ *STP1-ZZ*	This work
M5525	*MAT***a** *SUC2 mal gal2 CUP1 ura3 gap1*Δ *rts1*Δ *grr1*Δ *STP1-ZZ*	This work

the amino acid-sensing pathway (4, 8). Subsequent cleavage of internalized Gly-His satisfies the growth requirement for histidine (39). Thus, growth on Gly-His reflects activation of the signaling pathway, so that growth in the absence of an inducing amino acid reveals that the amino acid-sensing pathway is turned on constitutively. The constitutive mutants thus found were tested for constitutive induction of the *BAP2* gene, encoding a branched-chain amino acid permease, using the β-galactosidase reporter assay. After this secondary screen the mutants included some with transposon insertions in the *RTS1* gene. *RTS1* encodes a regulatory subunit of protein phosphatase 2A (PP2A). We subsequently studied the role of this gene in SPS-mediated amino acid signaling.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used in this study are listed in Table 1 and are all derived from strain M4054 (*MAT***a** *ura3 gap1*Δ) (19), which in turn is derived from S288C. The *HIS3* and *LEU2* genes were disrupted in M4054; the resulting strain was named M4605 and was used as the host for the transposon mutagenesis library, as described below. The histidine-independent strain M5397 (*MAT***a** *ura3 HIS3 gap1*Δ *rts1*Δ) was constructed by crossing M4599 (*MAT***a** *ura3 his3 gap1*Δ *rts1*Δ) with the isogenic X2180-1b (*MAT*α). Spores with the desired genotype (*ura3 HIS3*) were tested for the *gap1* phenotype on minimal citrulline medium (20, 43).

The *RTS1*/*rts1* genotype was tested on SD plates supplemented with uracil (1% [wt/vol] succinic acid, 0.6% [wt/vol] NaOH, 0.17% [wt/vol] Bacto yeast nitrogen base without amino acids, 2% Bacto agar, 2% [wt/vol] glucose, 20 mg/liter uracil), at the center of which a filter was placed with 4 mg of the toxic dipeptide L-leucyl-L-ethionine, as described previously (8). Equal amounts of cells were suspended in water, and 10 μl of each strain was streaked towards the center of the plate with the filter disk. *rts1*Δ strains are sensitive to L-leucyl-L-ethionine in this test.

To check for a functional SPS amino acid-sensing system, strains were plated on yeast-peptone-dextrose medium (YPD) supplemented with metsulfuron methyl (30).

Strains M5431, M5437, M5470, and M5471 were constructed by PCR amplification of the *loxP-kanMX-loxP* cassette from plasmid pUG6 (21) with primer overhangs complementary to the parts immediately upstream and downstream of the open reading frame (ORF) to be deleted. Transformants were selected on YPD containing 300 mg/liter Geneticin G-418 sulfate and were tested for correct deletion by diagnostic PCR. The *kanMX* marker was removed when necessary as described previously (21).

Strains M5443, M5444, M5445, M5447, M5474, M5501, M5524, and M5525 were constructed by replacing in situ the *STP1* stop codon by 2,159 bp of a construct carrying a doublet of the immunoglobulin G-binding Z domain of the *Staphylococcus aureus* protein A and the *kanMX* marker (40, 41). Transformants were selected with Geneticin and tested by PCR.

Transposon-based mutagenesis. Mutagenesis was based on insertion of the prokaryotic mTn*3* transposon. Propagation of the mutagenized, *LEU2*-based library, transformation, and isolation of recombinants were carried out essentially as described previously (46). Thirty thousand colonies were screened, and 11 mutants were selected according to the following criteria: their ability to grow on SC medium devoid of leucine before being tested for constitutive amino acid uptake on SD medium supplemented with uracil and 0.5 g/liter Gly-His dipeptide; their ability to grow on SD plus Ura plus 30 mg/liter Ile plus 20 mg/liter His plus 60 mg/liter Val plus 100 mg/liter metsulfuron methyl; and finally by their constitutively active *BAP2* promoter.

β-Galactosidase reporter assay. Strains of interest were transformed with plasmid pRB108 or pTD17 (7, 42), centromere-based plasmids in which 1.0 kb of the amino acid-inducible *AGP1* promoter and 683 bp of the *BAP2* promoter, respectively, are fused to *Escherichia coli lacZ*. Transformants were grown overnight in SD medium to an optical density at 600 nm of 0.1 to 0.3. The cultures were then divided in aliquots to which different compounds were added: L-citrulline (Sigma) to a final concentration of 5 mM or an equal volume of water, and L-leucine to a final concentration of 100 μM. Each treatment was performed in duplicate on aliquots originating from the same culture. β-Galactosidase assays were performed as described previously (7). Miller unit values were calculated as follows: (optical density at 420 nm × 1,000)/(cell volume [ml] × reaction time [min] × optical density at 600 nm at harvest).

