Role of Gcn4p in nutrient-controlled gene expression in *Saccharomyces cerevisiae*

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Summary

Under amino acid starvation conditions, the bakers' yeast *Saccharomyces cerevisiae* activates a system called "General control of amino acid biosynthesis". Gcn4p, the transcription factor of this system induces the expression of more than 50 genes involved in the different amino acid biosynthetic pathways. In this thesis it could be shown that during simultaneous limitation of amino acids and nitrogen the general control is not activated. More exactly, even a decrease of the Gcn4p activity was detected, which was traced back onto a reduction of the Gcn4 protein amount in the cell. This decrease of the intracellular concentration was caused by translational control of the *GCN4* mRNA, which was able to repress even a 2-fold increase of the *GCN4* transcription rate. Furthermore during nitrogen starvation conditions no correlation between the stature of eIF-2 phosphorylation and *GCN4* expression was observed. For this reason an involvement of the already known mechanism of translational regulation of *GCN4* mRNA could be excluded. Rather a factor is postulated, which is situated downstream of eIF-2 and has a regulatory effect on initiation of translation.

Although it could be proven that the Gcn4p activity is repressed during amino acid starvation, a *gcn4Δ* mutant strain was not able to form pseudohyphae any more. This indicated to a dependence of pseudohyphal growth on the presence of Gcn4p. Furthermore it was detected that the transcription of *FLO11*, which is a flocculin gene necessary for pseudohyphal growth, was activated by amino acid limitation under normal nitrogen concentrations as well. This activation is Gcn4p-dependent and leads to an improved cell-cell adhesion. Gcn4p as well as the transcription factor of the cAMP pathway, Flo8p are necessary for *FLO11* expression during amino acid limitation. On the other hand the transcription factors of the MAPK pathway, Ste12p and Tec1p, which are important under nitrogen starvation conditions as well have only a minor importance.

By comparative transcriptome analysis of a yeast wild type and a *gcn4Δ* mutant strain, which were grown under amino acid limitation conditions, 225 genes were identified, which were Gcn4p-dependent activated during amino acid starvation conditions. Not only genes of amino acid or nucleotide metabolism, for which a Gcn4p-dependent transcription was already known, but also several other genes were identified, which are involved in completely different cellular processes. A Gcn4p-

dependent activation of transcription could be detected for genes of carbon, fatty acid and phosphorus metabolism, as well as for genes coding for chromatin structure determining proteins (e.g. histones).

Zusammenfassung

Unter Aminosäure-Mangelbedingungen schaltet die Bäckerhefe *Saccharomyces cerevisiae* die sogenannte „Allgemeine Kontrolle der Aminosäure-Biosynthese“ ein. Gcn4p, der Transkriptionsfaktor dieses Systems, aktiviert die Expression von über 50 Genen, die in die verschiedenen Aminosäure-Biosynthesewege involviert sind. Im Rahmen dieser Arbeit konnte gezeigt werden, daß bei gleichzeitiger Limitierung von Aminosäuren und Stickstoff, die Allgemeine Kontrolle der Aminosäure-Biosynthese nicht aktiviert wird. Vielmehr konnte eine Abnahme der Gcn4p-Aktivität beobachtet werden, die auf eine Verminderung der Gcn4-Proteinmenge zurückzuführen war. Ursache für diese Abnahme der intrazellulären Proteinkonzentration war die translationelle Kontrolle der *GCN4*-mRNA, die selbst eine unter diesen Bedingungen auftretende zweifache Erhöhung der *GCN4*-Transkriptionsrate repremieren konnte. Außerdem wurde gezeigt, daß unter Stickstoff-Mangelbedingungen keine Korrelation zwischen dem Phosphorylierungsstatus von eIF-2 und der *GCN4*-Expression mehr gegeben war. Dadurch konnte ausgeschlossen werden, daß der bereits bekannte Mechanismus der translationellen Kontrolle der *GCN4*-mRNA unter diesen Bedingungen eine Rolle spielt. Vielmehr wird ein zusätzlicher Faktor postuliert, der unterhalb von eIF-2 direkt auf die Translationsinitiation wirkt.

Obwohl nachgewiesen werden konnte, daß die Gcn4p-Aktivität unter Stickstoff-Mangelbedingungen repremiert wird, war eine *gcn4Δ*-Mutante nicht mehr in der Lage, Pseudohyphen zu bilden. Dies zeigt eindeutig, daß eine Abhängigkeit des Pseudohyphen-Wachstums von der Anwesenheit von Gcn4p gegeben ist. Darüber hinaus wurde festgestellt, daß die Transkription des für das Pseudohyphen-Wachstum notwendigen Flocculin-Gens, *FLO11* auch durch Aminosäure-Mangel unter normalen Stickstoff-Konzentrationen aktiviert werden kann. Diese Aktivierung ist Gcn4p-abhängig und führt zu einer verstärkten Zell-Zell Adhäsion. Neben Gcn4p ist für die *FLO11*-Expression unter Aminosäure-Mangelbedingungen auch der Transkriptionsfaktor des cAMP-Weges, Flo8p notwendig. Hingegen spielen die für die *FLO11*-Expression unter Stickstoff-Mangelbedingungen gleichfalls wichtigen Transkriptionsfaktoren des MAPK-Weges, Ste12p und Tec1p, eine eher untergeordnete Rolle.

Durch vergleichende Transkriptom-Analyse eines Hefe-Wildtyp-Stammes und eines *gcn4Δ*-Deletionsstammes, die unter Aminosäure-Mangelbedingungen gewachsen waren, konnten 225 Gene identifiziert werden, die unter Aminosäure-Mangelbedingungen Gcn4p-abhängig induziert werden. Neben Genen des Aminosäure- oder Nukleotid-Metabolismus, für die eine Gcn4p-abhängige Transkription bereits bekannt war, konnten auch eine Vielzahl von Genen identifiziert werden, die in völlig andere zelluläre Prozesse involviert sind. So wurde eine Gcn4p-abhängige Transkriptionsaktivierung für Gene des Kohlenstoff-, Fettsäure- und Phosphat-Stoffwechsels, aber auch für Gene, die für Chromatinstruktur determinierende Proteine codieren (z.B. Histone), nachgewiesen.

Chapter I

Introduction

Regulation of morphology of the bakers' yeast *Saccharomyces cerevisiae* in dependence on the nutritional conditions.

Life cycle of *Saccharomyces cerevisiae.*

The budding yeast *S. cerevisiae* is able to change its morphology and interconvert between a unicellular and a multicellular filamentous growth type. Furthermore, the organism can grow in a haploid or a diploid form (Figure 1). In the laboratory, yeast strains are usually cultivated on media containing a fermentable carbon source and sufficient nitrogen, such as glucose and ammonium, respectively. On these media haploid cells are sticky and smaller than diploid cells, but their overall morphology is similar. They show a unicellular ellipsoid cell morphology, called yeast form (YF). During growth of diploids, depletion of both nutrients leads to sporulation (meiosis), whereas limitation of only one nutrient source either favors growth arrest (carbon starvation) or induces filamentous growth (nitrogen starvation) as chains of elongated cells, called pseudohyphae (PH). During prolonged growth of haploids on complex media with glucose limitation, the so-called haploid invasive growth is observed, characterized by the development of small microfilaments, which stick on the surface of the agar plate (Cullen & Sprague, 2000).

Additionally, both cellular forms can be distinguished by their budding pattern (Mösch, 2000): diploid yeast form cells exhibit a bipolar budding pattern, which switches to a unipolar distal mode during pseudohyphal growth. This budding at the opposite pole of the birth end is essential for establishment of a filamentous growth mode. Haploid cells always bud unipolar at the proximal pole, that means, budding is constrained to the mother-daughter neck region.

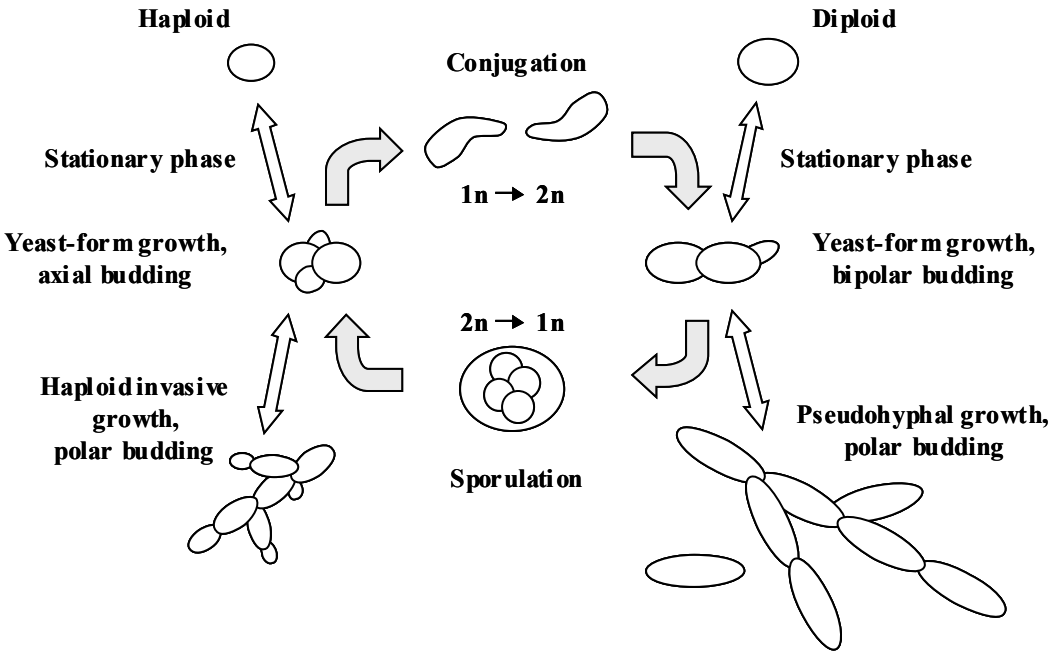

Figure 1. Life cycle of the bakers' yeast *Saccharomyces cerevisiae*.
Haploid as well as diploid cells can adopt yeast form vegetative growth, invasive growth or stationary phase arrest. Upon nitrogen limitation, diploid cells switch their morphology and grow invasively as multicellular filaments called pseudohyphae. Haploid invasive growth occurs during prolonged growth on complex media with glucose limitation leading to the formation of small microfilaments. Haploid cells (1n) conjugate to form diploids (2n) and these can sporulate to form haploids. General nutrient limitation results in stationary phase arrest in the haploid as well as in the diploid form.

Pseudohyphal growth of *Saccharomyces cerevisiae*.

As described before, the development of pseudohyphae in *S. cerevisiae* demands at least two stimuli, starvation for nitrogen and the presence of a fermentable carbon source. The switch from the yeast form to pseudohyphal growth is accompanied by alterations in several distinct cellular processes: (i) Cell morphology changes from ellipsoidal shaped yeast form cells to long, thin pseudohyphal cells. (ii) The budding pattern of the cells is altered from bipolar to unipolar distal, resulting in linear filamentous chains of cells. (iii) Furthermore, cell separation switches from complete to incomplete division, which means that cells remain attached to each other and form long multicellular chains. (iv) The process of cell division is modified during pseudohyphal development as well. All pseudohyphal growth occurs during the budded period (G2-phase) of the cell cycle, so that mitosis is restricted until the bud has reached the cell

size of the mother cell. Consequently, both cells bud synchronously in the following cycle without G1-delay (Mösch, 2000).

Taken together, these changes enable pseudohyphae to grow invasive, in contrast to superficial growth of yeast form cells (Figure 2).

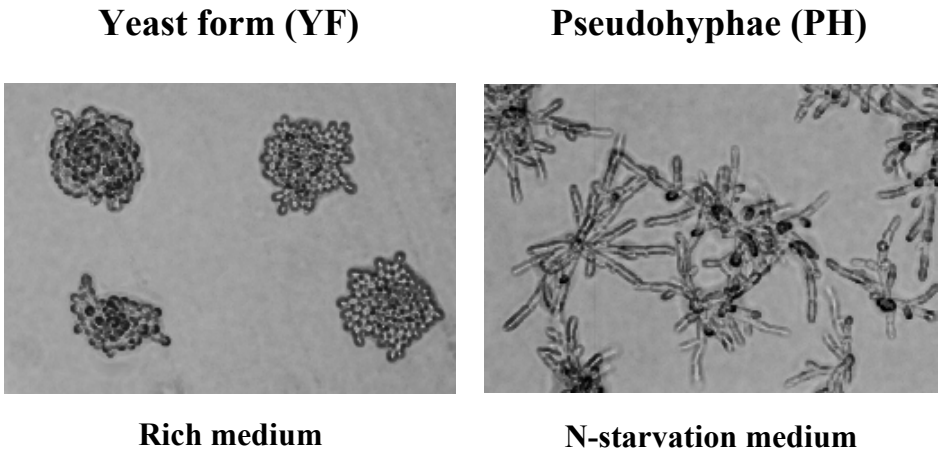

Figure 2. Comparison of the growth behavior of yeast form and pseudohyphal cells of *S. cerevisiae* (according to Mösch, 2000).
Diploid *S. cerevisiae* cells were streaked on either rich medium or nitrogen starvation medium to obtain single colonies. Microcolonies of cells growing as yeast form (YF) or as pseudohyphae (PH) were photographed after 17 h of incubation at 30°C.

Environmental stimuli and sensing systems.

However, yeast cells must be able to sense both, abundant fermentable carbon source as well as nitrogen deprivation to undergo pseudohyphal development. Easy utilizable nitrogen sources like ammonium or arginine suppress pseudohyphal formation in standard concentrations, whereas standard amounts of proline or uracil are permissive for the formation of pseudohyphae (Gimeno *et al*., 1992). The sensor systems that differentiate between diverse nitrogen compounds and control pseudohyphal growth are largely unknown. For sensing ammonium availability the membrane-bound high-affinity ammonium permease Mep2p is already described (Lorenz & Heitman, 1998). Strains with deletions in the *MEP2* gene are unable to form pseudohyphae under ammonium starvation conditions, suggesting that Mep2p, besides its function in ammonium uptake, additionally transduces the signal to intracellular signaling pathways.

Besides nitrogen, carbon is the other nutrient crucial for pseudohyphal differentiation. It has to be fermentable like glucose, galactose or raffinose and should be available in surplus to prevent cells from sporulation. Membrane-bound or membrane-associated sensors regulating pseudohyphal development in response to the presence of fermentable carbon sources are also unknown up to now. Nevertheless, it has been reported that components of the Ras/cAMP pathway are involved in the perception of extracellular glucose concentrations (Broach, 1991). Under conditions of nitrogen starvation, activation of the small GTP-binding protein Ras2p indeed induces hyperfilamentous growth. This indicates that Ras2p may be a transmitter that regulates pseudohyphal development in response to glucose availability. A possible sensor for the Ras2p dependent glucose signal could be the membrane-bound Gpr1p/Gpa2p complex (Lorenz *et al*., 2000). Recently, a suppression of pseudohyphal development was detected in *GPR1* deletion strains grown on glucose, whereas on media containing maltose this suppression was not observed, implicating an involvement of Gpr1p in glucose perception (Lorenz *et al*., 2000). The influence of other stimuli, such as osmolarity, pH or warmth on pseudohyphal development of *S. cerevisiae* has not yet been investigated, whereas these environmental conditions are known to be important for dimorphism of pathogenic fungi like *Candida albicans* (Soll, 1997).

Haploid strains also change their growth phenotype depending on environmental stimuli. Contrary to diploid strains, it is believed that this morphological change occurs under non-starvation conditions (Banuett, 1998; Madhani & Fink, 1998). Most recent studies demonstrated that haploid invasive growth is also induced by starvation conditions suggesting glucose limitation as stimulus for the invasive growth behavior (Cullen & Sprague, 2000; Madhani, 2000). This is supported by the observation that invasive growth on agar plates containing 2 % glucose does not appear until a few days of growth, when the glucose concentration has been reduced by consumption that limitation conditions are evident.

Signaling pathways.

The regulation of pseudohyphal development is a complex process, involving at least two separate, but interconnected signaling pathways (Figure 3) (Pan *et al*., 2000; Rupp *et al*., 1999). One is the cAMP pathway, which was identified in a yeast strain showing a hyperfilamentous growth phenotype due to a dominant activated Ras2 protein

(Ras2^{Val19}p) (Gimeno *et al*., 1992). This small GTP-binding protein is known to elevate the intracellular cAMP levels by stimulating the adenylyl cyclase Cyr1p. High levels of cAMP in turn remove the inhibitory subunit Bcy1p, from one of the three catalytic subunits of proteinkinase A, Tpk1p, Tpk2p and Tpk3p, respectively (Broach, 1991). Although for pseudohyphal differentiation only Tpk2p is necessary, all three subunits are redundant for viability (Robertson & Fink, 1998). One of the Tpk2p target proteins is the transcriptional repressor Sfl1p, which negatively regulates transcription of *FLO11*, encoding a cell surface flocculin that is strictly required for flocculation and pseudohyphal growth (Lo & Dranginis, 1998). An additional target of Tpk2p is Flo8p, a transcription factor acting positively on *FLO11* transcription (Rupp *et al*., 1999). Mutants in the *FLO8* gene have been shown to be unable to form pseudohyphae. Nevertheless, not all genetic backgrounds of *S. cerevisiae* are able to form pseudohyphae, e.g. *S288C* strains are naturally defective in pseudohyphal development, due to a mutation in the *FLO8* gene that leads to the formation of an unfunctional polypeptide (Liu *et al*., 1996). Conclusively, *S288C* strains transformed with an intact *FLO8* gene regain the ability for pseudohyphal growth, suggesting a quite important role of Flo8p in pseudohyphal differentiation. Another activator of the cAMP pathway is the Gpr1/Gpa2 protein complex, which possibly senses glucose availability (see before) (Kübler *et al*., 1997; Lorenz & Heitman, 1997; Lorenz *et al*., 2000).

The other signal transduction pathway known to be involved in regulating pseudohyphal growth is the MAPK (mitogen-activated protein kinase) cascade that is also important for signaling during the mating process of haploid *S. cerevisiae* cells. Four proteins of this MAPK cascade are functional in the pheromone response as well as in pseudohyphal formation, the p65^{PAK} kinase homolog Ste20p, Ste11p (MAPKKK), Ste7p (MAPKK), and the transcription factor Ste12p (Liu *et al*., 1993). For signaling during filamentous growth, the MAPK of the pheromone response Fus3p is replaced by Kss1p (Cook *et al*., 1997; Madhani *et al*., 1997). Upon non-induced conditions, unphosphorylated Kss1p inhibits the transcription factor Ste12p via Dig1p and Dig2p, and thus prevents the activation of Ste12p-dependent pseudohyphal development (Cook *et al*., 1996). During activation of the MAPK cascade, Kss1p is phosphorylated and stimulates Ste12p, which promotes transcription of the target genes. Nevertheless, Ste12p requires an additional transcription factor for the activation of pseudohyphae inducing genes (Gavrias *et al*., 1996). This transcription factor is Tec1p. Ste12p and

Tec1p are able to bind as a heterodimer to specific *cis* sequences termed filamentation responsive elements (FREs) (Madhani & Fink, 1997). FREs are not only necessary but also sufficient to direct pseudohyphal specific gene expression. They are present in the promoter regions of at least two genes required for pseudohyphal development, *TEC1* and *FLO11*.

Upstream components that activate the MAPK cascade during pseudohyphal differentiation comprise the small GTP-binding proteins Ras2p and Cdc42p (Mösch & Fink, 1997; Mösch *et al*., 1996; Roberts *et al*., 1997). In addition to these components, the two yeast homologs of 14-3-3 proteins, Bmh1p and Bmh2p, are implicated in the filamentation MAPK cascade. These proteins appear to regulate transcriptional induction and pseudohyphal cell elongation independently of each other, and their action may be exerted by interactions with the Ste20 protein (Roberts *et al*., 1997). Interestingly, under conditions of nitrogen limitation Ras2p is involved in both pathways, the cAMP pathway as well as the MAPK cascade. A variety of other proteins are also involved in the regulation of pseudohyphal differentiation such as Phd1p and Sok2p (Gimeno & Fink, 1994; Ward *et al*., 1995). Genetic studies indicated that Phd1p activates pseudohyphal growth, whereas Sok2p seems to be an antagonist of Phd1p. Up to now it is not known, if Phd1p and Sok2p act in a linear pathway or operate on the same target.

Furthermore, proteins important for cell morphology like Bud8p and Bud9p are involved in pseudohyphal development (Taheri *et al*., 2000). These membrane-bound proteins determine cell polarity and therefore the budding pattern of the cell. But the target sequences of Bud8p and Bud9p are still unknown.