Western blot analysis. Cells carrying a fusion (Stp1-ZZ) of an immunoglobulin G-binding domain of the *Staphylococcus aureus* protein A to the C terminus of Stp1p (40) were cultivated in SD supplemented with 20 mg/liter uracil to the early exponential phase. Cells were harvested and proteins were extracted under denaturing conditions with NaOH and β-mercaptoehanol before being enriched by precipitation with trichloroacetic acid. Protein concentrations were determined using the Bio-Rad protein assay. Proteins were separated on NuPAGE 10% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Chemiluminescent immunodetection was performed as described previously (41). The extent of binding of the antibody against horseradish peroxidase was quantified using the Image Quant software 5.0 (Molecular Dynamics) with a local median-based background correction.

RESULTS

Isolation of mutants constitutive in amino acid signaling. In order to select mutants with increased activity of the amino acid-sensing pathway, a screen was designed in which a *his3 leu2* derivative of the reference strain M4054 was subjected to transposon mutagenesis. The host strain was transformed with a mixture of yeast genomic fragments representing a mutagenized *LEU2*-based plasmid library (46). Mutants of interest were selected using three different screening criteria. First, we took advantage of the fact that dipeptide uptake (39), as well as branched-chain amino acid uptake (7), is very low in cells grown on minimal ammonium medium in the absence of leucine. Dipeptides, including Gly-His, are taken up by the peptide transporter Ptr2p (39), which is under the transcriptional control of the amino acid-sensing pathway (4). Mutants with constitutive signaling were thus selected on SD medium supplemented with the Gly-His dipeptide, which, upon intracellular cleavage, provides the histidine required for growth (39). A subsequent selection was carried out on SD supplemented with the amino acids Ile, His, and Val, and the sulfonylurea herbicide metsulfuron methyl. This type of compound inhibits synthesis of branched-chain amino acids and therefore prevents growth of cells unable to take up branched-chain amino acids from the medium (53), such as mutants deficient in amino acid signaling (29, 30). Finally, colonies of interest were tested for constitutive acitivity of the *BAP2* promoter.

Among the 11 mutants thus isolated, three had transposon insertions in the PP2A subunit-encoding gene *RTS1* (34). The remaining transposons were localized in *VPS15*, *VPS34*, and *VPS16*, coding for proteins involved in vacuolar protein sorting, and *STH1*, coding for the ATPase subunit in the chromatin remodeling complex RSC. Two others had mutations in ORFs with unknown function, *YFR044C* and *YFR045W*. Two mutants were not mapped. In the present work, the relationship between the phenotype and the locus affected by transposon insertion was analyzed in more detail for the *RTS1* gene.

Deletion of *RTS1* results in constitutive activation of *BAP2* and *AGP1* transcription. Since transposon insertion very often completely inactivates a gene, we wished to analyze the behavior of a strain deleted for *RTS1*. One of the selection criteria used in the original screen was the ability to take up Gly-His on SD medium, thereby promoting growth of a histidine-requiring strain because of intracellular cleavage of the dipeptide. To find out if an *rts1*Δ strain exhibits the same phenotype, we deleted the *RTS1* ORF in a *his3* derivative of the reference strain M4054. Indeed, the lack of *RTS1* enabled growth on the dipeptide as a histidine source (not shown).

In order to be able to analyze the *rts1*Δ mutation in the absence of extracellular amino acids or dipeptides, we also constructed a *HIS3 rts1*Δ strain (M5397). First we wished to confirm the activation of target promoters for the amino acid signaling by testing for growth on YPD supplemented with metsulfuron methyl; the strain grew, as expected (not shown). Then we tested whether this activation was constitutive by yet another criterion, using the dipeptide L-leucyl-L-ethionine. If this dipeptide is taken up, it is cleaved to yield the toxic methionine analogue L-ethionine. Also, uptake of the dipeptide L-leucyl-L-ethionine is mediated up by the peptide transporter Ptr2p (39) and is thus under transcriptional control of the amino acid-sensing pathway (4). Insensitivity to L-leucyl-L-ethionine therefore reflects that the pathway is not activated, while growth inhibition in the absence of an inducing amino acid indicates constitutive activation of the amino acid-sensing pathway. The *rts1*Δ strain M5397 was unable to grow on minimal medium in the presence of L-leucyl-L-ethionine, suggesting constitutive activity of the signaling pathway (not shown).

TABLE 2. *AGP1* promoter activity in wild-type cells and *rts1*Δ cells in response to leucine and citrulline

Inducer	Mean β-galactosidase activity[a] ± SEM	
	M4054 (wild type)	M5397 (*rts1*Δ)
L-Leucine (100 μM)		
−	1.5 ± 0.1	10.5 ± 0.4
+	8.4 ± 1.0	9.9 ± 0.9
L-Citrulline (5 mM)		
−	0.1 ± 0.1	11.4 ± 0.6
+	8.7 ± 1.4	13.5 ± 0.4

[a] Activity in Miller units in the absence (−) and 40 minutes after the addition (+) of inducer.

We also investigated activation of the promoter of the broad-specificity AAP gene *AGP1* using a fusion of the promoter to the *E. coli lacZ* gene. Cells were grown in SD medium and incubated for 40 min with or without 100 μM L-leucine. The experiment was repeated with 5 mM L-citrulline or an equal volume of water. These concentrations have been previously found to fully or almost fully induce the *AGP1* promoter (16, 40). The results show that *AGP1* transcription in *rts1*Δ cells is constitutive, i.e., unaffected by leucine or citrulline addition. In the wild-type control experiment, *AGP1* promoter activation was low in the absence of amino acids and increased strongly in response to either leucine or citrulline (Table 2).

Transcriptional induction of AAP genes was previously shown to involve Stp1p endoproteolysis (3). We used the Stp1-ZZ construct previously described (40), in which the C terminus of Stp1p was fused to a doublet of the IgG-binding Z domain of the *S. aureus* protein A. This insert was integrated in the wild-type strain and the *rts1*Δ strain, resulting in strains M5447 (40) and M5474, respectively. The fraction of processed Stp1p in *rts1*Δ cells in the absence of leucine was greater than in wild-type cells (30% versus 13%), whereas addition of leucine led to almost complete (above 90%) Stp1p processing in both wild-type and *rts1*Δ cells (Table 3 and Fig. 1). Quantification of antibody binding showed that the relative amount of processed Stp1p in the absence of inducer was more than doubled in *rts1*Δ cells compared to wild-type cells.

TABLE 3. Quantification of processed Stp1-ZZ in wild-type and *rts1*Δ cells in response to leucine[a]

Strain	Mean amt of processed Stp1-ZZ ± SEM	
	Without inducer	With inducer
M5447 (wild type)	12.8 ± 1.1	90.2 ± 0.4
M5474 (*rts1*Δ)	30.4 ± 5	92.7 ± 1.6

[a] Samples were taken in the absence and 20 minutes after addition of 100 μM L-leucine. Relative amounts of full-length and processed Stp1-ZZ were determined by performing the experiment twice.

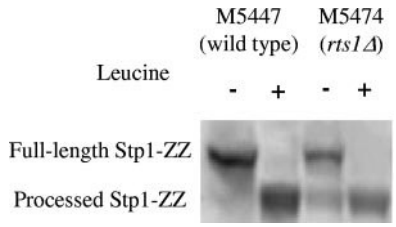

FIG. 1. Quantitative Western analysis of Stp1-ZZ processing in wild-type and *rts1*Δ cells in response to 100 μM L-leucine.

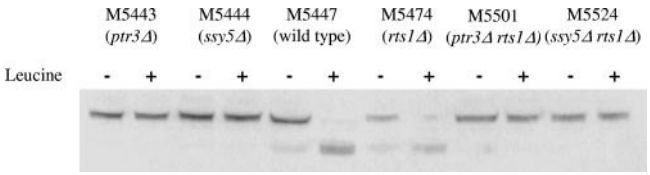

FIG. 2. Quantitative Western analysis of Stp1-ZZ processing in *rts1*Δ, *ssy5*Δ, and *ptr3*Δ single mutants and in *ssy5*Δ *rts1*Δ and *ptr3*Δ *rts1*Δ double mutants, in the absence (−) and 20 min after the addition (+) of 100 μM L-leucine. Upper band: unprocessed Stp1-ZZ; lower band: processed Stp1-ZZ.