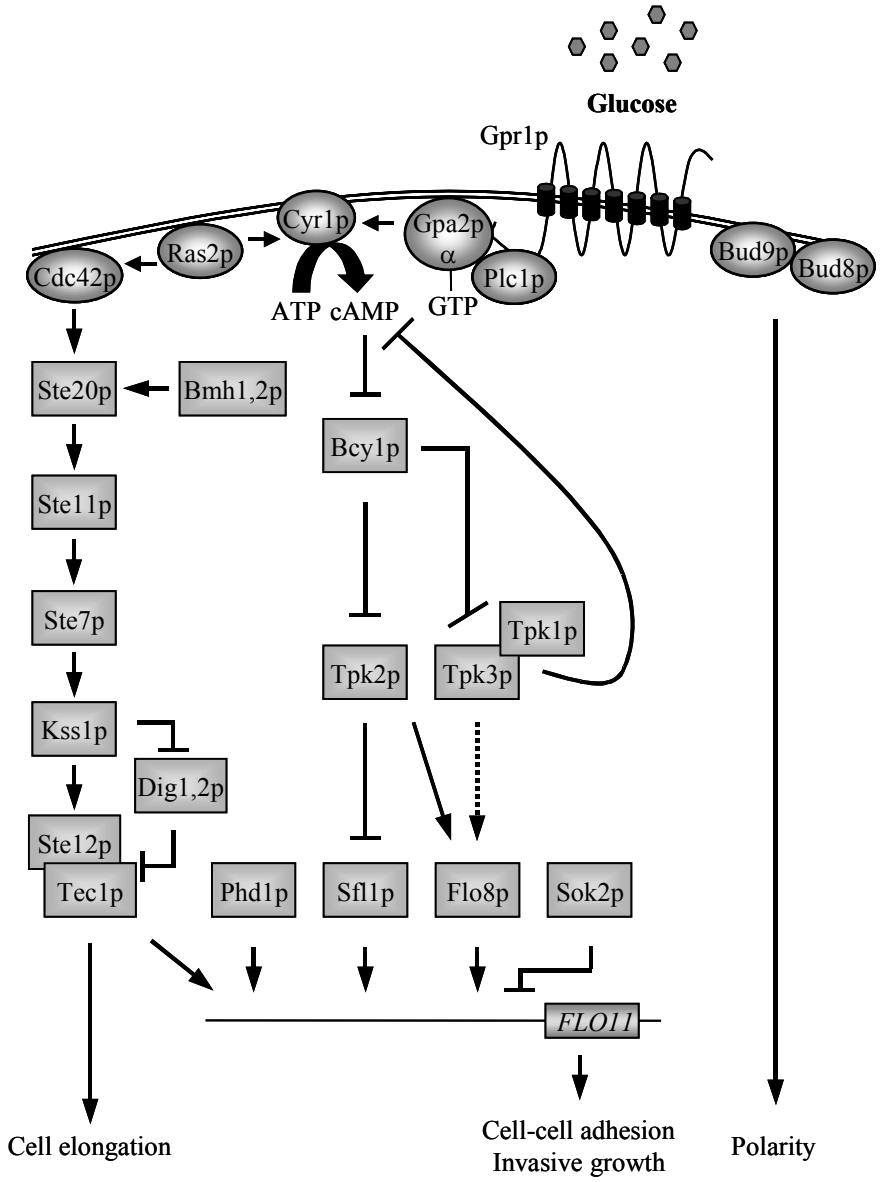

Figure 3. Model of signaling pathways regulating pseudohyphal development in *S. cerevisiae* .
See text for details.

***FLO11* is indispensable for filamentous growth.**

The *S. cerevisiae* genome contains a family of cell-wall proteins related to the adhesins of pathogenic fungi. One branch of this protein family, encoded by genes including *FLO1, FLO5, FLO9* and *FLO10,* is called the 'flocculins' (Caro *et al*., 1997), as these proteins promote cell-cell adhesion to form multicellular clumps that sediment out of solution (Teunissen & Steensma, 1995). The *FLO1, FLO5, FLO9* and *FLO10* genes share considerable sequence homology. A second group of Flo family members

has a domain structure similar to that of the first group, but with quite distinct primary sequences. This second group includes three proteins, Flo11p, Fig2p and Aga1p (Guo *et al*., 2000). Fig2p and Aga1p are induced during mating (Erdman *et al*., 1998), whereas Flo11p is required for diploid pseudohyphal formation and haploid invasive growth (Lo & Dranginis, 1998; Roberts & Fink, 1994). *FLO11* expression is controlled by the cAMP as well as the MAPK pathway, which supports the importance of regulating *FLO11* expression in response to different environmental signals (Rupp *et al*., 1999). Strains impaired in pseudohyphae formation, like *tpk2Δ*, *flo8Δ*, *ste12Δ* or *tec1Δ* null mutants, exhibit decreased *FLO11* expression levels, too.
Contrarily, deletion of inhibitors of pseudohyphal formation, like Sfl1p, results in an activation of *FLO11* expression (Robertson & Fink, 1998). Other transcription factors necessary for the regulation of pseudohyphal development also bind in the *FLO11* promoter region such as Phd1p. To make all these interactions possible, that means to integrate all these signals, the *FLO11* promoter is extraordinary large comprising up to 3000 kb, thus making it to the largest promoter known in yeast (Rupp *et al*., 1999).

Taken together, *FLO11* displays an excellent reporter gene for studying the influence of nutrients and other environmental stimuli on pseudohyphae formation. Nevertheless, the whole process of pseudohyphal development is much more complex involving the activation of several other genes. For instance, the MAPK pathway is additionally required for cell elongation, whereas the cAMP pathway modulates the transition from bipolar to unipolar budding, too.

Why studying pseudohyphal development in *Saccharomyces cerevisiae*?

S. cerevisiae is a model organism for studying regulatory mechanisms, because cells are able to grow in a haploid as well as a diploid form so that genetic studies are relative simple to carry out. Furthermore, the genome of the yeast strain *S288C* is sequenced completely and many protocols and tools for the manipulation of the organism are available, forming a good base for investigations. Additionally, many proteins and signal transduction pathways are conserved throughout yeast and higher eukaryotes, which are not so easy to handle. So, results obtained from experiments in yeast may help elucidating and understanding the function of proteins and complex pathways in higher eukaryotes much faster.

One example for the elucidation of a complex regulatory system is the pseudohyphal differentiation of the bakers' yeast *S. cerevisiae*, which has features in common with dimorphism of pathogenic fungi, in which dimorphic transition is often correlated with pathogenicity (Soll, 1997). The molecular models drawn from pseudohyphal growth in *S. cerevisiae* have turned out to be true for human and plant pathogens as well. Homologs of the G-proteins, the protein kinases and the transcription factors required for pseudohyphal signaling in *S. cerevisiae* have been found to control hyphal development of *Candida albicans, Cryptococcus neoformans* or *Ustilago maydis* (Alspaugh *et al*., 1997; Banuett & Herskowitz, 1994; Kohler & Fink, 1996). So the cAMP pathway as well as the MAPK cascade are involved in morphogenesis of these organisms, although they are activated during different environmental stimuli and have been showed to differ in the regulatory details as well as the final targets (Gancedo, 2001). But it is thought that elucidation of the regulatory pathways involved in pseudohyphal development of *S. cerevisiae* may help to understand why some fungi are opportunistic pathogens of higher organisms and *S. cerevisiae* is not.

Regulation of amino acid biosynthesis in the yeast *Saccharomyces cerevisiae.*

General control of amino acid biosynthesis.

In bacteria, starvation for any amino acid triggers the activation of the biosynthetic pathway leading to the formation of the limiting amino acid, whereas in many fungi a system is activated, which is called 'cross-pathway control' or in *S. cerevisiae*, 'general control of amino acid biosynthesis'. By its transcription factor Gcn4p the general control activates more than 50 genes involved in several different amino acid biosynthetic pathways, as well as tRNA- and purine biosynthesis (Braus, 1991; Hinnebusch, 1992). The lack of just one amino acid or an amino acid imbalance causes the derepression of the genes underlying the general control (Hinnebusch, 1992). As *S. cerevisiae* can synthesize all 20 amino acids *de novo* and as the basal transcriptional level of the amino acid biosynthetic genes is sufficient to supply the cell with all amino acids, *S. cerevisiae* is able to grow even on minimal medium without leading to the activation of the general control. For this reason in the laboratory, analogues of amino

acid precursors are used to artificially create amino acid limitation by feedback inhibition of the corresponding enzymes. The most frequently used analogues are 3-amino-triazole (3AT), a false feedback inhibitor in histidine biosynthesis (Dever, 1997; Klopotowski & Wiater, 1965) and 5-methyl-tryptophan an analogue interfering with tryptophan biosynthesis (Dever, 1997; Schürch *et al*., 1974). Alternatively, a strain carrying a 'leaky' mutation in an amino acid biosynthetic gene can be cultured on minimal medium, resulting in amino acid starvation for the respective amino acid. Both methods induce a strong response resulting in reduced growth rates or even no growth. Supplementing minimal medium with e.g. leucine results in an intracellular amino acid imbalance and thus leads to the activation of the general control without significantly affecting the growth behavior. But this induction is weaker than that triggered by the first methods.

Activation of amino acid biosynthetic genes via the final effector of the general control, Gcn4p, results in a 2- to 10-fold increased protein expression (Hinnebusch, 1992).

The reason, why *S. cerevisiae* responds to limitation for a single amino acid by increasing the transcription of several different amino acid biosynthetic genes is not obvious. One possibility is that in nature limitation for just one amino acid rarely occurs, so that the activation of more than one amino acid biosynthesis gene by the same pathway is more economical for the cell. However, the general control must be an efficient tool for the regulation of amino acid biosynthesis, because otherwise one should believe that the organism has adopted another mechanism for its regulation during evolution.

General structure of DNA binding transcription factors.

Typically, transcription factors consist of two distinct domains, which are different in function and can be physically separated from each other (Keegan *et al*., 1986; Ptashne, 1988). One of them is required for the interaction with the basic transcription machinery (activation domain), whereas the other is responsible for recognition of and binding to the target sequences (DNA binding domain). Generally, transcription factors are only active in the form of oligomeres like dimers or tetramers. The domains necessary for dimerization are usually located close to the DNA binding domain and they together possess a characteristic 3-dimensional structure (Pabo & Sauer, 1992). The majority of

prokaryotic and eukaryotic transcription factors can be classified into three different groups. Transcription factors having (I) a helix-turn-helix motif, (II) a zinc-finger motif or (III) coiled-coiled structures. The latter group also comprises factors with the so-called alkaline leucine zipper domain and helix-loop-helix motifs. All these structural elements of transcription factors consist of α-helices determining the overall structure of the protein. Furthermore, transcription factors generally contain additional regions such as phosphorylation sites and/or sites determining protein stability (e.g. PEST-regions) and cellular localization.

By which way the activation domains of the transcription factors stimulate transcription is not yet known. Probably the activation of transcription is achieved by releasing the inhibitory effect of chromatin (Dudley *et al*., 1999; Kingston *et al*., 1996), which may allow binding of the basic transcription machinery onto the DNA (Ptashne & Gann, 1997; Pugh, 1996; Stargell & Struhl, 1996).

Gcn4p is the final effector of the general control in *S. cerevisiae*.

GCN4 encodes a polypeptide comprising 281 amino acids, which is the prototype of the family of alkaline leucine zipper transcription factors (Hinnebusch, 1984; Thireos *et al*., 1984). The sixty C-terminal amino acids are sufficient for dimerization and DNA binding (Hope & Struhl, 1986). This region contains the zipper domain as well as the alkaline DNA binding domain (Figure 4). Gcn4p specifically binds as homodimer on a 9 bp palindromic nucleotide sequence (5'-ATGA(C/G)TCAT-3') called Gcn4-Protein Responsive Element (GCRE) (Arndt & Fink, 1986; Hope & Struhl, 1987; Oliphant *et al*., 1989). Recent investigations of Hollenbeck & Oakley (2000) revealed, that *in vitro* a homodimer of Gcn4p can also bind to a GCRE half site with high affinity. Additionally to the DNA binding domain, an activation domain exists, which spans approximately half of the protein (Drysdale *et al*., 1995). This activation domain extends from amino acid 17 to 144 and can be divided further into two subdomains, the N-Terminal Activation Domain (NTAD, amino acid 17 to 98) and the Central Acidic Activation Domain (CAAD, amino acid 107 to 144), which have almost identical activation potentials. In addition to mainly acidic amino acids, some hydrophobic amino acids are necessary for an efficient stimulation of transcription (Drysdale *et al*., 1995; Drysdale *et al*., 1998). As Gcn4p is a transcription factor that can activate more than 50 genes, a very strict regulation in *GCN4* expression itself is required, in order to avoid

unnecessary energy consumption by the cell. Therefore, Gcn4p contains a so-called PEST-region (Rechsteiner & Rogers, 1996), which is responsible for the instability of the protein. Whereas the half-life of Gcn4p is about 5 min under non-starvation conditions, it increases to more than 30 min upon amino acid limitation (Kornitzer *et al*., 1994). Deletion of the PEST-region consequently leads to a stabilization of the protein. Additionally, a Pho85-dependent phosphorylation on threonine 165 is necessary for ubiquitination of Gcn4p by the SCF complex finally leading to rapid protein degradation (Meimoun *et al*., 2000). Furthermore upon amino acid starvation conditions, a 2-fold increase of mRNA transcription occurs, resulting in an elevated *GCN4* expression after 3-4 h of starvation (Albrecht *et al*., 1998). Nevertheless, main regulation of *GCN4* expression is achieved by translational regulation of the *GCN4* mRNA and will be described later on.

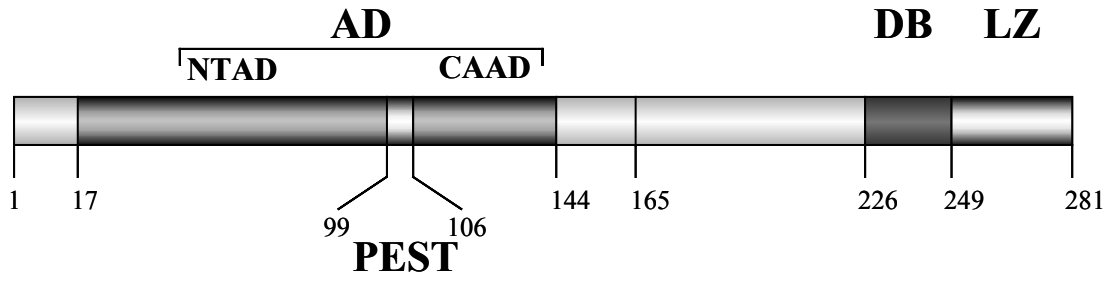

Figure 4. Schematic structure of Gcn4p.
The activation domain (AD) consists of two subdomains the N-Terminal Activation Domain (NTAD, aa 17 to 98) und the so-called Central Acidic Activation Domain (CAAD, aa 107 to 144), which mainly contain acidic and hydrophobic amino acids important for activation of Gcn4p. These two domains are separated by the PEST-region (aa 99-106), which is responsible for the instability of Gcn4p during non-starvation conditions. The C-terminal part of the protein comprises the domains necessary for DNA binding (DB, aa 226 to 249) and dimerization (LZ, Leucine Zipper, aa 249-281). The region ranging from aa 144 to 226 is postulated to contain sites of phosphorylation by which DNA binding activity might be regulated. Additionally, threonine 165 represents a site of phosphorylation for the protein kinase Pho85p which is prerequisite for rapid protein degradation. Numbers correspond to the amino acid (aa) positions in the protein.

Initiation of translation and its regulation in eukaryotes.

Translational control is an important step in regulation of gene expression in eukaryotes (Hershey *et al*., 1996). Most of the known events concern the initiation of translation. Initiation of protein biosynthesis consists of three steps leading to the

complete construction of 80S ribosomes attached to the mRNA molecule. (I) Formation of the pre-initiation complex, (II) binding of this complex to the mRNA, followed by scanning of the mRNA transcript towards the 3'-end and (III) formation of the 80S ribosome at the start codon (Pain, 1996). Several eukaryotic initiation factors (eIFs) are involved in all those steps, of which eIF-2 is the most important one. eIF-2 consists of three different subunits (α, ß and γ) in a stochiometric relation of 1:1:1. The eIF-2 activation level is regulated via a serine residue within a highly conserved phosphorylation site in the α-subunit (Clemens, 1996). The eIF-2 ß-subunit shows homologies to known zinc-finger DNA binding motifs (Donahue *et al*., 1988). As zinc-finger proteins are known to be involved in mRNA binding, it is assumed that a direct contact between eIF-2ß and the mRNA molecule is likely. The γ-subunit of eIF-2 is thought to contain a GDP/GTP binding site (Erickson *et al*., 1997). For a continuous initiation of translation it is necessary to recycle the initiation factors. Regeneration of the inactive eIF-2-GDP complex to the active eIF-2-GTP complex is performed by the guanine nucleotide exchange factor eIF-2B (Figure 5). The exchange of the nucleotide GDP by GTP allows the binding of a further initiation tRNA (Met-$tRNA_i^{Met}$) to eIF-2-GTP and this complex is competent again for a further cycle of initiation.

Regulation of translational initiation in eukaryotes is achieved by phosphorylation of eIF-2 (Figure 5). When exposed to stress or starvation conditions, eukaryotic cells react by repressing the general protein biosynthesis, usually achieved by reduction of the intracellular eIF-2-GTP concentration (Hershey *et al*., 1996). The eIF-2-GTP complex is the active form of eIF-2, because only in this form binding of a tRNA initiation molecule is possible. Hydrolysis of GTP to GDP leads to the release of an inactive eIF-2-GDP complex at the end of each initiation cycle. A specific phosphorylation of the eIF-2 α-subunit results in a higher affinity of eIF-2α to the guanine nucleotide exchange factor eIF-2B (Rowlands *et al*., 1988), which means that less molecules of eIF-2B are available for the exchange reaction of GDP to GTP. As eIF-2 is present in the cell in higher concentrations than eIF-2B (Scorsone *et al*., 1987), phosphorylation of eIF-2 affects a reduced eIF-2-GTP level, which lowers the rate of translational initiation of protein biosynthesis.

In the bakers' yeast the protein kinase Gcn2p is responsible for the phosphorylation of eIF-2 (Clemens, 1996), which happens in response to amino acid or purine limitation (Dever *et al*., 1992; Rolfes & Hinnebusch, 1993). The guanine nucleotide exchange

factor eIF-2B consists of 5 different subunits, Gcn3p, Gcd7p, Gcd2p, Gcd1p and Gcd6p. While Gcd1p and Gcd6p are responsible for the nucleotide exchange reaction, Gcn3p, Gcd7p and Gcd2p combine the regulatory function in dependence on the phosphorylation state of eIF-2α (Pavitt *et al*., 1997; Pavitt *et al*., 1998).

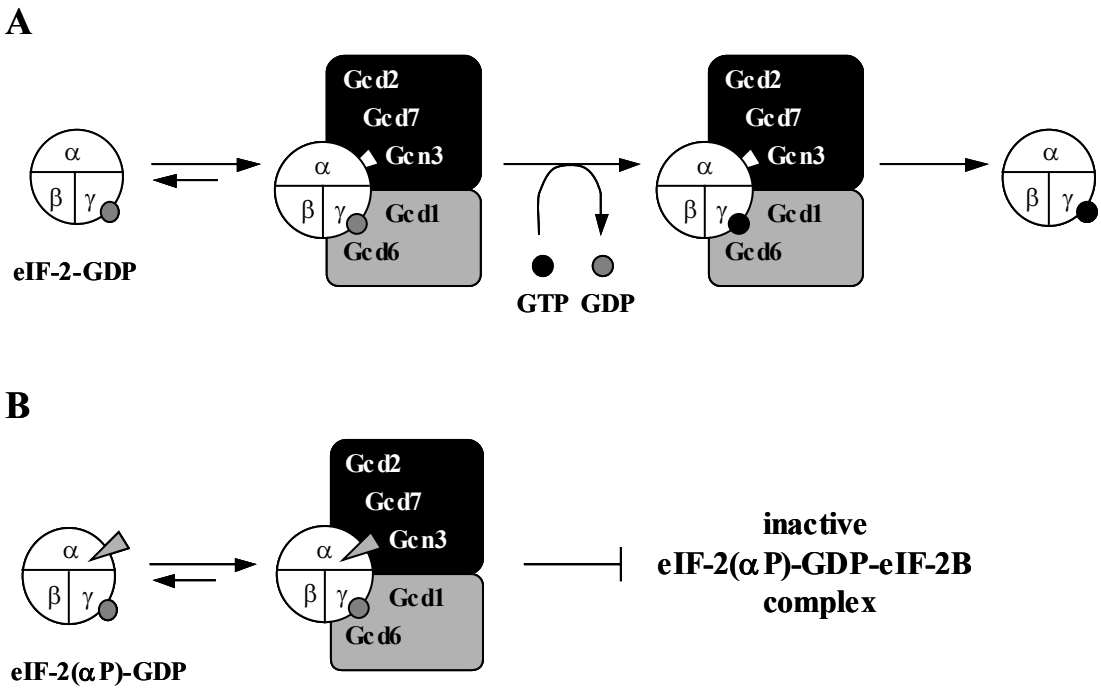

Figure 5. Model for the GDP/GTP nucleotide exchange reaction at eIF-2 catalyzed by the guanine nucleotide exchange factor eIF-2B (according to Pavitt *et al*., 1998).
The eukaryotic initiation factor eIF-2 and the guanine nucleotide exchange factor eIF-2B are depictured by a circle and two rectangles, respectively. eIF-2 consists of 3 subunits (α, ß and γ), while eIF-2B contains 5 subunits, Gcd2p, Gcd7p, Gcn3p, Gcd1p and Gcd6p. The α-subunit is the regulatory subunit of eIF-2, the regulatory subunits of eIF-2B (Gcd2p, Gcn7p and Gcn3p) are printed in black. Small circles represent GDP (gray) or GTP (black), respectively. Phosphorylation of eIF-2α is indicated by a gray triangle.

(A) GDP/GTP nucleotide exchange at non-phosphorylated eIF-2.
The primary contact between the eIF-2-GDP-complex and eIF-2B happens at the surface of the regulatory subunit complex of eIF2B (Gcd2p, Gcd7p and Gcn3p) and the α-subunit of eIF-2. A hypothetical change in the conformation of eIF-2B makes a precise contact between the catalytic subunits of eIF-2B (Gcd1p and Gcd6p) and the γ-subunit of eIF-2 possible allowing the exchange of GDP to GTP.

(B) Inhibition of the nucleotide exchange by phosphorylation of eIF-2.
A phosphorylation of the eIF-2-GTP complex at a serine residue of the α-subunit leads to the binding of this complex onto the regulatory sub-complex of eIF-2B with high affinity. This inhibits the change in conformation of eIF-2B and the nucleotide exchange is retarded. For this reason less eIF-2-GTP is available in the cell leading to a retardation of the reinitiation of the ribosome.

Translational control of *GCN4* expression.

In the yeast *S. cerevisiae* as well as in higher eukaryotes protein biosynthesis is inhibited during starvation or stress conditions. Thus nutrient resources are saved and the cell division process is slowed down. The reduction of the protein biosynthesis rate is achieved by phosphorylation of eIF-2. Under amino acid starvation conditions the sensor kinase of the general control, Gcn2p, senses the accumulation of uncharged tRNAs in the cell (Lanker *et al*., 1992) (Figure 6). The kinase domain of Gcn2p then phosphorylates eIF-2α (Wek *et al*., 1995; Zhu *et al*., 1996), leading to a decreased eIF-2-GTP concentration in the cell (see before). The phosphorylation of eIF-2 leads to an improved translation of *GCN4* mRNA in *S. cerevisiae* under conditions where translation of other transcripts is usually reduced. This contradictory response is mediated by the extraordinarily large untranslated 5'-region of *GCN4* mRNA that comprises 4 small upstream open reading frames (uORFs). These 4 uORFs affect an improved translation efficiency under amino acid limitation conditions.

At the onset of translation initiation the small ribosomal subunit binds to the 5'-end of the *GCN4* mRNA and forms, together with eIF-2α-GTP and the initiation tRNA (Met-$tRNA_i^{Met}$), a competent initiation complex at the first AUG-codon that is reached while scanning the mRNA. During initiation at the first uORF, eIF-2-bound GTP is hydrolyzed to GDP. The ribosome dissociates after reaching the stop codon, leaving about half of the small subunits attached to the mRNA transcript. For a further initiation the eIF-2-GDP complex has to become recycled to an eIF-2-GTP complex, otherwise being unable to reinitiate. Under non-starvation conditions the exchange of GDP to GTP by the guanine nucleotide exchange factor eIF-2B happens before the small subunit of the ribosome reaches the start codon of the fourth uORF (Figure 6A) resulting in translation of this uORF. After translation the ribosome dissociates again, so that the coding sequence of *GCN4* will not be translated (Hinnebusch, 1997). Consequently, under non-starvation conditions the four uORFs block efficient translation of *GCN4* mRNA.

Contrarily, upon amino acid starvation conditions, uncharged tRNAs accumulate in the cell and stimulate the sensor kinase Gcn2p to phosphorylate the α-subunit of eIF-2-GDP. This phosphorylation inhibits eIF-2B, so that eIF-2-GDP is not efficiently recycled to eIF-2-GTP resulting in a low intracellular eIF-2-GTP level. Therefore, the time required for the reconstitution of 40S subunits is prolonged. The 40S subunits,

which remained attached to the mRNA transcript after translation of uORFI move further downstream and are not able to reinitiate at uORFIV. Instead of translation of uORFIV, reinitiation at the start codon of the *GCN4* ORF takes place and, consequently, expression of *GCN4* (Figure 6B).

The destruction of all four uORFs results in a high Gcn4p expression under non-amino acid starvation conditions. Studies of Mueller and Hinnebusch (1986) suggested that especially the first and the fourth uORF are necessary for translational control, as deletion of the second or/and the third uORF had only a weak effect on *GCN4* mRNA translation. Why *S. cerevisiae*, nevertheless, possesses four uORFs on its *GCN4* mRNA is still unknown.

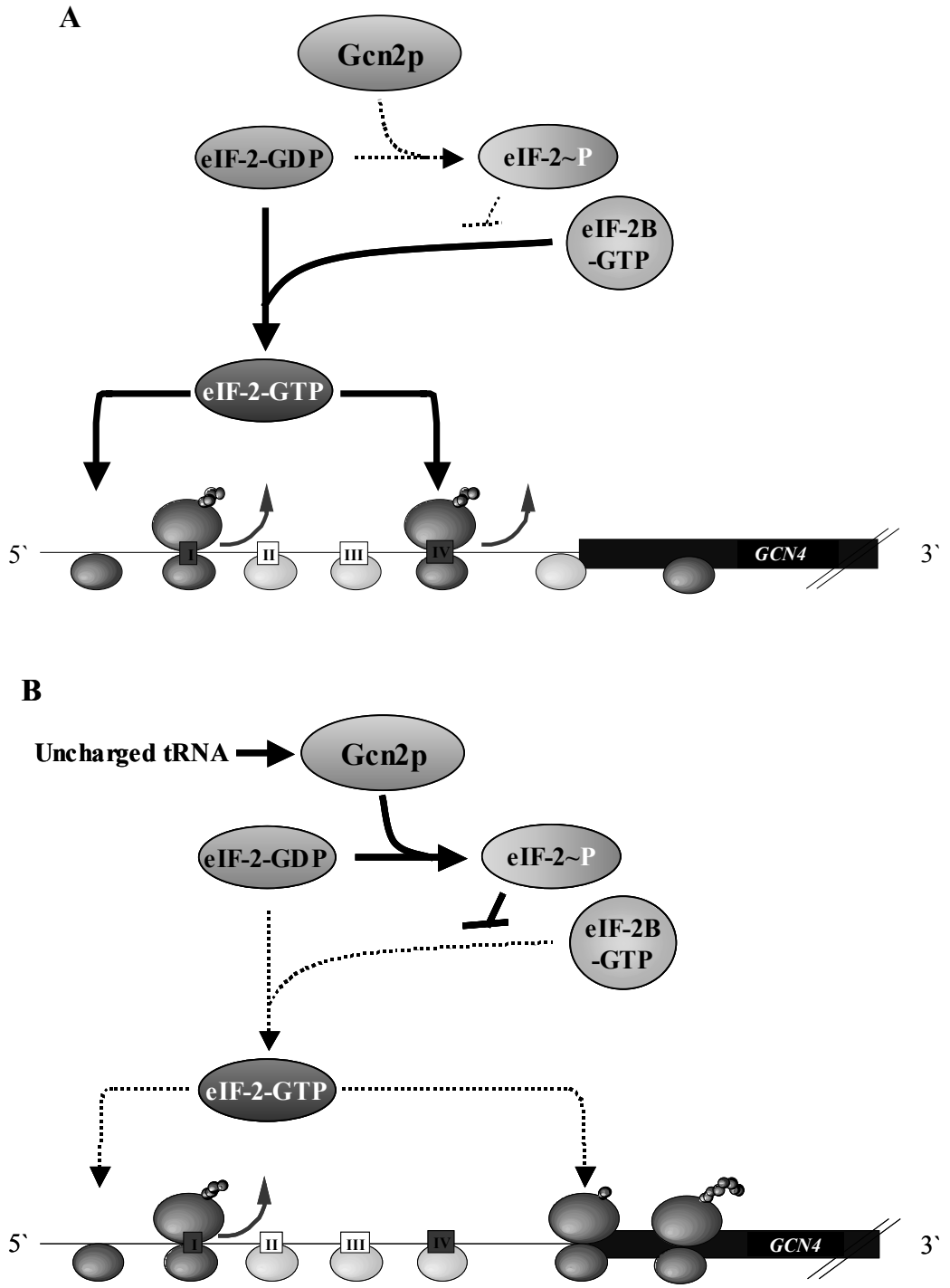

Figure 6. Postulated mechanism for translation regulation of *GCN4* mRNA during (A) non-starvation conditions and (B) amino acid starvation.
A *GCN4* mRNA transcript containing the four small uORFs upstream of the *GCN4* ORF is drawn schematically. The small ellipses represent the 40S subunits of the ribosome, the bigger ones the 60S subunits, forming together an 80S ribosome. The 40S subunits are printed in dark gray when they are competent for initiation, otherwise they are printed in light gray. 40S subunits are competent for initiation when they are associated with eIF-2-GTP and an initiation tRNA (Met-tRNA$_i^{Met}$).

(A) During non-starvation conditions, the eIF-2-GDP complex is efficiently recycled to eIF-2-GTP by the guanine nucleotide exchange factor eIF-2B (represented by the bold arrows) leading to a high level of eIF-2-GTP in the cell. Consequently, a competent 40S initiation complex can be produced, which forms an 80S ribosome when it reaches the start codon of uORFI, and protein biosynthesis starts. During this process GTP is hydrolyzed to GDP. By reaching the stop codon of uORFI the 80S ribosome dissociates, leaving about half of the 40S subunits attached to the mRNA transcript (light gray ellipse). The non-competent 40S subunits move downstream, but when reaching uORFII and III they are not able to reinitiate. For this reason these uORFs are not translated. The exchange of GDP by GTP by the guanine nucleotide exchange factor eIF-2B makes a reinitiation at the start codon of uORFIV possible. After translation of this ORF the ribosome dissociates again. So the ORF of *GCN4* will not be translated as no competent 40S subunits are available.

(B) Uncharged tRNAs accumulate in the cell during amino acid starvation, which interact with the histidyl-tRNA synthetase domain of the sensor kinase Gcn2p (bold arrow). This stimulates the kinase activity of Gcn2p leading to the phosphorylation of the α-subunit of eIF-2-GDP (bold arrow). Phosphorylation of eIF-2-GDP inhibits the guanine nucleotide exchange factor eIF-2B, so that eIF-2-GDP is not efficiently recycled to eIF-2-GTP (dotted arrow). Consequently, a low level of eIF-2-GTP is present in the cell, which means that the reconstitution rate of the 40S subunits is reduced. The 40S subunits, which remain attached to the mRNA transcript after the translation of uORFI, move further downstream. When they reach uORFIV they are not able to reinitiate, so that this uORF is not translated. Therefore, reinitiation happens at the start codon of *GCN4*, which leads to the expression of *GCN4*.

Aim of this work.

Upon amino acid starvation conditions, expression of the transcription factor of the general control of amino acid biosynthesis, Gcn4p is activated, leading to elevated expression of amino acid biosynthetic genes in *S. cerevisiae*. As amino acid metabolism is part of the general nitrogen metabolism of the cell, the aim of this work was to elucidate the effect of nitrogen starvation on the activity/expression of Gcn4p. When the cell is unable to produce amino acids due to a lack of nitrogen, by which way does Gcn4p regulate the amino acid biosynthesis? Another interesting question was, how the cell perceives that it is waste of energy to start the production of amino acids during nitrogen limitation.

On the other hand, yeast cells switch their morphology during nitrogen starvation conditions and form thin, elongated cells called pseudohyphae. As nitrogen starvation also generates amino acid starvation conditions, it should be investigated whether the transcription factor Gcn4p is involved in triggering pseudohyphal development and, if it is so, by which means Gcn4p influences this process. Furthermore, the identification of a Gcn4p-dependent activation of the transcription/expression of the cell-wall-associated flocculin Flo11p during nitrogen starvation in the second part of this thesis was the reason for the decision to perform genome-wide transcriptome analysis of a wild type in comparison to a *gcn4*Δ strain, in order to identify further genes regulated by Gcn4p.

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Chapter II

Repression of *GCN4* mRNA translation by nitrogen starvation in *S. cerevisiae*

SUMMARY

Saccharomyces cerevisiae activates a regulatory network called 'general control' that provides the cell with sufficient amounts of protein precursors during amino acid starvation. We investigated how starvation for nitrogen affects the 'general control' regulatory system, because amino acid biosynthesis is part of nitrogen metabolism. Amino acid limitation results in the synthesis of the central transcription factor Gcn4p, which binds to specific DNA binding motif sequences called Gcn4-protein responsive elements (GCREs) that are present in the promoter regions of its target genes. Nitrogen starvation increases *GCN4* transcription, but efficiently represses expression of both a synthetic *GCRE6::lacZ* reporter gene and the natural amino acid biosynthetic gene *ARO4*. Repression of Gcn4p-regulated transcription by nitrogen starvation is independent of the ammonium sensing systems that include Mep2p and Gpa2p or Ure2p and Gln3p, but depends on the four uORFs in the *GCN4* mRNA leader sequence. Efficient translation of *GCN4* mRNA is completely blocked by nitrogen starvation, even when cells are simultaneously starved for amino acids and eIF-2α is fully phosphorylated by Gcn2p. Our data suggest that nitrogen starvation regulates translation of *GCN4* by a novel mechanism that involves the four uORFs, but that still acts independently of eIF-2α phosphorylation by Gcn2p.

INTRODUCTION

In baker's yeast *Saccharomyces cerevisiae*, starvation for a single amino acid induces the transcription of more than 50 genes encoding enzymes involved in several amino acid biosynthetic pathways, amino acid tRNA synthetases (Meussdoerffer & Fink, 1983; Mirande & Waller, 1988) or enzymes of purine biosynthesis (Mösch *et al*., 1991). This genetic system is called ´general control of amino acid biosynthesis´ (Hinnebusch, 1992). Amino acid limitation results in the synthesis of the transcription factor Gcn4p (Ellenberger *et al*., 1992), which binds to specific Gcn4-protein responsive elements (GCRE) present in the promoter regions of its target genes. Gcn4p stimulates transcription of its target genes by a factor of two to ten (Hope & Struhl, 1986). Whereas some target genes are regulated via several GCREs in their promoter regions like e.g. *HIS3* or *TRP4* (Braus *et al*., 1989; Hill *et al*., 1986; Hope & Struhl, 1985; Mösch *et al*., 1990), others contain only a single GCRE site that is both essential and sufficient for regulation by Gcn4p as found for the *ARO4* gene (Künzler *et al*., 1992).

The best understood regulatory mechanism of Gcn4p expression is translational control of its mRNA, which requires four small upstream open reading frames (uORFs) present in the *GCN4* 5´-untranslated region (Hinnebusch, 1984; Thireos *et al*., 1984). When cells are growing under non-starvation conditions, ribosomes translate the first uORF, reinitiate at uORF 2 to 4 and are unable to recognize the *GCN4* start codon (Hinnebusch, 1997). In cells starved for amino acids, ribosomes that have translated the first uORF reinitiate at the *GCN4* translational start site leading to high expression of Gcn4p. Inactivation of the four uORFs in the GCN4 leader by either deletion or mutation of the four ATG start codons uncouples translational control of *GCN4* from the general control system, leading to high expression of Gcn4p already under non-starvation conditions (Mueller & Hinnebusch, 1986). Translational regulation of *GCN4* depends on the sensor kinase Gcn2p, which includes an N-terminal protein kinase domain and a C-terminal amino acyl tRNA synthetase like domain (Roussou *et al*., 1988; Wek *et al*., 1989). Under amino acid starvation conditions, Gcn2p detects uncharged tRNAs and in response phosphorylates the α-subunit of eIF-2 (eukaryotic initiation factor 2). Phosphorylated eIF-2 inhibits translation of most mRNAs mediated by the inhibition of the guanine nucleotide exchange activity of eIF-2B. This allows

ribosomes to scan past the remaining uORFs in the *GCN4* 5'-untranslated region and translate the mRNA of the *GCN4* gene (Dever *et al*., 1992; Wek *et al*., 1995).

Amino acid biosynthesis is part of the general nitrogen metabolism of yeast cells. Ammonium is among the inorganic nitrogen sources in nature that support optimal growth of yeast cells (Wiame *et al*., 1985). Three ammonium permeases - Mep1p, Mep2p and Mep3p - are known that control uptake of ammonium into the cytoplasm (Marini *et al*., 1997). Deletion of all three *MEP* genes renders yeast cells inviable on media containing less than 5 mM ammonium sulfate as the sole nitrogen source. In addition to its functions as ammonium permease, Mep2p is thought to act as an ammonium sensor protein in a signaling system that controls pseudohyphal development of diploid *Σ1278b* strains, because diploid *mep2Δ* mutant strains are unable to form pseudohyphae in response to nitrogen starvation. Pseudohyphal development of *mep2Δ* mutants can be restored by expression of dominant activated forms of Gpa2p or Ras2p, GTP-binding proteins that both regulate intracellular cAMP levels (Kübler *et al*., 1997; Lorenz & Heitman, 1997). These studies have led to the model that Mep2p is a central ammonium sensor protein that activates Gpa2p and cAMP-dependent protein kinase in response to nitrogen starvation. The proteins Ure2p and Gln3p are two additional important regulators in the ammonium utilization pathway and are required under all conditions (ter Schure *et al*., 2000).

As amino acid biosynthesis is part of the general nitrogen metabolism in yeast, we investigated how starvation for nitrogen affects the general control system of amino acids. We found that deprivation of a suitable nitrogen source efficiently counteracts the activation of Gcn4p-controlled gene expression. Expression of a synthetic *GCRE6::lacZ* reporter gene and natural amino acid biosynthetic genes as e.g. *ARO4* are suppressed when cells are starved for nitrogen, whereas starvation for glucose has only minor effects on Gcn4p-controlled gene expression. We find *GCRE*-mediated repression although the mRNA level of *GCN4* strongly increases, suggesting that nitrogen starvation specifically suppress translation of *GCN4*. The four intact uORFs in the *GCN4* mRNA leader sequence are necessary components for the repression mechanism, whereas eIF-2α phosphorylation, the ammonium sensing system that includes Mep2p or Gpa2p, and the proteins Ure2p or Gln3p are not required.

Materials & Methods

Yeast Strains and Growth Conditions.

All strains used in this study are derivatives of the *S. cerevisiae* strain background *Σ1278b* with exception of RH1376, RH2407, RH2446 and RH2520, which derive from the *S288c* background (Table 1). Deletion mutants for *GCN2* (*gcn2Δ*) were obtained by using the *gcn2Δ* deletion plasmids pME1658 or pME1659, respectively (Table 2). Plasmids pME1105 and pME1660 were used for constructing *gcn4Δ* and *gpa2Δ* mutant strains. *Aro3Δ* and *aro4Δ* mutants were obtained by using plasmids pME1756 and pME1757. *Aro4Δ* strains were transformed with a linear fragment carrying the *ARO4m* mutant gene that was excised from plasmid pME643 to construct *ARO4m*-strains. *Mep1Δ* and the *mep2Δ* mutant strains were gifts from G. R. Fink (Whitehead Institute, Cambridge, Massachusetts). The plasmids pME1911 and pME1912 were used to delete the genes *URE2* and *GLN3*, respectively. *Ras2Δ* mutants were constructed using plasmid pras2*Δ*::HIS3 (Mösch *et al*., 1999). A *gcn3Δ* strain, GP3153 (Pavitt *et al*., 1997), was kindly provided by A. G. Hinnebusch (National Institute of Child Health and Human Development, Bethesda, Maryland). The *GCRE6*::*lacZ*::*URA3* reporter gene cassette was introduced by transformation with the integrative *GCRE6*::*lacZ* reporter construct pME1112. Transformations were carried out using the lithium-acetate yeast transformation method (Ito *et al*., 1983). All gene deletions, integrations or replacements were confirmed by Southern blot analysis (Southern, 1975).