Remarkably, Stp1p processing to the extent of 30% is sufficient to generate an activity of the *AGP1* promoter comparable to that observed in wild-type cells after amino acid addition (compare Table 2 and Table 3). In fact, this is expected from previous comparisons of dose-response relationships using quantification of Stp1p processing versus *AGP1* promoter activity as the read-out (40, 41). The sensing of L-leucine exhibits a 50% effective concentration of 12 μM under the growth conditions studied when Stp1p processing is monitored, but only 1 μM when *AGP1* promoter-driven *lacZ* expression is measured (40). The most obvious interpretation is that saturation of the promoter activity occurs already at modest levels of activated Stp1p.

Epistasis relationships. In order to further investigate the role of *RTS1*, we constructed strains M4600 (*ssy1*Δ *rts1*Δ), M5437 (*ptr3*Δ *rts1*Δ), M5439 (*ssy5*Δ *rts1*Δ), M5470 (*grr1*Δ *rts1*Δ), and M4608 (*stp1*Δ *stp2*Δ *stp3*Δ *rts1*Δ). *STP3* is homologous to *STP1* and *STP2*, and Helge A. Andersen (personal communication) has found that deletion of *STP3* further reduces the low, remaining activity of the amino acid signaling pathway in an *stp1*Δ *stp2*Δ mutant. All of the resulting strains were sensitive to metsulfuron methyl on YPD, and all were insensitive to L-leucyl-L-ethionine (not shown), suggesting that the amino acid-sensing pathway was inactive.

β-Galactosidase assays were performed on extracts from cells in which the *E. coli lacZ* gene was placed under the control of the *AGP1* promoter. β-Galactosidase measurements confirmed that the multiple mutants behaved similarly to the single mutants deficient in signaling, i.e., *AGP1* transcription was very low even in the presence of extracellular amino acids (Table 4). Using the Stp1-ZZ construct, we also measured relative amounts of processed and full-length Stp1p in each of the strains, in the absence and presence of amino acids. Stp1p was exclusively present as the full-length protein in all single and multiple mutants (Fig. 2). In other words, *ssy1*, *ptr3*, *ssy5*, and *grr1* are epistatic over *rts1*.

DISCUSSION

In this work we have attempted to identify potential negative regulators of the amino acid-sensing pathway. For this purpose we have designed a screen allowing selection of mutants with constitutive activity of the pathway. The screen described in this report was performed in the absence of inducer in cells lacking *GAP1*, and we investigated transcription levels of a *lacZ* reporter gene placed under the control of the amino acid-inducible promoters of the *AGP1* and *BAP2* genes, which are known targets of the SPS-mediated pathway. The genes that were disrupted by transposon insertion encode proteins that could be involved at any level of the signaling.

The isolated mutants include some in which the transposon insertion disrupted the *RTS1* gene. This gene encodes a regulatory subunit of PP2A, which is a major serine/threonine phosphatase involved in several nutrient-induced signaling pathways, in cell growth control and cell division control (9, 35, 45, 56). Protein phosphatase 2A exists in several isoforms and is mostly present in cells as a heterotrimeric complex, consisting of a catalytic (C) subunit, encoded by *PPH21*, *PPH22*, or *PPH3* (45, 49), a scaffolding subunit (A), encoded by *TPD3* (55), and a regulatory subunit (B or B′), encoded by *CDC55* (22) and *RTS1* (47, 48), respectively. The B and B′ subunits are believed to regulate the activity of PP2A by determining its cellular location and modifying the substrate specificity of the C subunit (31, 54). *CDC55* is required for correct cell cycle checkpoint control (35), and *cdc55*Δ cells display a cold-sensi-

TABLE 4. *AGP1* promoter activity in cells deleted of genes encoding positive factors in amino acid signaling and/or *RTS1* in the absence and presence of inducing amino acids

Inducer	Mean β-galactosidase activity[a] ± SEM						
	M4054 (wild type)	M5397 (*rts1*Δ)	M4600 (*ssy1*Δ *rts1*Δ)	M5437 (*ptr3*Δ *rts1*Δ)	M5439 (*ssy5*Δ *rts1*Δ)	M5471 (*grr1*Δ *rts1*Δ)	M4608 (*rts1*Δ *stp1*Δ *stp2*Δ *stp3*Δ)
L-Leucine (100 μM)							
−	1.7 ± 0.1	10.1 ± 0.2	1.1 ± 0.1	−0.1 ± 0.0	−0.8 ± 0.0	0.1 ± 0.0	
+	7.2 ± 0.6	9.6 ± 0.2	1.1 ± 0.0	−0.1 ± 0.0	−0.6 ± 0.0	−0.1 ± 0.0	
L-Citrulline (5 mM)							
−	0.1 ± 0.0		0.95 ± 0.1				0.0 ± 0.0
+	11.3 ± 1.2		0.83 ± 0.1				0.0 ± 0.0

[a] Activity in Miller units in the absence (−) and 40 minutes after the addition (+) of inducer.

tive phenotype but no phenotype at elevated temperatures (22). *RTS1* was found in two independent genetic screens, as a multicopy suppressor of *hsp60*(Ts) mutant alleles (48), and later as a *R*ox *T*hree *S*uppressor (12); *rts1*Δ cells are thermosensitive and exhibit a typical *cdc* mutant phenotype (47).