For log-phase measurements (non-starvation conditions), strains were cultivated in liquid synthetic minimal medium (SD) overnight at 30 °C, diluted and cultivated for 8 h before assaying enzymatic activities or isolation of total RNA. For amino acid starvation, 3-amino-triazole (3AT) was added to cultures grown to mid-log phase to a final concentration of 10 mM and cells were further incubated for 6 – 8 h before all assays. For nitrogen starvation, cells grown to mid-log phase were washed with 2 % glucose and incubated for 24 h in liquid SD medium containing only 50 µM ammonium sulfate (instead of 50 mM) as the sole nitrogen source. Carbon starvation was measured by incubation in medium with only 0.05 % glucose for 8 h.

Plasmids.

All plasmids used in this study are listed in Table 2. The Kanamycin resistant cassette (*kan*^{r}) was amplified by PCR from plasmid pUG6 (Guldener *et al*., 1996) using the two primers UG6-1 (5'- CGCGGATCCGAACGCGGCCGCCAGCTGAAGC -3') and UG6-2 (5'- CGCGGATCCCGCATAGGCCACTAGTGGATCTG -3') and subsequent insertion of the PCR product into plasmid pBluescriptKS$^+$ (Stratagene) to obtain plasmid pME1765. Deletion cassettes for *GCN2* were created by replacement of *GCN2* coding sequences by either *LEU2* (pME1658) or *kan*^{r} (pME1659) as selectable markers. To obtain plasmids pME1660, pME1756, pME1911 or pME1912, *GPA2*, *ARO3*, *URE2* or *GLN3* open reading frames were replaced by the *kan*^{r} gene as selectable marker. Plasmid pME2157 was constructed by homologous recombination in yeast using p180 and a linear *ura3::TRP1 URA3*-disruption cassette (provided by Yona Kassir, Technion, Haifa, Israel).

Northern Hybridization Analysis.

Total RNA from yeast was isolated following the protocol described by Cross and Tinkelenberg (Cross & Tinkelenberg, 1991). RNA was separated on 1.4 % agarose gel containing 3 % formaldehyde and transferred onto nylon membranes by electroblotting. Gene specific probes were ^{32}P-radiolabeled with the MBI Fermentas HexaLable™ DNA Labelling Kit. Hybridizing signals were quantified using a BAS-1500 Phosphor-Imaging scanner (Fuji).

ß-Galactosidase Assay.

Assays were performed with extracts of cultures grown on liquid media. Specific ß-galactosidase activity was normalized to the total protein (Bradford, 1976) in each extract and equalized (OD_{415} x 1.7) / (0.0045 x protein concentration x extract volume x time) (Rose & Botstein, 1983). Assays were performed for at least three independent transformants, and the mean value is presented. The standard errors of the means were below 15 %.

Western Hybridization Analysis.

Strains were cultivated as described above. Crude protein extracts were prepared in the presence of a protease inhibitory mix (Drysdale *et al*., 1995). Routinely, 10 µg of crude protein extracts were separated on a 12 % polyacrylamide gel, and proteins were transferred onto a nitrocellulose membrane by electroblotting. Gcn4p was visualized using ECL technology (Amersham) after incubation of membranes with a polyclonal rabbit anti-Gcn4p antibody (Albrecht *et al*., 1998; Hoffmann *et al*., 1999) and a peroxidase-coupled goat anti-rabbit IgG secondary antibody. Expression of Aro7p was used as internal standard in all measurements and Aro7p was visualized using a specific anti-Aro7p antiserum (Hoffmann *et al*., 1999; Schnappauf *et al*., 1997). Gcn4p and Aro7p signals were quantified using the Molecular Analyst software (Bio-Rad) as described previously (1). The Gcn2p, eIF-2α and eIF-2α~P Western analyses were carried out using polyclonal antibodies that specifically recognize Gcn2p, eIF-2α (both kindly provided by Alan Hinnebusch (Romano *et al*., 1998)) or the phosphorylated form of eIF-2α, eIF-2α~P (BIOSOURCE INTERNATIONAL, Camarillo, USA). The eIF-2α and eIF-2α~P Western were quantified by using a Image Station 440CF (Kodak, New Heaven, USA) for detecting luminescence of ECL technology and Kodak 1D Image Analysis Software (Kodak, New Heaven, USA) for quantification of the obtained signals.

Gel Retardation Assay.

Gel retardation assays using crude yeast extracts or Gcn4p purified from *E. coli* were performed as described (Braus *et al*., 1989). As DNA probe a ^{32}P-labeled synthetic GCRE-fragment was used that was obtained by annealing of two synthetic oligonucleotides GCRE-1A (5'-GATCTGCTCGAGATGACTCATTTTTTGATCAA TT-3') and GCRE-1B: (5'-TTGATCAAAAAATGAGTCATCTCGAGCAGATCTT-3'). Essentially, 10 µg of crude protein extract were mixed with 10 fmole of ^{32}P-radiolabeled probe, separated on a native 6 % polyacrylamide gel, and visualized by autoradiography. Protein-DNA complexes were quantified using a BAS-1500 Phosphor-Imaging scanner (Fuji).

TABLE 1. Yeast strains used

Strain	Genotype	Source
RH2396	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG*	This study
RH2397	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 leu2::hisG/leu2::hisG his3::hisG/HIS3 trp1::hisG/trp1::hisG gcn2Δ::LEU2/gcn2Δ::LEU2*	This study
RH2398	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 leu2::hisG/leu2::hisG his3::hisG/HIS3 trp1::hisG/trp1::hisG gcn4Δ::LEU2/gcn4Δ::LEU2*	This study
RH2399	*MAT***a**/α *ura3-52/ura3-52 trp1::hisG/trp1::hisG*	This study
RH2400	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 leu2::hisG/LEU2 his3::hisG/his3::hisG trp1::hisG/trp1::hisG ras2Δ::ura3::HIS3/ras2Δ::URA3*	This study
RH2401	*MAT***a**/α *ura3-52/ura3-52 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG gcn2Δ::LEU2/gcn2Δ::LEU2*	This study
RH2402	*MAT***a**/α *ura3-52/ura3-52 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG gcn4Δ::LEU2/gcn4Δ::LEU2*	This study
RH2403	*MAT***a**/α *ura3-52 /ura3-52 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG gcn4Δ::LEU2/gcn4Δ::LEU2 gcn2Δ::kanR/gcn2Δ::kanR*	This study
RH2404	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG gpa2Δ::kanR/gpa2Δ::kanR*	This study
RH2405	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/his3::hisG leu2::hisG/leu2::hisG mep1Δ::HIS3/mep1Δ::LEU2*	This study
RH2406	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/his3::hisG leu2::hisG/leu2::hisG mep2Δ::HIS3/mep2Δ::LEU2*	This study
RH2407	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3* (*S288c*-background)	This study
RH2445	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/his3::hisG leu2::hisG/leu2::hisG mep1Δ::HIS3/mep1Δ::HIS3 mep2Δ::LEU2/mep2Δ::LEU2*	This study
RH2446	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 ADE2/ade2-101 TRP1/trp1-Δ901 SUC2/suc2-Δ9 LEU2/leu2-3 leu2-112 gcn4Δ/gcn4-101* (*S288c*-background)	This study
RH2485	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG aro3Δ::kanR/aro3Δ::kanR*	This study
RH2486	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG aro3Δ::kanR/aro3Δ::kanR ARO4m/ARO4m*	This study

RH2489	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG ure2Δ::kanR/ure2Δ::kanR*	This study
RH2490	*MAT***a**/α *ura3-52/ura3-52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG gln3Δ::kanR/gln3Δ::kanR*	This study
GP3153	*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 gcn3Δ::LEU2*	(Pavitt *et al*., 1997)
RH2520	*MAT***a** *ura3-52* (*S288c*-background)	This study

TABLE 2. Plasmids used

Plasmid	Description	Source
pME1105	Cassette for full deletion of *GCN4*-open reading frame (*LEU2*)	(Albrecht *et al*., 1998)
pME1658	Cassette for full deletion of *GCN2*-open reading frame (*LEU2*)	This study
pME1659	Cassette for full deletion of *GCN2*-open reading frame (*kan*^{r})	This study
pME1660	Cassette for full deletion of *GPA2*-open reading frame (*kan*^{r})	This study
pME1756	Cassette for full deletion of *ARO3*-open reading frame (*kan*^{r})	This study
pME1757	Cassette for full deletion of *ARO4*-open reading frame (*LEU2*)	G. H. Braus
pME1911	Cassette for full deletion of *URE2*-open reading frame (*kan*^{r})	This study
pME1912	Cassette for full deletion of *GLN3*-open reading frame (*kan*^{r})	This study
pras2Δ::URA3	Cassette for full deletion of *RAS2*-open reading frame (*URA3*)	(Mösch *et al*., 1999)
pME1765	pBluescriptKS$^+$ containing *kan*^{r}-cassette	This study
pME643	Integration-cassette for *ARO4m*	(Künzler *et al*., 1992)
pRS314	*TRP1*-marked centromere vector	(Sikorski & Hieter, 1989)
pME1092	2.8-kb fragment containing *GCN4* in pRS314	(Albrecht *et al*., 1998)
pME1098	2.8-kb fragment containing *GCN4m* in pRS314	(Albrecht *et al*., 1998)
pME1108	Integrative *ΔUAS*::*lacZ* reporter construct	(Albrecht *et al*., 1998)
pME1112	Integrative *GCRE6*::*lacZ* reporter construct	(Albrecht *et al*., 1998)
p180	*GCN4*::*lacZ* reporter construct on centromere vector (*URA3*)	(Hinnebusch, 1985)

pME2157	*GCN4*::*lacZ* reporter construct on centromere vector (*TRP1*)	This study
p227	*GCN4m*::*lacZ* reporter construct on centromere vector	(Mueller & Hinnebusch, 1986)
B2389	*ura3::TRP1* marker exchange construct	Yona Kassir
Ep69	*GCN3* on centromere vector	(Hannig & Hinnebusch, 1988)
p2304	*GCN3* on 2μm vector	(Yang & Hinnebusch, 1996)

RESULTS

Gcn4p-dependent reporter gene expression is repressed by nitrogen starvation.

We examined the effects of nitrogen starvation on expression of a Gcn4p-specific reporter gene *GCRE6*::*lacZ* that contains six GCRE-binding sites for Gcn4p in front of a *CYC1*::*lacZ* minimal promoter. Efficient expression of *GCRE6*::*lacZ* requires the presence of Gcn4p and accurately reflects the Gcn4p-transcriptional activity in the cell (Albrecht *et al.*, 1998). Two different genetic backgrounds of *S. cerevisiae*, *S288c* and *Σ1278b*, were chosen for these measurements. The *Σ1278b* background is ideal for measuring the effects of nitrogen starvation, because *Σ1278b* strains are highly responsive to changes in the ammonium concentrations in the environment. However, most studies addressing Gcn4p-activity and regulation by the general control system in the past were performed using the *S288c* background. Thus, the *GCRE6*::*lacZ* gene was integrated into the *URA3* locus of wild-type control and *gcn4Δ* mutant strains of both genetic backgrounds. ß-galactosidase activities of resulting strains were determined under three different growth conditions, mid-log phase (no starvation), starvation for amino acids by addition of the histidine analogue 3AT and starvation for nitrogen by 1000-fold reduction of the ammonium sulfate concentration (Figure 1). We found that under non-starvation conditions (mid-log phase) expression of the *GCRE6*::*lacZ* reporter was approximately 4-fold higher in the *Σ1278b* than in the *S288c* strain. However, in the absence of Gcn4p (in a *gcn4Δ* mutant) or under conditions of amino acid starvation when the general control system is fully activated, expression of the *GCRE6*::*lacZ* gene was almost identical in both genetic backgrounds. Thus, the maxima and minima of the measured values representing the maximal Gcn4p dependent gene expression and the Gcn4p independent basal expression are identical. However, the basal activity of Gcn4p under non-starvation conditions is at a significantly higher level in the *Σ1278b* background. This is in agreement with a recent study reporting a higher basal Gcn4p-activity in a *Σ1278b* strain when compared to a strain, which carries the SP1 background (Stanhill *et al*., 1999). When cells were starved for nitrogen, however, GCRE-driven gene expression was drastically reduced in strains with both genetic backgrounds. Expression of the *GCRE6::lacZ* gene was decreased 11-fold in *Σ1278b* and 3.3-fold in the *S288c* strains, respectively. The absolute amounts of ß-galactosidase

activities measured under these conditions were similar with 25 units in the *Σ1278b* or 22 units in the *S288c* strain. In the absence of *GCN4*, expression of *GCRE6*::*lacZ* was not further reduced by nitrogen starvation.

Results in this section show that Gcn4p-dependent gene expression is repressed by nitrogen starvation in different genetic backgrounds.

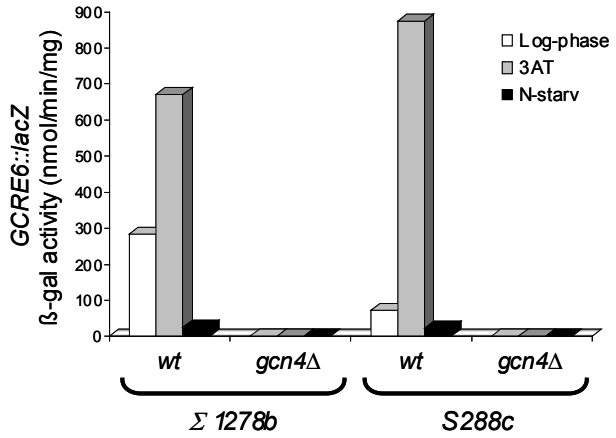
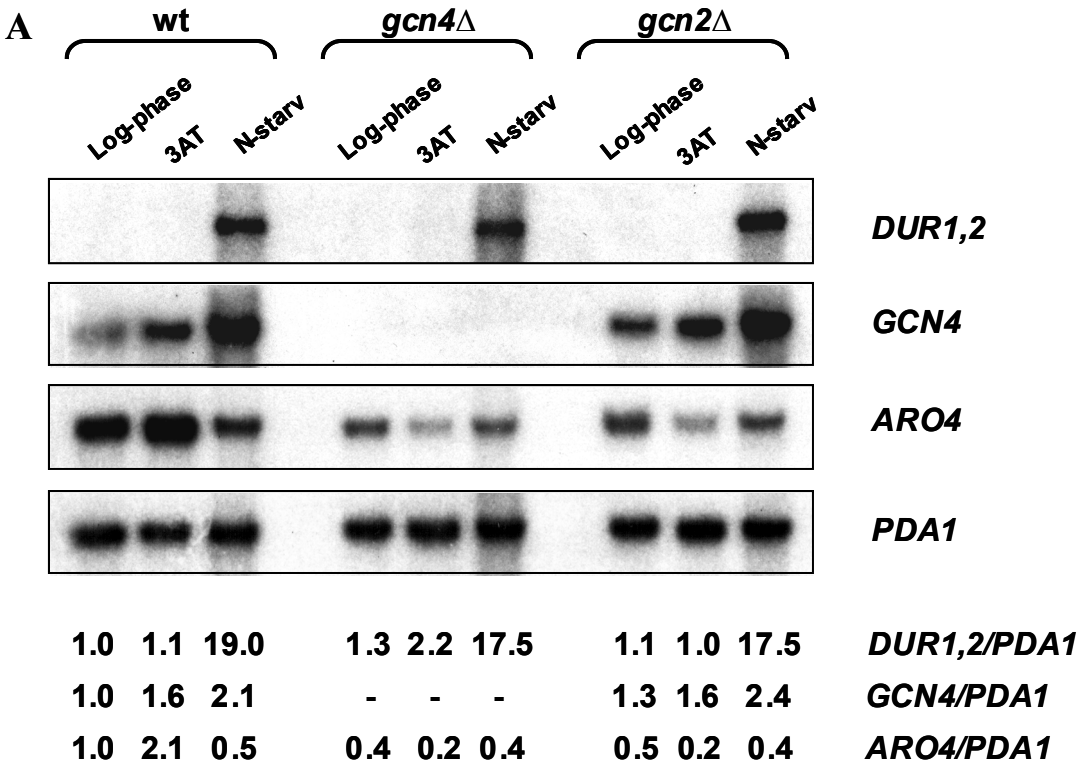


Figure 1. Gcn4p-dependent gene expression is repressed by nitrogen starvation. Expression of the *GCRE6::lacZ* reporter gene was measured in *Σ1278b* yeast strains RH2396 (wt) and RH2398 (*gcn4Δ*) and in *S288c* strains RH2407 (wt) and RH2446 (*gcn4Δ*) under different nutritional conditions. Cultures grown to mid-log phase (Log-phase) were used for assaying non-starvation conditions. Amino acid starvation was induced by addition of 3-amino-triazole (3AT) to 10 mM and nitrogen starvation (N-starv) by growth on minimal medium containing 50 µM ammonium sulfate as the sole nitrogen source. Strains RH2396 and RH2398 were carrying the *TRP1* centromeric plasmid (pRS314) to obtain Trp+ prototrophy. ß-Galactosidase activities are given in nanomoles per minute per milligram. Bars depict means of at least three independent measurements with a standard deviation not exceeding 15 %.

Nitrogen starvation represses Gcn4p-dependent transcription of the amino acid biosynthetic *ARO4* gene.

We next examined the effects of nitrogen starvation on expression of the general control regulated amino acid biosynthetic gene *ARO4* and of *GCN4* itself. For this purpose, steady-state mRNA levels of *ARO4* and *GCN4* were measured under different nutritional conditions and compared to expression of two control genes *DUR1,2* and *PDA1*. Whereas *ARO4* and *GCN4* are genes known to be regulated by amino acid starvation, *DUR1,2* was used as a control gene that is strongly up-regulated by nitrogen starvation (Erdman *et al*., 1998). *PDA1* was chosen as a control gene that is unaffected by starvation conditions (Wenzel *et al*., 1995). Total RNAs of a control strain or of

gcn2Δ and *gcn4Δ* mutant strains which had either been grown to mid-log phase (no starvation) or which had been starved for amino acids or for nitrogen, were isolated and expression of *DUR1,2*, *GCN4*, *ARO4* and *PDA1* mRNA was monitored by quantitative Northern hybridization analysis (Figure 2A). Amino acid starvation induced by addition of 3AT increased mRNA levels of *ARO4* by a factor of 2.1 when compared to *PDA1*. No induction of *ARO4* by 3AT was observed in the two general control mutant strains *gcn2Δ* or *gcn4Δ*, respectively. As observed previously, a 1.6-fold increase in the *GCN4* transcript levels was detected under these conditions (Albrecht *et al.*, 1998). In contrast, transcription of the amino acid catabolic gene *DUR1,2* was not affected by the addition of 3AT. Thus, starvation for amino acids stimulates transcription of genes required for amino acid biosynthesis, whereas amino acid catabolic genes do not appear to be affected.



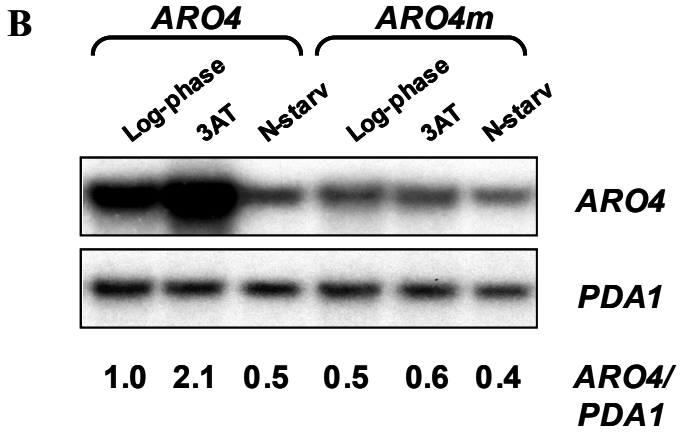


Figure 2. Gcn4p-dependent but not basal expression of *ARO4* is repressed by nitrogen starvation. (A) Expression of *DUR1,2*, *GCN4*, *ARO4* and *PDA1* under different nutritional conditions. Total RNA was prepared from yeast strains RH2396 (wt), RH2397 (*gcn2Δ*) and RH2398 (*gcn4Δ*) all carrying the *TRP1* centromeric vector pRS314 and grown to mid-log phase (Log-phase) or starved for either amino acids (3AT) or for nitrogen (N-starv). For measurements of *DUR1,2*, *GCN4*, *ARO4* and *PDA1* transcript levels, 10 µg of total RNA from each sample was subjected to a Northern-Hybridization analysis. Signals were quantified using a BAS-1500 Phosphor-Imaging scanner. Numbers given indicate relative expression levels of *DUR1,2*, *GCN4* and *ARO4* when compared to *PDA1* as internal standard and with a value for mid-log phase expression set to 1. (B) Nitrogen starvation-induced repression of *ARO4* transcription is mediated by Gcn4p and a single GCRE site in the *ARO4* promoter. Total RNA was prepared from yeast strains RH2485 (*ARO4*) and RH2486 (*ARO4m*) grown to mid-log phase (Log-phase) or starved for either amino acids (3AT) or for nitrogen (N-starv). *ARO4* and *PDA1* transcript levels were analyzed as described above. Numbers given indicate relative expression levels of *ARO4* when compared to *PDA1* as internal standard and with a value for mid-log phase expression set to 1.

When cells were starved for nitrogen, transcription of *DUR1,2* was induced 19-fold independently of either *GCN2* or *GCN4*. Similarly, steady state mRNA levels of *GCN4* were enhanced 2-fold under nitrogen starvation conditions, even in the absence of *GCN2*. This suggests that *GCN4* is transcriptionally regulated by nitrogen via factors that also control expression of *DUR1,2*. However, when cells were starved for nitrogen *ARO4* transcript levels dropped by a factor of two compared to mid-log phase. Other general control regulated genes as e.g. *HIS7* also showed decreased mRNA levels under nitrogen starvation (data not shown). Repression of *ARO4* by nitrogen starvation was depending on either *GCN2* or *GCN4*, because *ARO4* transcript levels remained unchanged under this condition in both *gcn2Δ* and *gcn4Δ* mutant strains. Thus, starvation for nitrogen only represses the Gcn4p-dependent transcription of *ARO4*. To

confirm this finding, we also measured expression of an *ARO4* mutant gene (*ARO4m*), whose single GCRE-site present in its promoter is inactivated by two point mutations and whose expression therefore is independent of Gcn4p (22). Under non-starvation conditions, *ARO4m* mRNA levels were decreased 2-fold when compared to expression of wild-type *ARO4* (Figure 2B). In addition, *ARO4m* expression was no longer inducible by 3AT. Thus, expression of *ARO4m* is comparable to expression of wild-type *ARO4* in a *gcn4Δ* mutant background. Importantly, starvation for nitrogen did not affect expression of the *ARO4m* mutant gene, corroborating that the drop of *ARO4* expression in nitrogen starved cells is due to a loss of the Gcn4p-dependent transcription of the gene.

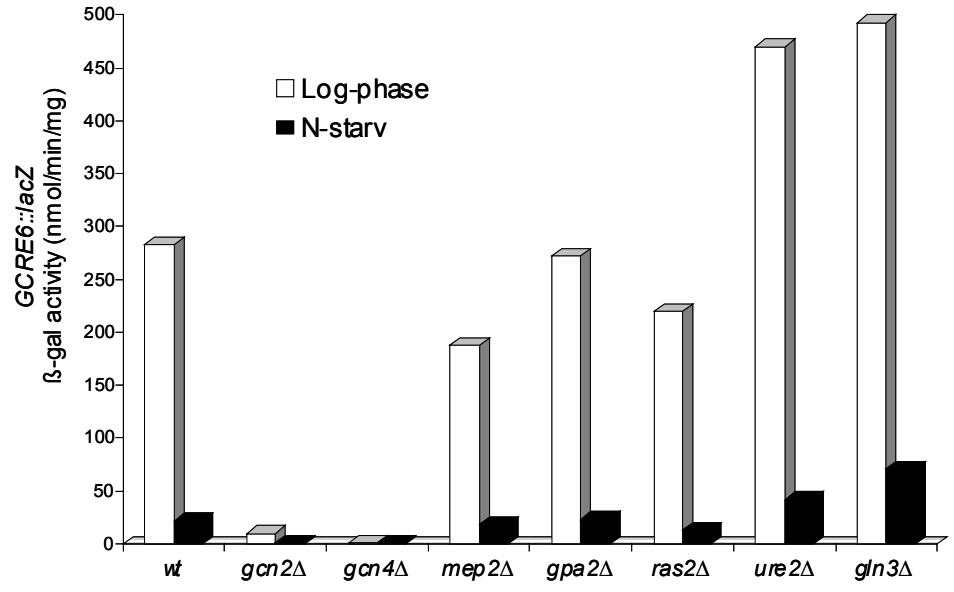
In summary, results in this section show that starvation for nitrogen increases *GCN4* transcription but represses transcription of *ARO4*, an amino acid biosynthetic gene that is induced by amino acid starvation. Moreover, repression affects specifically the Gcn4p-dependent transcription of *ARO4*, as no repression by nitrogen starvation can be observed in strains lacking elements of the general control system, like e.g. *GCN2* or *GCN4*, or when the GCRE-element of the *ARO4* promoter was inactivated. In contrast, the amino acid catabolic gene *DUR1,2* is not induced by amino acid starvation, but is strongly inducible by nitrogen starvation in a general control-independent manner.

Repression of Gcn4p-regulated transcription by nitrogen starvation is independent of the ammonium sensing and utilization system controlled by *MEP2, GPA2, URE2* or *GLN3*.

We tested whether repression of Gcn4p-dependent transcription by nitrogen starvation depends on elements of the general control system like the sensor kinase Gcn2p, or whether the ammonium sensing and signaling system that includes the Mep2 high affinity ammonium permease and the G-alpha subunit Gpa2p (Lorenz & Heitman, 1998) is involved. We also investigated the role of the two regulators for the ammonium utilization, Ure2p and Gln3p (ter Schure *et al*., 2000). For this purpose, the *GCRE6*::*lacZ* reporter was integrated into the *URA3* locus of *gcn2Δ*, *mep2Δ, gpa2Δ, ure2Δ* and *gln3Δ* mutant strains, respectively. We also measured *GCRE6*::*lacZ* reporter activity in a *ras2Δ* mutant strain, because the Mep2p/Gpa2p system has been postulated to exert its effects via cAMP. Expression of *GCRE6*::*lacZ* was reduced 28-fold in a strain lacking *GCN2* in non-starvation medium, yet was still repressible to a certain

extent by nitrogen starvation (Figure 3A). This indicates that nitrogen starvation induced repression of Gcn4p-dependent transcription might involve loss of Gcn2p-activity. In contrast, inactivation of neither *MEP2*, *GPA2,* nor *RAS2* significantly affected expression of *GCRE6*::*lacZ* under conditions of high or low ammonium concentrations (Figure 3A), indicating that these signaling components are not directly involved in mediating nitrogen starvation induced repression of Gcn4p-dependent transcription. Under nitrogen starvation in *ure2Δ* or *gln3Δ* mutant strains a comparable decrease of Gcn4p activity to the wild-type cells was detected, suggesting that Ure2p and Gln3p are also not involved in the observed repression of *GCN4*. Nevertheless, both mutant strains, which are impaired in the ammonium regulation, showed an increase of the ß-galactosidase activity of the *GCRE6*::*lacZ* reporter of about 60 % in log-phase even under non-nitrogen starvation conditions. This increase might be caused by the poor ability of the cells to utilize ammonium resulting in amino acid starvation.

A



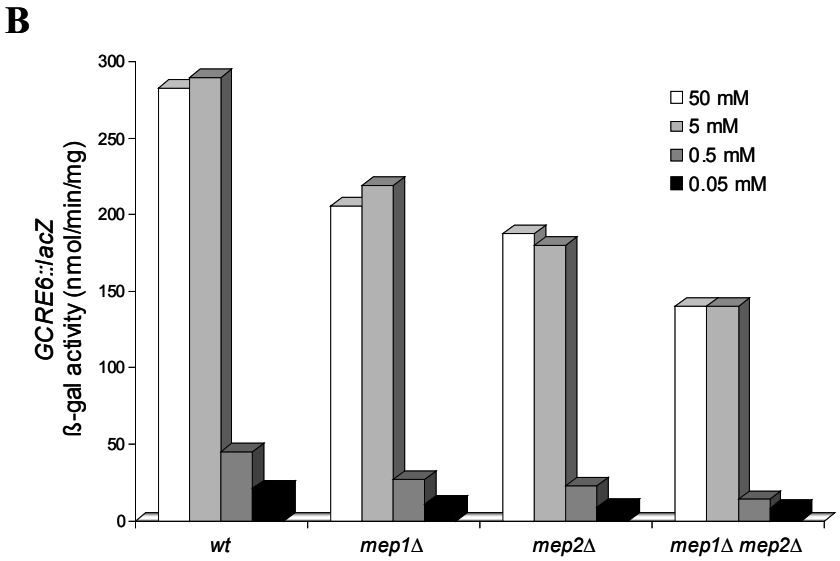


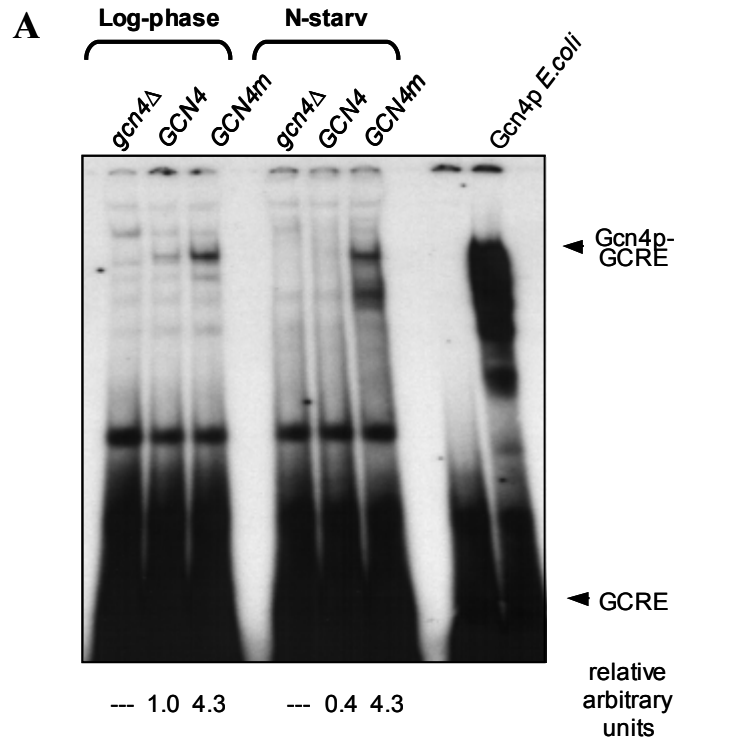
Figure 3. Regulation of Gcn4p-dependent gene expression by ammonium. (A) Repression of Gcn4p-dependent gene expression by nitrogen starvation is independent of *MEP2*, *GPA2*, *RAS2*, *URE2* and *GLN3*. Expression of the *GCRE6::lacZ* reporter gene was measured in yeast strains RH2396 (wt), RH2397 (*gcn2Δ*), RH2398 (*gcn4Δ*), RH2406 (*mep2Δ*), RH2404 (*gpa2Δ*), RH2400 (*ras2Δ*), RH2489 (*ure2Δ*) and RH2490 (*gln3Δ*) carrying plasmid pRS314 under non-starvation (Log-phase) or under nitrogen starvation (N-starv) conditions. (B) Gcn4p-regulated gene expression depends on ammonium uptake by Mep1p and Mep2p. Expression of *GCRE6::lacZ* was measured in yeast strains RH2396 (wt), RH2405 (*mep1Δ*), RH2406 (*mep2Δ*) and RH2445 (*mep1Δ mep2Δ*) carrying plasmid pRS314 in minimal medium containing 50 mM, 5 mM, 0.5 mM or 0.05 mM ammonium sulfate as the sole nitrogen source. In (A) and (B), bars depict means of at least three independent measurements of ß-galactosidase activities with a standard deviation not exceeding 15 %.

Expression of *GCRE6*::*lacZ* was further determined in dependence on different ammonium sulfate concentrations and on the presence of different combinations of the ammonium transporters Mep1p and Mep2p, to test whether intracellular ammonium concentrations might be a trigger for repression of Gcn4p-dependent gene expression. Double mutant strains carrying deletions of both *MEP1* and *MEP2* have been shown to exhibit reduced ammonium uptake (Lorenz & Heitman, 1998; Marini *et al*., 1997). We postulated that *mep1Δ mep2Δ* double mutant strains were more sensitive to a lack of ammonium in the growth medium with respect to loss of *GCRE6*::*lacZ* reporter activity, if intracellular ammonium was a trigger for repression of Gcn4p transcriptional activity. Accordingly, we found that expression of *GCRE6*::*lacZ* was significantly lower in the

mep1Δ mep2Δ double mutant when compared to a wild-type strain in the presence of both high and low amounts of ammonium sulfate (Figure 3B). Expression in the single *mep1Δ* or *mep2Δ* mutants was also reduced to a certain extent. Our results indicate that reduced ammonium uptake and consequently reduced intracellular ammonium concentrations are a trigger for repression of Gcn4p-dependent transcription under nitrogen starvation conditions.

Gcn4p DNA-binding activity is not affected by nitrogen starvation.

Our finding that Gcn4p-dependent transcription via GCRE-elements is repressed 11-fold by nitrogen starvation whereas *GCN4* mRNA levels increase by a factor of two, prompted us to closer examine the mechanism of repression. For this purpose, we measured both Gcn4p DNA-binding activity and the amount of Gcn4 protein in cells grown in medium containing either high or low concentrations of ammonium. *gcn4Δ* mutant strains with a chromosomally integrated *GCRE6*::*lacZ* reporter gene and carrying either a control plasmid or *GCN4* on a centromeric vector were grown in non-starvation or in nitrogen starvation medium. Crude protein extracts were prepared and analyzed for Gcn4p DNA binding activity by gel mobility shift assays with a synthetic GCRE-fragment (Figure 4A). In addition, the amount of Gcn4p present in the extracts was determined by Western hybridization analysis using a specific anti-Gcn4p antibody (Figure 4B). Purified Gcn4p heterologously expressed in *E. coli* was used as a control in both cases. We found a roughly 2.5-fold decrease in both Gcn4 protein levels and the amount of synthetic GCRE-fragment bound by Gcn4p present in crude extracts when cells were starved for ammonium. *GCRE6*::*lacZ* reporter activity was reduced 14-fold in these strains (Figure 4C). These results indicate that repression of Gcn4p-dependent transcription induced by nitrogen starvation is in part due to a decrease in the amount of intracellular Gcn4p. In addition, the DNA binding activity of Gcn4p does not appear to be affected by ammonium in the medium, because loss of Gcn4p DNA binding activity is paralleled by a decrease of intracellular Gcn4 protein levels.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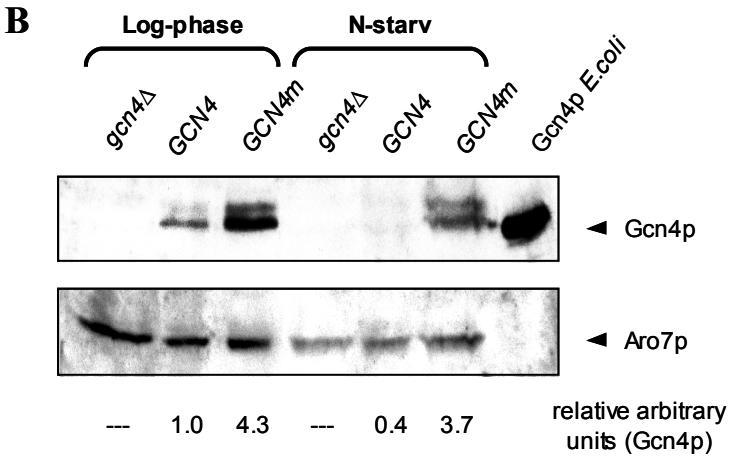
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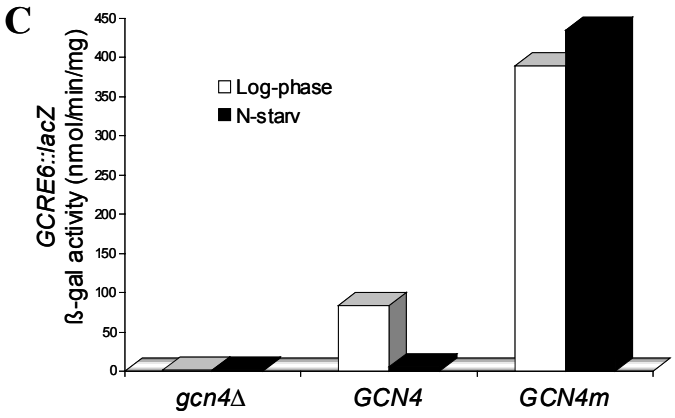
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GCRE6::lacZ
ß-gal activity (nmol/min/mg)
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400
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Log-phase
N-starv
gcn4Δ
GCN4
GCN4m

Figure 4. Regulation of Gcn4p DNA-binding activity, Gcn4p protein levels and Gcn4p transcriptional activity by nitrogen starvation. (A) Gel retardation assays. Crude protein extracts were prepared from yeast strain RH2398 (*gcn4Δ*) carrying either the control plasmid pRS314 (*gcn4Δ*), plasmid pME1092 expressing wild-type *GCN4* (*GCN4*) or plasmid pME1098 expressing the *GCN4m* mutant allele with inactivated uORFs in the *GCN4* leader sequence grown under non-starvation (Log-phase) or under nitrogen starvation conditions. *In vitro* DNA-binding activity of Gcn4p present in crude protein extracts was measured by mixing 10 µg of protein extracts from each sample with 10 fmole of a synthetic ^{32}P-end-labeled DNA fragment carrying a single GCRE site and separation of protein-DNA complexes on a native 6 % acrylamide gel. Gcn4p purified from *E. coli* was used as positive control. Specific Gcn4p-GCRE complexes are indicated (bound) and signals were quantified using a BAS-1500 Phosphor-Imaging scanner (Fuji). Numbers represent the amount of ^{32}P-end-labeled DNA fragment bound to Gcn4p in arbitrary units with a value for the strain carrying the *GCN4* wild-type plasmid grown under non-starvation conditions set to 1. (B) Amount of Gcn4p present in protein extracts isolated in (A) was analyzed by Western blot analysis using a polyclonal anti-Gcn4p antibody. As an internal control, protein levels of Aro7p were measured in the same extracts using a polyclonal anti-Aro7p antibody. Signals for Gcn4p and Aro7p were quantified using the Molecular Analyst software from Bio-Rad. Numbers represent Gcn4p levels relative to Aro7p with a value for a *wt GCN4* expressing strain set to 1. (C) Expression of the *GCRE6::lacZ* reporter gene was measured in strains described in (A) under non-starvation (Log-phase) and nitrogen starvation (N-starv) conditions. ß-Galactosidase activities are given in nanomoles per minute per milligram. Bars depict means of at least three independent measurements with a standard deviation not exceeding 15 %.

Translational repression of *GCN4* mRNA by nitrogen starvation requires the uORFs in the *GCN4* leader sequence.

We determined whether the decrease in intracellular amounts of Gcn4p upon ammonium limitation might be due to a change in the translational efficiency of *GCN4* mRNA, because translational control of *GCN4* mRNA is a well-documented mechanism for regulation of *GCN4* expression (Hinnebusch, 1984; Hinnebusch, 1985; Hinnebusch, 1990; Hinnebusch, 1992). Mutations in the start codons of the upstream open reading frames (uORFs) in the *GCN4* leader sequence are known to uncouple *GCN4* mRNA from translational control by the general control system upon amino acid starvation. We reasoned that if translation of *GCN4* was affected by nitrogen starvation, translation of a *GCN4* mRNA with inactivated uORFs in the upstream leader sequence should be unaffected by ammonium concentrations in the growth medium. As a consequence, Gcn4 protein levels and *GCRE6*::*lacZ* reporter gene expression should be identical in strains grown with or without limitation for ammonium. We measured Gcn4p DNA binding activity, Gcn4 protein levels and *GCRE6*::*lacZ* reporter activity in

a strain expressing a *GCN4* mutant allele (*GCN4m*) carrying point mutations that inactivate all four uORFs in the *GCN4* upstream leader under high and low ammonium conditions (Figure 4). We found that in a strain expressing the *GCN4m* allele both Gcn4p binding to synthetic GCRE-DNA fragments and intracellular Gcn4p levels were increased 4.3-fold under non-nitrogen starvation conditions, when compared to *GCN4* with intact uORFs. As a consequence, expression of *GCRE6*::*lacZ* reporter was induced 4.6-fold. Importantly, a significant decrease in neither Gcn4p binding to GCREs, intracellular Gcn4p levels nor expression of *GCRE6*::*lacZ* was detected under nitrogen starvation conditions when Gcn4p was translated from the *GCN4m* mRNA. Thus, mutations in the uORFs not only uncouple *GCN4* expression from translational control by the general control system, but as well from repression by nitrogen starvation. This indicates that ammonium regulates intracellular Gcn4p levels by a translational control mechanism involving the upstream open reading frames of the *GCN4* mRNA.