We identified *RTS1* as a negative component of the SPS-mediated amino acid-sensing pathway. Deletion of *RTS1* results in constitutive transcription of *AGP1* and *BAP2*. Moreover, *ssy1*, *ptr3*, *ssy5*, and *grr1* were found to be epistatic over *rts1*. These results indicate that the PP2A is involved in the SPS-mediated pathway and suggest that a dephosphorylation step is required to down-regulate signaling in the absence of extracellular amino acids. Rts1p associated with Tpd3p and the C subunit might thus dephosphorylate one of the SPS proteins. Alternatively, it could dephosphorylate the Stp transcription factors, resulting in a conformational change that perhaps limits its accessibility of the cleavage site to the protease, thereby impairing Stp1p and Stp2p proteolysis and activation in the absence of amino acids.

While PP2A appears to down-regulate amino acid signaling, a corresponding kinase should be involved in the activation of the pathway. Casein kinase I is a candidate for this activity, since the amino acid-sensing pathway is inactive in temperature-sensitive mutants affected in the casein kinase I genes *YCK1* and *YCK2*, and these strains exhibit loss of Stp1p processing (1). Casein kinase I and PP2A are known to act on the same substrate in *Xenopus* embryos (17) and we propose that they do so in yeast as well. This hypothesis is substantiated by the finding that the yeast Cdc55p subunit forms a complex with casein kinase I (23). Moreover, casein kinase I has also been reported to be involved in the glucose-sensing pathway (50), which mediates transcriptional induction of hexose transporter (*HXT*) genes in response to extracellular glucose and which shares many similarities with the amino acid-sensing pathway. Casein kinase I is indeed required for phosphorylation of Mth1, which is then targeted for degradation by SCF^{Grr1} (36, 50). Thus, glucose induction of the *HXT1* promoter is deficient in a *yck1*Δ *yck2*(Ts) mutant; in addition, this mutant lacks glucose-induced degradation of Mth1p and Std1p (36).

Mth1p and Std1p interact with the transcriptional repressor Rgt1p in the absence of glucose: the resulting complex binds promoter DNA, thereby repressing transcription from the *HXT* genes (33, 52). Likewise, Stp1p is phosphorylated in a casein kinase I-dependent way prior to its endoproteolytic activation (1). SCF^{Grr1} recognizes phosphorylated substrates; this is the case for Mth1p (50) and for the G_1 cyclin Cln2p (25). Although Stp1p is phosphorylated by casein kinase I (1), there is no reason to invoke it as a target for SCF^{Grr1}. A possible target for SCF^{Grr1} is Ptr3p, which in fact has been found to be subject to posttranslational modification, exhibiting a slower-migrating band (13) that might be due to ubiquitination. Alternatively, the target for SCF^{Grr1} could be a protein not yet known to be part of the pathway. Further work is needed to propose which protein is a target for PP2A associated with Rts1p. There may, however, be cross talk between the amino acid-sensing and the target of rapamycin (TOR) pathways, since N. Eckert-Boulet (unpublished data) has observed that treatment of *rts1*Δ cells with rapamycin enhanced the constitutive signal, also at the level of Stp1-ZZ processing.

Interestingly, it has recently been found that PP2A is involved in regulating the induction of *HXT1* by glucose and that Cdc55p is the regulatory subunit involved. The 14-3-3 proteins Bmh1p and Bmh2p also appear to be involved (51). These observations add to existing similarities between the glucose induction pathway and the amino acid induction pathway. It will be interesting to see whether Bmh1p and Bmh2p also are involved in the SPS-mediated pathway and whether Rts1p interacts with casein kinase I like Cdc55p does.

In summary, we have shown that Rts1p, one of the two known regulatory subunits of PP2A, is a down-regulator of the SPS-mediated amino acid-sensing pathway. This finding illustrates a new role in nutrient-induced signaling for PP2A and further highlights the reuse of components in different signaling pathways.

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