Since Yang and co-workers recently found (Yang *et al*., 2000) that glucose starvation in *S288c* cells resulted in an activation of the *GCN4* expression 4-fold or more, we investigated whether this effect exists also in *Σ1278b* cells. We used the same *GCN4*::*lacZ* fusion construct as previously described (Yang *et al*., 2000), and found that glucose starvation activates *GCN4* expression only 2-fold in *Σ1278b* cells (Figure 5), but much more (4-fold) in *S288c* yeast cells (data not shown). Therefore, our *S288c* data confirmed the results of Yang *et al*. (Yang *et al*., 2000). In contrast to glucose starvation, nitrogen starvation only slightly decreased *GCN4*::*lacZ* expression but drastically reduced *GCRE6*::*lacZ* expression by a factor of 10. Therefore, nitrogen and glucose starvation affect *GCN4* expression in opposite manners. Surprisingly, the increased *GCN4* expression during glucose starvation does not result in an increased expression of Gcn4p regulated target promoters. The *GCRE6*::*lacZ* reporter decreases in a time course after glucose starvation by a factor of two. This suggests that the expressed Gcn4p might be modified or destabilized under glucose starvation conditions.

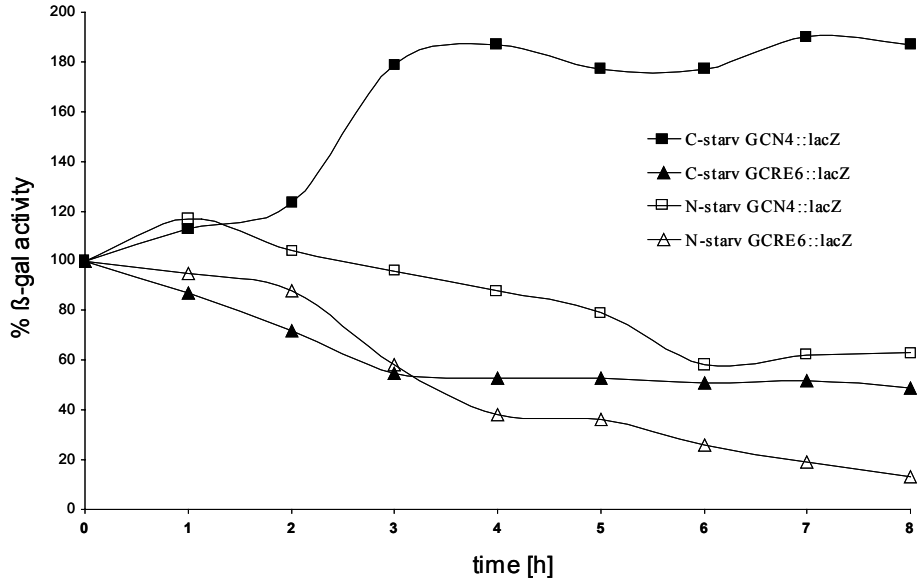


Figure 5. Time course of different ß-galactosidase reporter constructs under carbon and nitrogen starvation conditions. The yeast strains were incubated overnight transferred into fresh medium and collected after 4 h of growth. These cells served as inoculation for the starvation media (time-point: 0 h). At time 0 h the ß-galactosidase activity was set to 100 % for each strain. In the strain RH2399 the *GCN4*::*lacZ* reporter activity was tested under glucose limitation (■) and nitrogen limitation (□), respectively. Additionally the *GCRE6*::*lacZ* fusion gene was measured in the strain RH2396 under glucose limitation (▲) and nitrogen limitation (Δ). All values result from at least three independent measurements with a standard deviation not exceeding 15 %.

We further measured expression of *GCN4*::*lacZ* fusion constructs in more detail to corroborate the model that starvation for nitrogen affected translational efficiency of *GCN4* mRNA. *GCN4*::*lacZ* fusions are an accurate measure for translational efficiency of *GCN4* mRNA when compared to *GCN4* transcript levels (Hinnebusch, 1985). Wild-type strains as well as mutant strains lacking either *GCN2* (*gcn2Δ*) or *GCN4* (*gcn4Δ*) or both (*gcn2Δ*/*gcn4Δ*) were transformed with plasmids carrying either a *GCN4*::*lacZ* fusion construct or a *GCN4m*::*lacZ* fusion with mutated uORFs. ß-galactosidase activities of resulting strains were measured under both non-starvation and nitrogen starvation conditions (Figure 6).

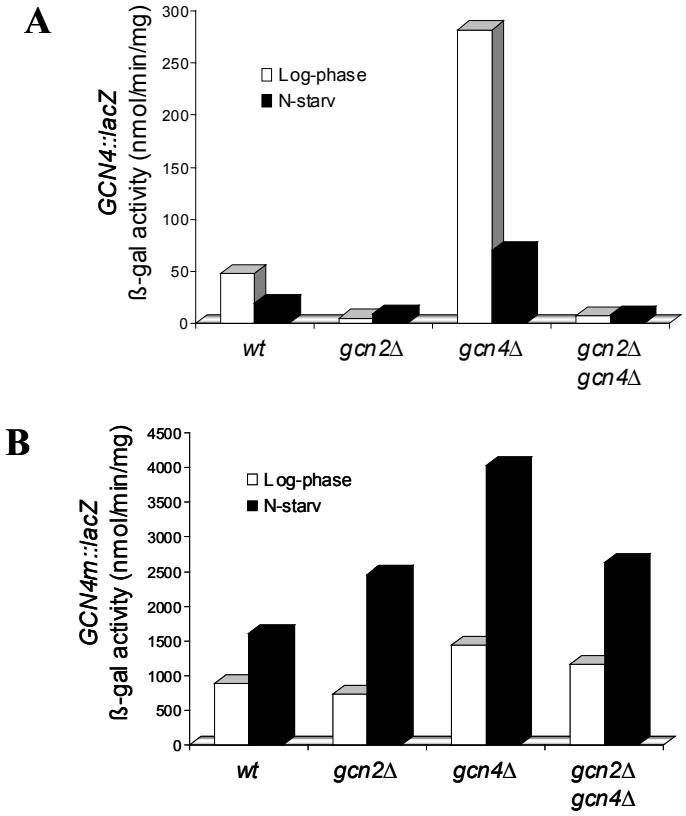


Figure 6. Repression of *GCN4* expression by nitrogen starvation depends on the uORFs in the *GCN4* leader sequence. (A) Expression of a *GCN4*::*lacZ* fusion gene was measured in yeast strains RH2399 (wt), RH2401 (*gcn2Δ*), RH2402 (*gcn4Δ*) and RH2402 (*gcn2Δ gcn4Δ*) under non-starvation (Log-phase) and nitrogen starvation (N-starv) conditions after 8 hours. (B) Expression of a *GCN4m::lacZ* fusion gene with inactivated uORFs in the *GCN4* leader sequence was measured in identical strains as described in (A). In (A) and (B), bars depict means of at least three independent measurements of ß-galactosidase activities with a standard deviation not exceeding 15 %.

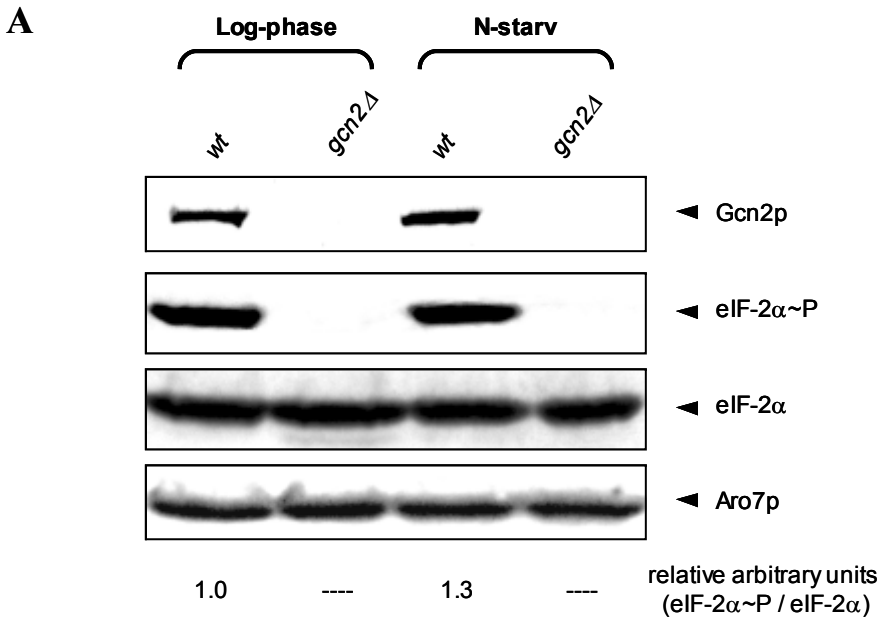
In a wild-type strain with an intact general control system, expression levels of *GCN4*::*lacZ* decreased 2-fold when cells were starved for ammonium. When *GCN2* was lacking from strains (*gcn2Δ* or *gcn2Δ*/*gcn4Δ* double mutants), *GCN4*::*lacZ* expression decreased by a factor of 9.6 under nitrogen non-starvation conditions and 2.1-fold under starvation in comparison to the wild type. However, no significant differences between non-starvation and nitrogen starvation conditions were measured in *gcn2Δ* strains. Interestingly, deletion of *GCN4* itself led to a 5.9-fold induction of *GCN4*::*lacZ* expression. This induction is due to the lack of any amino acids in the medium used (SD medium). It is known that Gcn4p is required for basal expression of genes involved in different amino acid biosynthetic pathway like arginine. A lack of the Gcn4p

transcription factor in the cell and amino acid starvation in the medium induces the general control system. In strains lacking both *GCN4* and *GCN2*, no induction of *GCN4*::*lacZ* could be measured, corroborating the interpretation that the 5.9-fold higher *GCN4*::*lacZ* expression levels in *gcn4Δ* mutants are due to an activated general control system. However, nitrogen starvation repressed *GCN4*::*lacZ* expression by a factor of 4 even in the *gcn4Δ* mutant background with an activated general control system. This indicates that nitrogen starvation efficiently counteracts amino acid starvation-induced activation of *GCN4* expression. Moreover, the repression mechanism takes place at the level of *GCN4* translation. Measurements with the *GCN4m*::*lacZ* construct corroborated this interpretation. Expression of this construct was no longer dependent on the presence of either *GCN2* or *GCN4*, because any of the strains used show comparable expression levels of *GCN4m*::*lacZ*. More importantly, no repression but even a roughly 2-fold induction of *GCN4m*::*lacZ* was detected when strains were starved for nitrogen. This corresponds to the increase in *GCN4* mRNA levels as shown in Figure 2. Thus, repression of *GCN4*::*lacZ* expression by nitrogen starvation requires the uORFs present in the *GCN4* upstream leader, again suggesting a translational mechanism of repression.

Nitrogen starvation blocks *GCN4* mRNA translation under simultaneous amino acid starvation conditions in an eIF-2α~P independent manner.

Gcn2p and eIF-2α~P are positive trans-acting factors of general translational control mechanism of the *GCN4* mRNA (Hinnebusch, 1997). Nitrogen starvation might act via these factors to block translation. To test these possibilities, we measured the protein levels of Gcn2p and the relation between eIF-2α and eIF-2α~P. In addition, we determined the Gcn4p and *GCN4::lacZ* levels (Figure 7). We found that nitrogen starvation does not affect the Gcn2p level (Figure 7A). Surprisingly, a strong increase of eIF-2α phosphorylation could be detected in a nitrogen starved *S288c* strain after 24 h (Figure 7B). In contrast, only a weak increase was found in the *Σ1278b* strain (Figure 7A). An explanation for this finding may be the partially induced general control of *Σ1278b* strains (Stanhill *et al*., 1999), which can be observed by comparing the ß-galactosidase activity of a *gcn2Δ*- and a wild-type strain measured by the *GCRE::lacZ* reporter construct (Figure 3A). Accordingly, the phosphorylation level of eIF-2α is higher in log-phase in a *Σ1278b* strain than in a *S288c* strain (Figure 7), resulting in a

weaker increase of eIF-2α~P levels under nitrogen starvation. Expression of the *GCN4::lacZ* reporter construct was identical in strains grown to log-phase or starved for nitrogen (Figure 7B). Under 3AT conditions, however, *GCN4::lacZ* expression is 13-fold higher than under nitrogen starvation, whereas the phosphorylation levels of eIF-2α are comparable. Maximal phosphorylation of eIF-2α is achieved significantly faster under amino acid starvation than under nitrogen starvation conditions (Figure 7C). Thus, under nitrogen starvation condition, phosphorylation of eIF-2α does not correlate with the expression of the *GCN4::lacZ* reporter gene, implicating an additional regulatory mechanism for the translational control of *GCN4*. This conclusion is further supported by the fact that under simultaneous nitrogen and amino acid starvation conditions the phosphorylation level of eIF-2α was inducible nearly 2-fold, while the expression of *GCN4::lacZ* remained constant (Figure 7B). Furthermore, the effect caused by nitrogen starvation appears to overrule that of amino acid starvation.



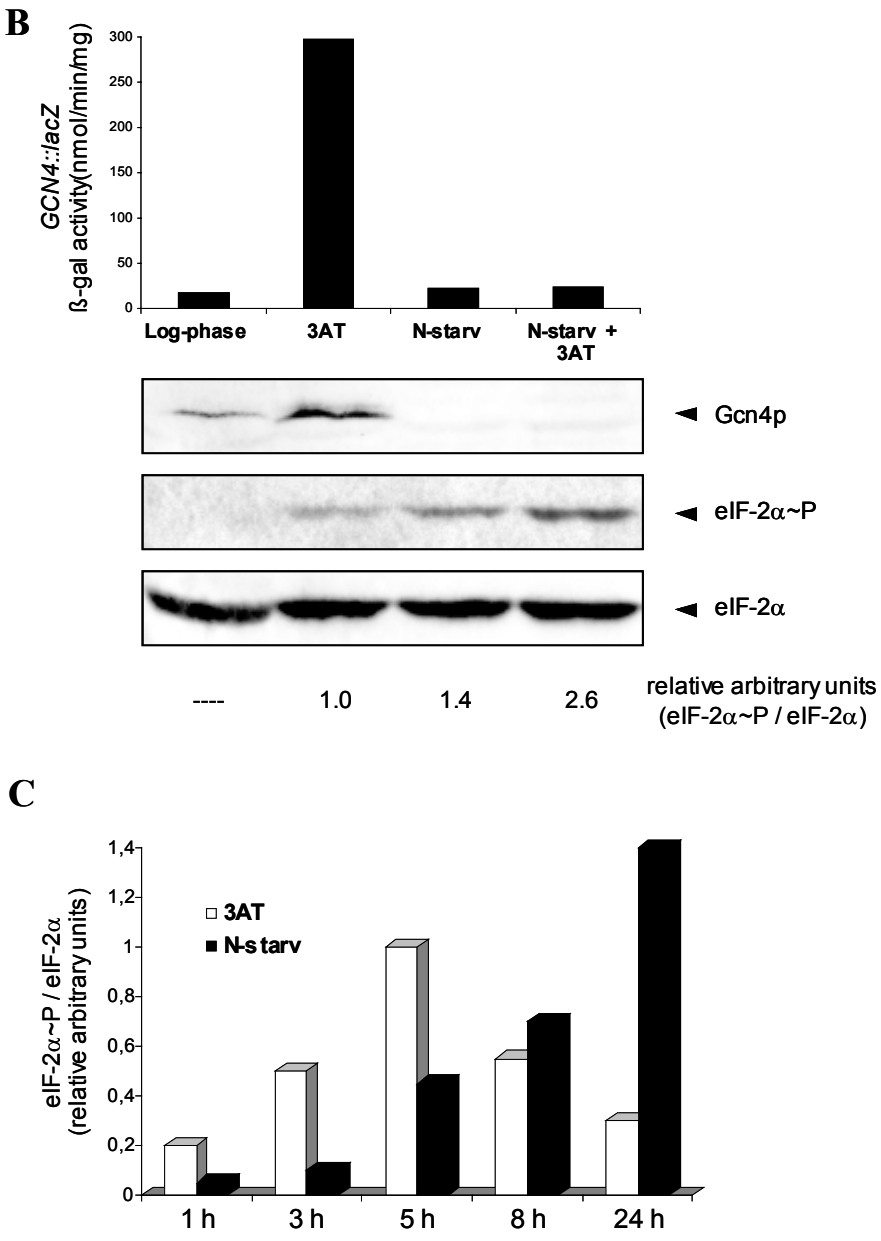


Figure 7. Regulation of Gcn2p and eIF-2α~P protein levels by nitrogen starvation. (A) Crude protein extracts were prepared from yeast *Σ1278b* strains RH2396 (wt) and RH2397 (*gcn2Δ*) carrying plasmid pRS314 grown under non-starvation (Log-phase) or under nitrogen starvation conditions (N-starv). The amount of Gcn2p, eIF-2α and eIF-2α~P protein present in isolated protein extracts were analyzed by Western blot analysis using a polyclonal anti-Gcn2p antibody, a polyclonal anti-eIF-2α antibody or a phosphorylation specific polyclonal anti-eIF-2α~P antibody. As an internal control, protein levels of Aro7p were measured in the same extracts using a polyclonal anti-Aro7p antibody. Signals for eIF-2α~P and eIF-2α were quantified and their relation is given below in relative arbitrary units. (B) Crude protein extracts from the strain

RH2520 (haploid, *S288c*), which carried the p180 plasmid, were prepared. Cells from an overnight culture were diluted and incubated for 5 h under non-starvation conditions (Log-phase) or amino acid starvation conditions by adding 10 mM 3AT (3AT). For nitrogen starvation (N-starv) or nitrogen starvation with additional 3AT (N-starv + 3AT) yeast cells were incubated under nitrogen starvation for 24 h and then divided up in two cultures. One culture was mixed with 10 mM 3AT, while the other remained unchanged, and cultures were further incubated for 5 h before harvesting. From all cultures the ß-galactosidase activities were measured. Bars depict means of at least three independent measurements of ß-galactosidase activities with a standard deviation not exceeding 15 %. From the same cultures the protein levels of Gcn4p, eIF-2α and eIF-2α~P were analyzed by Western hybridization using specific antibodies. The ratio between eIF-2α~P and eIF-2α was determined and is shown at the bottom. (C) A time course of the eIF-2α phosphorylation status was performed under amino acid starvation (3AT) and under nitrogen starvation conditions (N-starv) using strain RH2520 and the cultivation procedures described in Figure 7B. Bars represent the ratio between eIF-2α~P and eIF-2α after 1, 3, 5, 8 and 24 h of starvation, respectively. Arbitrary units were chosen with a value of 1 for the ratio measured after 5 h of amino acid starvation.

We also measured the Gcn4p levels under all different conditions (Figure 7B) to test the correlation between the amount of Gcn4p in the cells and the expression of the *GCN4::lacZ* reporter construct. In non-nitrogen starvation medium, Gcn4p levels and ß-galactosidase activities correlate well. Under nitrogen starvation conditions, however, where comparable ß-galactosidase activities as in log-phase were measured, only very low levels of Gcn4p were detectable, indicating a destabilization of Gcn4p. In addition, expression of the *GCN4m::lacZ* reporter construct increased under nitrogen starvation conditions (data not shown), suggesting that both a higher instability of Gcn4p and the translational control are involved in the decrease of the Gcn4p level under nitrogen starvation.

We further tested, whether *GCN3* might be involved in translational repression of *GCN4* under nitrogen starvation conditions. Gcn3p is part of the regulatory eIF-2B subcomplex that is inhibited in its guanine nucleotide exchange activity of eIF-2α by phosphorylated eIF-2α (Pavitt *et al*., 1997). Expression of *GCN4::lacZ* was measured in strains lacking *GCN3* or carrying *GCN3* on a high-copy vector (Figure 8), because nitrogen starvation might lower expression of *GCN3*, an effect that should be corrected by high-copy expression of *GCN3*. However, even in strains carrying *GCN3* on a high-copy number plasmid, expression of *GCN4::lacZ* under nitrogen starvation conditions was as low as in the control strain (Figure 8). This suggests that *GCN3* is not required in the repression of *GCN4* mRNA translation by nitrogen starvation.

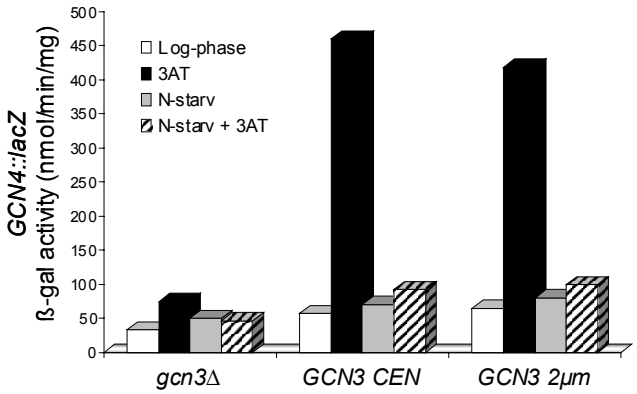


Figure 8. Influence of copy number variation of *GCN3* on expression of *GCN4::lacZ*. Expression of the *GCN4::lacZ* reporter gene was measured in yeast strain GP3153 carrying a control vector (*gcn3Δ*), *GCN3* on a CEN plasmid (*GCN3* CEN) or *GCN3* on a 2µm plasmid (*GCN3* 2µm) under different nutritional conditions. Strains were cultivated as described in Figure 7B, and ß-galactosidase activities were measured from all cultures. Bars depict means of at least three independent measurements of ß-galactosidase activities with a standard deviation not exceeding 15 %.

In summary, we detected a translational block of *GCN4* mRNA resulting in a decrease of the Gcn4p amount under nitrogen starvation conditions. This translational control mechanism appears to be independent of the known trans-acting elements Gcn2p, eIF-2α or Gcn3p, but still requires the four uORFs of the *GCN4* mRNA. Furthermore, nitrogen starvation conditions block an activation of the general control caused by 3AT without interfering with the phosphorylation of eIF-2α.

DISCUSSION

Yeast cells integrate many nutritional signals to adapt their metabolism for optimal growth and development. Nitrogen starvation requires the expression of genes for enzymes able to degrade nitrogen-containing compounds. Simultaneously, cells adapt morphologically by switching to a filamentous growth mode. In this study, we investigated how starvation for nitrogen affects the 'general control' regulatory network that induces amino acid biosynthetic gene expression when yeast cells are starved for amino acids. Because amino acid biosynthesis requires at least one aminotransferase reaction, this network is an important part of the general nitrogen metabolism. Yeast cells utilize ammonia exclusively by incorporation into glutamate and glutamine (ter Schure *et al*., 2000). This prompted us to analyse whether the general signal 'lack of nitrogen' includes the more specific signal 'lack of amino acid' and subsequently activates the general control system and its transcriptional activator Gcn4p. In contrast, we found the opposite effect that a general lack of any nitrogen source specifically and efficiently inhibits Gcn4p-mediated gene expression.

Several lines of evidence indicate that inhibition of general control-regulated gene expression by nitrogen starvation occurs at the level of *GCN4* mRNA translation. (i) We find that Gcn4 protein levels decrease in the absence of nitrogen, whereas *GCN4* transcript levels increase. (ii) Reduction of Gcn4p levels or *GCN4::lacZ* fusion expression can been observed only when translated from *GCN4* mRNA species carrying the uORFs in the leader sequence. (iii) Nitrogen starvation completely blocks amino acid starvation dependent induction of *GCN4::lacZ* expression and subsequently increased Gcn4p levels, depending on the presence of the uORFs. However, phosphorylation of eIF-2α by Gcn2p is not blocked by nitrogen starvation. In contrary we find that nitrogen starvation leads to even a stronger increase in eIF-2α phosphorylation, than amino acid starvation. Yet, starvation for nitrogen completely blocks efficient translation of *GCN4* mRNA, even under simultaneous amino acid starvation conditions. This suggests that nitrogen starvation blocks *GCN4* mRNA translation by an additional yet undiscovered mechanism. Because nitrogen starvation does neither affect Gcn2p or eIF-2α protein levels nor the ratio of eIF-2α~P / eIF-2α, a component of the general control system that acts downstream of eIF-2α might be

altered in function or expression. This result indicates an additional translational regulation mechanism using the same *cis*-elements.

Our study further suggests that nitrogen starvation, apart from repressing translation of *GCN4* mRNA, as well down-regulates the Gcn4 protein on a posttranslational level. This conclusion is based on two observations: (i) Whereas in the absence of *GCN2*, expression of *GCN4::lacZ* is not repressible by nitrogen starvation, expression of the *GCRE6::lacZ* reporter is still down regulated to a certain extent. (ii) When uORFs are absent in the *GCN4* leader, expression of *GCN4m::lacZ* (reflecting translation of *GCN4*) is induced 2-fold by nitrogen starvation, but both Gcn4 protein levels and expression of the *GCRE6::lacZ* reporter are not further induced. A simple explanation might be that nitrogen starvation reduces Gcn4 protein stability. Whereas amino acid starvation inhibits Gcn4p degradation by the ubiquitin-mediated proteolytic system (Kornitzer *et al*., 1994), nitrogen starvation might have opposite effects and activate proteolysis of Gcn4p.

A nitrogen sensing and signaling system has to induce translational repression of *GCN4*. At least two distinct developmental programs are known that are negatively regulated by the presence of ammonium, pseudohyphal development and meiosis. A signaling system that comprises Mep2p, Gpa2p and the cAMP-dependent protein kinase is thought to positively regulate pseudohyphal development when cells are starved for nitrogen (Kübler *et al*., 1997; Lorenz & Heitman, 1998). However, neither Mep2p nor Gpa2p seem to be involved in translational control of *GCN4*. In the presence of ammonium, yeast turns off the utilization of poor nitrogen sources, such as urea and proline. This phenomenon is referred as nitrogen catabolite repression (Hofman-Bang, 1999). The genetic system involves the function of the positively acting GATA transcription factor Gln3p that is negatively regulated by the repressor protein Ure2p in the presence of ammonium. However, neither *ure2Δ* nor *gln3Δ* strains show effects which implicate that these proteins are involved in *GCN4* repression under nitrogen starvation. The reduction of the intracellular ammonium concentration by deleting the ammonium permeases *MEP1* and *MEP2* led to a decrease of Gcn4p activity. This suggests the existence of an additional sensor system for the intracellular ammonium concentration, which is able to induce a signal for repression of the *GCN4* mRNA translation.

In summary, our studies show that yeast cells up-regulate amino acid biosynthesis only when enough nitrogen-containing precursors are available. Nitrogen starvation specifically represses activity of the general control system but does not affect the basal expression of amino acid biosynthetic genes. This demonstrates that amino acid biosynthesis is not completely shut down even under severe starvation conditions. The importance of amino acid biosynthesis under nutrient limiting conditions is also reflected by the regulatory mechanism that we found in this study. Although yeast cells abrogate up-regulation of amino acid biosynthesis when nitrogen sources are absent, mRNA levels for the transcriptional activator Gcn4p are kept at a higher level in order to rapidly induce translation of *GCN4* mRNA when nitrogen becomes available.

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Chapter III

Amino acid starvation and Gcn4p activate adhesion and *FLO11* gene expression in yeast

Summary

Cell-cell and cell-surface adhesion are required for invasive growth and pseudohyphal development, in bakers' yeast *Saccharomyces cerevisiae*. These morphogenetic events are induced by glucose or nitrogen starvation and require the cell surface protein Flo11p. We show that amino acid starvation is a nutritional signal that activates adhesion and expression of *FLO11* in the presence of glucose and ammonium. Activation depends on Flo11p and requires the elements of the general control system of amino acid biosynthesis, Gcn2p and Gcn4p. The elements of the cAMP pathway, Tpk2p and Flo8p are also necessary, but not Ste12p and Tec1p, known targets of the MAPK cascade. Promoter analysis of *FLO11* uncovered at least three upstream activation sequences (UASs) and one repression site (URS) that confer regulation by amino acid starvation. Gcn4p is required for activation of two UAS elements, although the *FLO11* promoter does not contain known Gcn4p-binding sites. Our study suggests that amino acid starvation in yeast is a nutritional signal that is transmitted leading to adhesion and *FLO11* gene expression by a signaling pathway, which is under control of Gcn4p.

Introduction

Adherence of cells to one another and to surfaces is the prerequisite for the formation of multicellular structures. In the yeast *Saccharomyces cerevisiae*, cell-cell and cell-surface adhesion are required for many developmental processes that include mating, invasive growth, biofilm formation and pseudohyphal development (Erdman *et al*., 1998; Guo *et al*., 2000; Mösch, 2000; Reynolds & Fink, 2001). Each of these events is initiated by distinct signals that are coupled to the expression of specific cell surface proteins by the corresponding signaling pathways (Erdman *et al*., 1998; Guo *et al*., 2000; Mösch, 2000; Reynolds & Fink, 2001). For instance, starvation for glucose causes invasive growth in haploid strains of *S. cerevisiae*, a developmental event that depends on expression of the cell surface protein Flo11p and that is under control of the MAPK and the cAMP pathways (Cullen & Sprague, 2000; Pan & Heitman, 1999; Roberts & Fink, 1994; Rupp *et al*., 1999). In diploid strains starvation for nitrogen induces pseudohyphal growth, a morphogenetic development that also requires Flo11p and functional MAPK and cAMP signaling pathways (Gimeno *et al*., 1992; Liu *et al*., 1993; Lo & Dranginis, 1998).

FLO11 belongs to a gene family that encodes glycosyl-phosphatidylinositol (GPI)-linked glycoproteins of domain structure similar to the adhesins of pathogenic fungi (Guo *et al*., 2000). Flo11p is localized to the cell surface and is required for nutritionally induced cell-cell and cell-surface adhesion during invasive growth, biofilm formation and pseudohyphal development (Guo *et al*., 2000; Lo & Dranginis, 1996; Mösch, 2000; Reynolds and Fink, 2001). The unusually large *FLO11* promoter is complex and integrates multiple inputs from the cAMP pathway, the MAPK cascade, the mating type and nutritional signals (Rupp *et al*., 1999). The transcription factor Flo8p is required for activation of *FLO11* by Tpk2p, the catalytic subunit of the cAMP-dependent protein kinase specifically involved in activation of invasive growth and pseudohyphal development (Pan & Heitman, 1999; Robertson & Fink, 1998; Rupp *et al*., 1999). Ste12p and Tec1p are further transcription factors that are important for *FLO11* regulation and transmit signals from the MAPK to sites within the *FLO11* promoter that are distinct from the Flo8p target sites (Lo & Dranginis, 1998; Rupp *et al*., 1999).

Amino acid biosynthesis is part of the general nitrogen metabolism of yeast (ter Schure *et al*., 2000). Starvation for a single amino acid induces a genetic system called

´general control of amino acid biosynthesis´ that activates transcription of more than 50 genes encoding enzymes involved in several amino acid biosynthetic pathways, amino acid tRNA synthetases (Meussdoerffer & Fink, 1983; Mirande & Waller, 1988) and enzymes of purine biosynthesis (Hinnebusch, 1992; Mösch *et al*., 1991). In this signaling system, the sensor kinase Gcn2p phosphorylates the translation elongation factor eIF2 in response to amino acid starvation, an event that results in efficient translation of *GCN4* that encodes the transcription factor Gcn4p (Hinnebusch, 1997). Gcn4p activates transcription of target genes by direct promoter binding at sequence-specific Gcn4p-responsive elements (GCREs).

Here, we report that amino acid starvation is a nutritional signal that activates adhesion and expression of *FLO11* in both haploid and diploid strains. Activation depends on Flo11p and requires Gcn2p, Gcn4p, Tpk2p and Flo8p, but not Ste12p and Tec1p. Promoter analysis of *FLO11* indicates that at least three upstream activation sequences (UASs) and one repression site (URS) confer regulation by amino acid starvation. Gcn4p is required for regulation of two UAS elements, although they do not contain any GCREs. Our study suggests that amino acid starvation is a potent nutritional signal for initiation of cell-cell and cell-surface adhesion in yeast, which is mediated to the *FLO11* promoter by a signaling pathway that is under control of Gcn4p.

Material & Methods

Yeast Strains and Growth Conditions

All strains used in this study are derivatives of the *S. cerevisiae* Σ*1278b* strain background (Table 1). Deletion mutants for *GCN2* (*gcn2Δ*) were obtained by using the *gcn2Δ* deletion plasmid pME1659 (Table 2). Plasmids pME1105 and pME1645 were used for constructing *gcn4Δ* and *tpk2Δ* mutant strains. *Flo8Δ* and *flo11Δ* mutants were obtained by using plasmids pME2155 and pME2156. *ste12Δ* mutant strain was a gift from G. R. Fink (Whitehead Institute, Cambridge, Massachusetts). Transformations were carried out using the lithium-acetate yeast transformation method (Ito *et al*., 1983). All gene deletions, integrations or replacements were confirmed by Southern blot analysis (Southern, 1975).

For non-starvation measurements, strains were cultivated in liquid synthetic minimal medium (YNB) (Guthrie & Fink, 1991) with additional arginine overnight at 30 °C, diluted and cultivated for 8 h before assaying enzymatic activities or isolation of total RNA. For amino acid starvation, 3-amino-triazole (3AT) was added to cultures grown to mid-log phase to a final concentration of 10 mM, and cells were further incubated for 6 – 8 h before all assays. For nitrogen starvation, cells grown to mid-log phase were washed with 2 % glucose and incubated for 24 h in liquid YNB medium with arginine containing only 50 µM ammonium sulfate (instead of 50 mM) as the sole nitrogen source. Qualitative pseudohyphal growth was assayed on SLAD plates (Gimeno *et al*., 1992). Solid media were prepared by using 2 % agar.

Plasmids

All plasmids used in this study are listed in Table 2. The *FLO8* deletion cassette was constructed by amplifying the plasmid backbone from the plasmid pHL129 (Liu *et al*., 1996) with the two primers OG33 (5'-GAAGATCTTCTACCACGGAATGCGTTTCC-3') and OG34 (5'-GAAGATCTCTGACATTTCGCTAAATTTGGG-3'). Into the new created *Bgl*II restriction site the kanamycin resistance cassette (*kan*^{r}) of pME1765 (Grundmann *et al*., 2001) was fused, resulting in the plasmid pME2155. Deletion cassettes for *FLO11* (pME2156) and *TPK2* (pME1645) were created by replacement of *FLO11* or *TPK2* coding sequences by *kan*^{r} as selectable marker.

Northern Hybridization Analysis

Total RNA from yeast was isolated following the protocol described by Cross and Tinkelenberg (Cross & Tinkelenberg, 1991). RNA was separated on 1.4 % agarose gel containing 3 % formaldehyde and transferred onto nylon membranes by electroblotting. Gene specific probes were ^{32}P-radiolabeled with the MBI Fermentas HexaLable™ DNA Labelling Kit. Hybridizing signals were quantified using a BAS-1500 Phosphor-Imaging scanner (Fuji).

ß-Galactosidase Assay

Assays were performed with extracts of cultures grown on liquid media. Specific ß-galactosidase activity was normalized to the total protein (Bradford, 1976) in each extract and equalized (OD_{415} x 1.7) / (0.0045 x protein concentration x extract volume x time) (Rose & Botstein, 1983). Assays were performed for at least three independent transformants, and the mean value is presented. The standard errors of the means were below 15 %.

Photomicroscopy

Pseudohyphal growth was viewed with a Zeiss Axiovert microscope and photographed using a Xillix Microimager digital camera with the Improvision Openlab software (Improvision, Coventry, UK).

Adhesive Assay

Strains were grown as patches at 30 °C. After incubation, plates were photographed and then carefully washed under a stream of water. The plate was photographed once again to document the remaining cells.

TABLE 1. Yeast strains used

Strain	Genotype	Source
RH2648	*MAT***a** *ura3-52*	This study
RH2649	*MAT***a** *ura3-52 gcn2Δ::kanR*	This study
RH2650	*MAT***a** *ura3-52 leu2::hisG gcn4Δ::LEU2*	This study
RH2651	*MAT***a** *ura3-52 leu2::hisG gcn4Δ::LEU2 gcn2Δ::kanR*	This study
RH2652	*MAT***a** *ura3-52 flo8Δ::kanR*	This study
RH2653	*MAT***a** *ura3-52 flo8Δ::kanR gcn2Δ::kanR*	This study
RH2654	*MAT***a** *ura3-52 leu2::hisG gcn4Δ::LEU2 flo8Δ::kanR*	This study
RH2662	*MAT***a** *ura3-52 flo11Δ::kanR*	This study
RH2656	*MAT***a**/α *ura3-52/ura3-52 his3::hisG/HIS3*	This study
RH2657	*MAT***a**/α *ura3-52/ura3-52 his3::hisG/HIS3 gcn2Δ::kanR/gcn2Δ::kanR*	This study
RH2658	*MAT***a**/α *ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/HIS3 gcn4Δ::LEU2/gcn4Δ::LEU2*	This study
RH2659	*MAT***a**/α *ura3-52/ura3-52 leu2::hisG/LEU2 his3::hisG/HIS3 tpk2Δ::kanR/tpk2Δ::kanR*	This study
L5627	*MAT***a**/α *ura3-52/ura3-52 leu2::hisG leu2::hisG ste12Δ::LEU2/ste12Δ::LEU2*	(Liu *et al*., 1993)
HMC267	*MAT***a**/α *ura3-52/ura3-52 trp1::hisG/TRP1 leu2::hisG/leu2::hisG tec1-101::Tn3::lacZ::LEU2/tec1-101::Tn3::lacZ::LEU2*	(Mösch & Fink, 1997)
RH2660	*MAT***a**/α *ura3-52/ura3-52 flo8Δ::kanR/flo8Δ::kanR*	This study
RH2661	*MAT***a**/α *ura3-52/ura3-52 flo11Δ::kanR/flo11Δ::kanR*	This study

TABLE 2. Plasmids used

Plasmid	Description	Source
pME1105	Cassette for full deletion of *GCN4*-open reading frame (*LEU2*)	(Albrecht *et al*., 1998)
pME1659	Cassette for full deletion of *GCN2*-open reading frame (*kan*^{r})	(Grundmann *et al*., 2001)
pME2155	Cassette for full deletion of *FLO8*-open reading frame (*kan*^{r})	This study
pME2156	Cassette for full deletion of *FLO11*-open reading frame (*kan*^{r})	This study
pME1645	Cassette for full deletion of *TPK2*-open reading frame (*kan*^{r})	This study
pME1765	pBluescriptKS$^+$ containing *kan*^{r}-cassette	(Grundmann *et al*., 2001)
pRS314	*TRP1*-marked centromere vector	(Sikorski & Hieter, 1989)
YEp355	*lacZ* shuttle vector	(Myers *et al*., 1986)
B3782	3 kb-*FLO11::lacZ* in YEp355	(Rupp *et al*., 1999)
p*flo11-1* to p*flo11-15*	200 bp deletions in B3782 from -1 to -200 bp, -200 to -400 bp until -2800 to -3000 bp	(Rupp *et al*., 1999)
pLG669Z	*lacZ* shuttle vector	(Guarente & Ptashne, 1981)
p*FLO11*-2/1 to p*FLO11*-15/14	440 bp sequence elements cloned into pLG669Z from -1 to -420 bp, -180 to -620 bp until -2580 to-2980 bp	(Rupp *et al*., 1999)

Results

Amino acid starvation activates adhesive growth and *FLO11* gene expression in the presence of glucose and ammonium.

We tested, whether starvation for amino acids activates adhesive growth and expression of *FLO11*. Haploid and diploid wild type and *flo11*Δ mutant strains were tested for adhesive growth on solid high ammonium medium with or without the addition of 3-amino-triazole (3AT), a histidine-analogue that induces amino acid starvation (Dever, 1997). As expected, haploid cells exhibited substrate adhesive growth under non-starvation conditions. Here, we found that amino acid starvation significantly enhanced this phenotype (Figure 1A). Haploid *flo11*Δ mutants were non-adhesive under all conditions tested. As previously described, diploid strains were non-adhesive under non-starvation conditions (Figure 1A). We found that diploids become highly adhesive when starved for amino acids even in the presence of high amounts of ammonium (Figure 1A). Substrate adhesion of diploids starved for amino acids is comparable to non-starved haploid strains. Deletion of *FLO11* blocked diploid adhesive growth under amino acid starvation conditions. These results show that amino acids starvation enhances adhesive growth of both haploid and diploid strains dependent on *FLO11*, but independent of the presence of ammonium.

Expression of a *FLO11-lacZ* reporter gene was measured in haploid and diploid strains starved for either nitrogen or amino acids, to determine the correlation between adhesive growth and expression of *FLO11*. A 3.6-fold increase in *FLO11-lacZ* expression was found in nitrogen-starved haploids when compared to non-starved cells, and a 59-fold increase was measured in diploid cells (Figure 1B). An induction of *FLO11*-expression by nitrogen starvation has previously been observed (Lo & Dranginis, 1998; Rupp *et al*., 1999). Here, we found that amino acid starvation led to a significant increase in the expression of *FLO11-lacZ* of 20.3-fold in haploid cells and 40.5-fold in diploid cells even when high amounts of ammonium are available (Figure 1B). *FLO11-lacZ* expression levels of non-starved haploids (52.1 units) corresponded to the levels found in diploid strains starved for nitrogen (100 units) or amino acids (68.8 units), and correlated with the degree of adhesive growth. Induction of *FLO11-lacZ* expression by the histidine-analogue 3AT was reversible by addition of histidine

(Figure 1B), demonstrating that amino acid starvation is the inducing signal for enhanced expression.

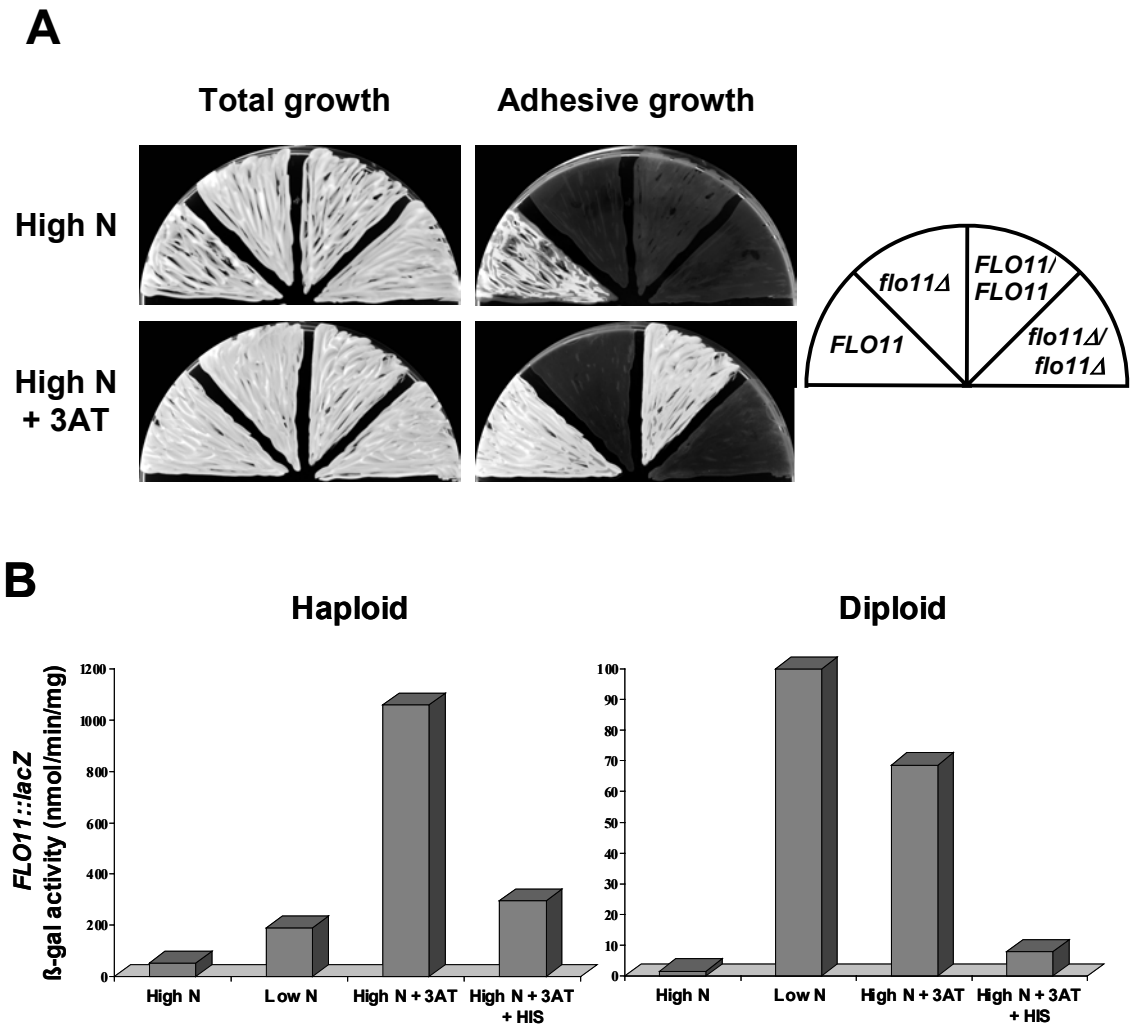


Figure 1. Regulation of adhesive growth and *FLO11* expression under different growth conditions.

(A) Haploid strains RH2648 (*FLO11*) and RH2662 (*flo11Δ*), and diploid strains RH2656 (*FLO11/FLO11*) and RH2661 (*flo11Δ/ flo11Δ*) carrying plasmid B3782 were streaked on YNB (High N) or YNB + 10 mM 3AT (High N + 3AT). Plates were incubated for 5 days (YNB), but only 1 day on YNB + 3AT and photographed before (Total growth) and after (Adhesive growth) cells were washed off the agar surface.

(B) Expression of the *FLO11::lacZ* reporter gene was measured in RH2648 (Haploid) and RH2656 (Diploid), carrying plasmid B3782, under different nutritional conditions. Cultures grown in minimal medium were used for assaying high ammonium conditions (High N). Amino acid starvation was induced by addition of 3AT to 10 mM (High N + 3AT) and nitrogen starvation (Low N) by growth on minimal medium containing 50 µM ammonium sulfate as the sole nitrogen source. As control that 3AT induces a histidine lacking we added 1 mM histidine to a 10 mM 3AT containing minimal medium (High N + 3AT + HIS). β-Galactosidase activities are given in nanomoles per minute per milligram. Bars depict means of at least three independent measurements with a standard deviation not exceeding 15 %.

The effect of amino acid starvation on transcript levels of *FLO11* was measured, to corroborate the data obtained with the *FLO11-lacZ* translational fusion. In haploid strains, amino acid starvation led to a 5.1-fold increase in *FLO11*-transcripts levels (Figure 2), thus correlating with *FLO11-lacZ* expression and the degree of adhesive growth. In non-starved diploid cells, no *FLO11*-transcripts were detectable in Northern-hybridization experiments (Figure 2). In 3AT-treated diploid cells, the amount of *FLO11*-transcripts increased to a level comparable to that found in non-starved haploids (Figure 2), correlating with *FLO11-lacZ* expression and adhesive growth.

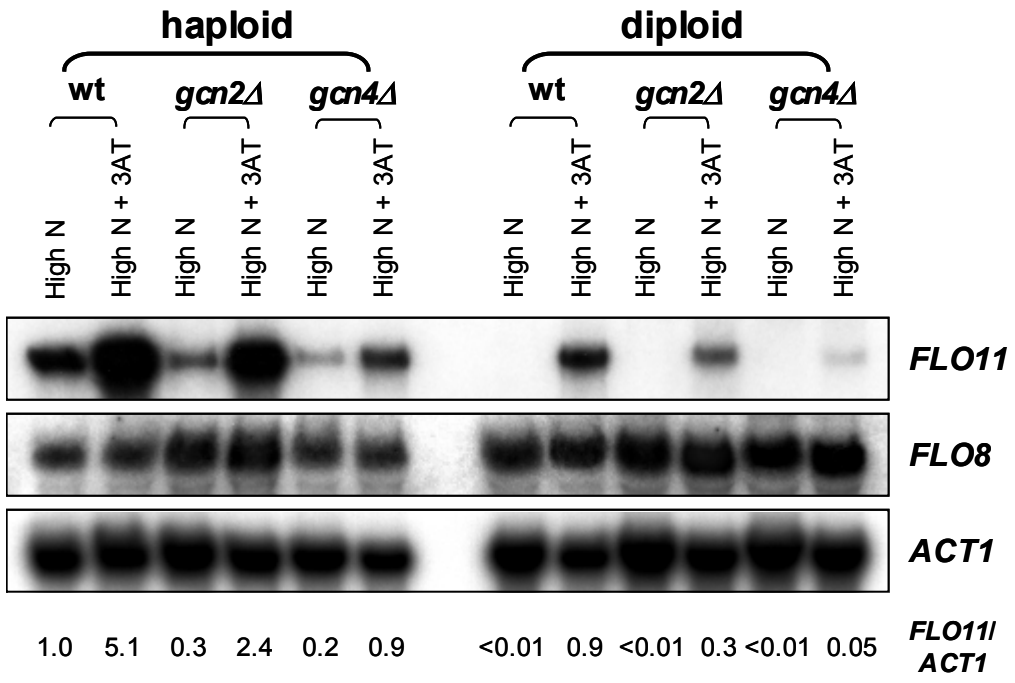


Figure 2. Regulation of *FLO11* expression in different strains.
Expression of *FLO11*, *FLO8* and *ACT1* under different nutritional conditions. Total RNA was prepared from haploid yeast strains RH2648 (wt), RH2649 (*gcn2Δ*), RH2650 (*gcn4Δ*) and the diploid yeast strains RH2656 (wt), RH2657 (*gcn2Δ*), RH2658 (*gcn4Δ*), all carrying the *FLO11* reporter construct B3782 and in non-starvation medium (High N) or starved for amino acids by adding 10 mM 3AT (High N + 3AT). For measurements of *FLO11*, *FLO8* and *ACT1* transcript levels, 10 µg of total RNA from each sample were subjected to a Northern hybridization analysis. Signals were quantified using a BAS-1500 Phosphor-Imaging scanner. Numbers given indicate relative expression levels of *FLO11* when compared to *ACT1* as internal standard and with a value for non-starvation expression of the haploid wild type set to 1.

The tryptophan-derivative 5-methyl-tryptophan (5MT) is a further amino acid analogue known to induce amino acid starvation (Dever, 1997). We also determined the effects of adding 5MT to high ammonium medium on adhesive growth and expression of *FLO11*. Results obtained by addition of 5MT were similar to that obtained by adding 3AT (data not shown).

Results in this section show that amino acid starvation conditions not only causes a strong increase in the expression of *FLO11* in haploid and diploid cells, but also enhances adhesive growth of both cell-types in a *FLO11*-dependent manner.

Haploid adhesive growth and expression of *FLO11* depend on *GCN2* and *GCN4*, elements of the general control system of amino acid biosynthesis.

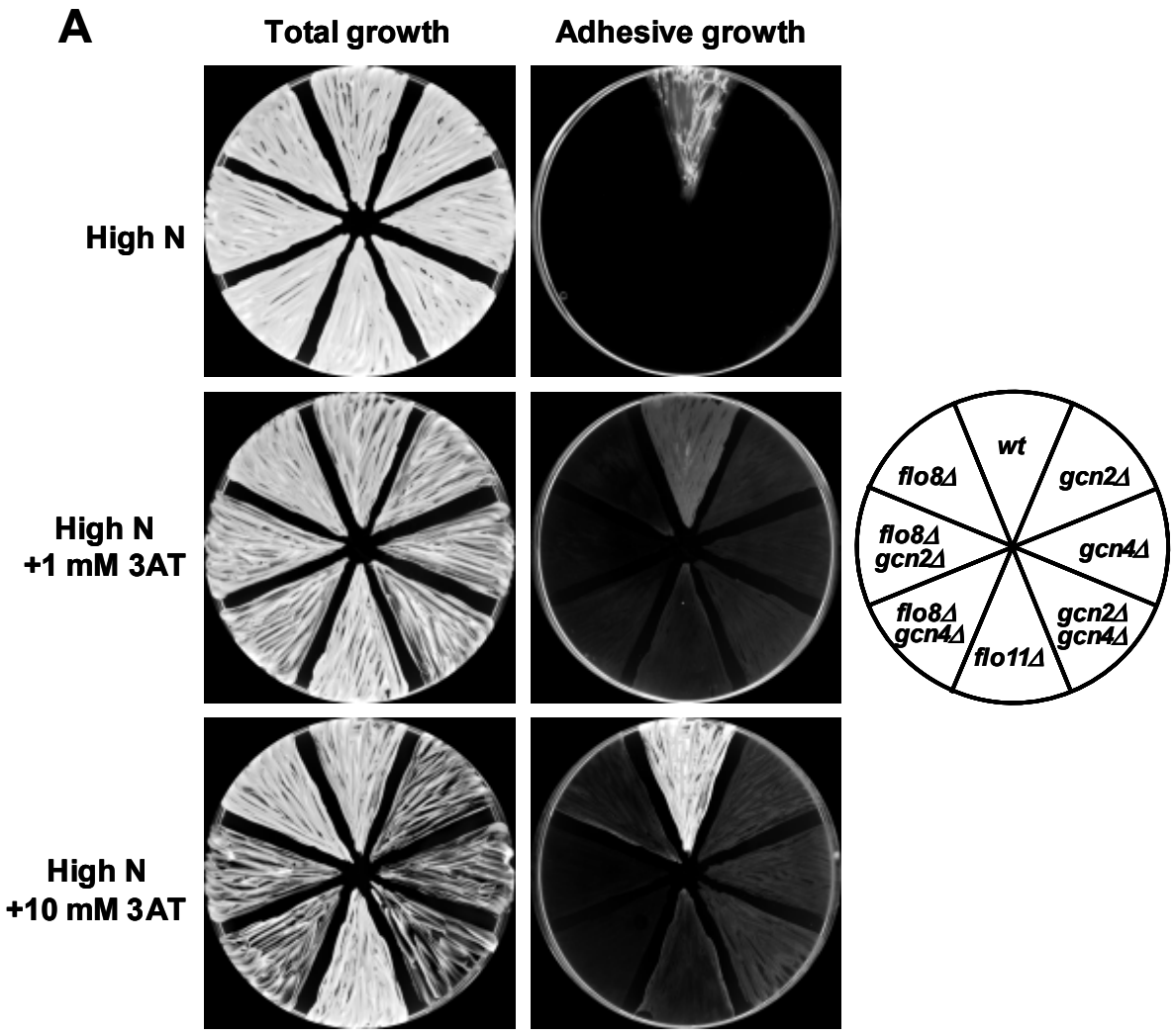
The requirement of the sensor kinase Gcn2p and the transcriptional activator Gcn4p for adhesive growth and expression of *FLO11* was analyzed in haploid *gcn2*Δ, *gcn4*Δ and *gcn2*Δ *gcn4*Δ mutant strains. Under non-starvation conditions, adhesive growth of haploid strains was markedly reduced in the absence of *GCN2* or *GCN4*, although effects were not as pronounced as that found for a *flo11*Δ strain (Figure 3A). The *gcn2*Δ *gcn4*Δ double mutant was indistinguishable from the single mutants. Amino acid starvation was induced by addition of either 1mM 3AT or 10 mM 3AT to the growth medium, because addition of 10 mM 3AT inhibits growth of strains lacking *GCN2* or *GCN4* (Figure 3A). Addition of 1 mM 3AT was sufficient to enhance adhesive growth of a control strain, without inhibiting growth of the *gcn2*Δ or *gcn4*Δ mutant strains (Figure 3A). Under these conditions, adhesive growth of haploid *gcn2*Δ or *gcn4*Δ mutants was reduced, although not to the degree found for a *flo11*Δ mutant.

Strains measured for adhesive growth were further assayed for expression of the *FLO11-lacZ* reporter gene and *FLO11*-transcript levels. Under non-starvation conditions, expression of *FLO11-lacZ* dropped from 52.1 units in a control strain to 11.5 units in the *gcn2*Δ, 11.5 units in the *gcn4*Δ and 11.4 units in the *gcn2*Δ *gcn4*Δ mutant strain, respectively (Figure 3B). This roughly 5-fold decrease in *FLO11-lacZ* expression in the *gcn* mutants correlates with the decrease in both *FLO11*-transcript levels (Figure 2) and the resulting adhesive growth behavior (Figure 3A). To induce amino acid starvation, 3AT was added to strains grown to logarithmic phase at concentrations of both 1 mM and 10 mM. Addition of 1 mM 3AT was sufficient to induce expression of *FLO11-lacZ* to levels (683 units) almost matching that obtained by

the addition of 10 mM 3AT (1059 units). Expression of *FLO11-lacZ* significantly decreased by deletion of *GCN2* (71.5 units), *GCN4* (67.3 units), or both (66.5 units) under 3AT conditions, corresponding to a roughly 16-fold drop in comparison to the expression measured in the control strain. Yet, expression of both *FLO11-lacZ* and *FLO11*-transcripts levels in the starved *gcn* mutant strains still comply with the levels obtained in a control strain under non-starvation conditions, a fact that explains the adhesive growth behavior of these strains on 3AT-containing medium (Figure 2 and Figure 3).

We further tested, whether high-level expression of Gcn4p is sufficient to induce adhesive growth and enhanced expression of *FLO11*. For this purpose, a mutant allele of *GCN4* (*GCN4m*) was expressed that carries point mutations inactivating all four uORFs in the *GCN4* upstream leader and causes high expression of Gcn4p under non-starvation conditions. However, expression of *GCN4m* was not sufficient to induce adhesive growth and did not lead to enhanced expression of *FLO11-lacZ* (data not shown), indicating that Gcn4p might control expression of *FLO11* by an indirect mechanism.

In summary, our results show that Gcn2p and Gcn4p, elements of the general control system of amino acids biosynthesis, are required for adhesive growth and efficient expression of *FLO11* in haploid cells.


A
Total growth
Adhesive growth
High N
High N
+1 mM 3AT
High N
+10 mM 3AT
wt
gcn2Δ
gcn4Δ
gcn2Δ
gcn4Δ
flo11Δ
flo8Δ
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flo8Δ
gcn2Δ
flo8Δ

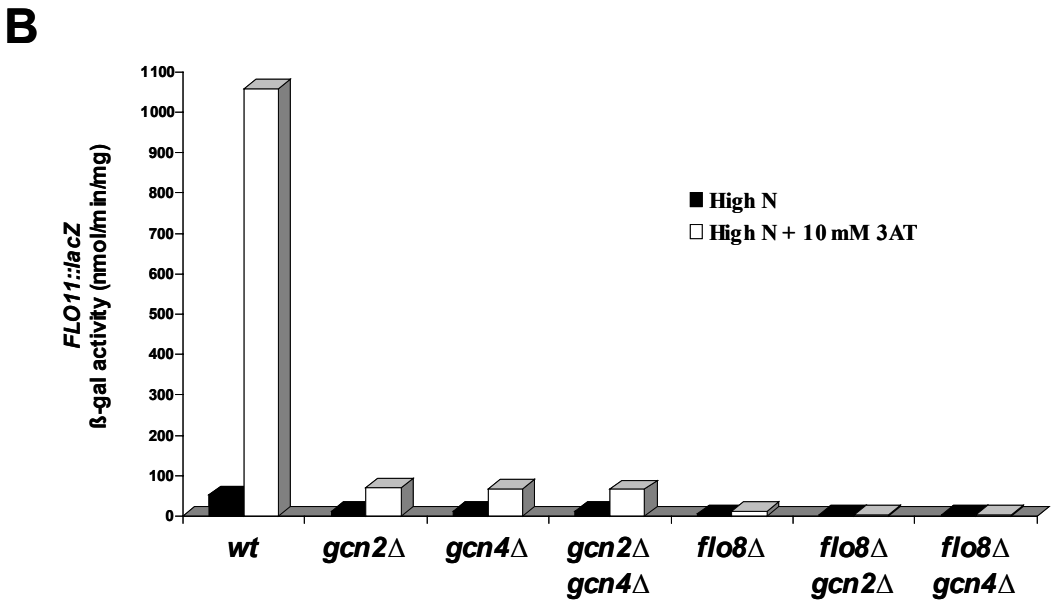

B
FLO11::lacZ
ß-gal activity (nmol/min/mg)
1100
1000
900
800
700
600
500
400
300
200
100
0
High N
High N + 10 mM 3AT
wt
gcn2Δ
gcn4Δ
gcn2Δ
gcn4Δ
flo8Δ
flo8Δ
gcn2Δ
flo8Δ
gcn4Δ

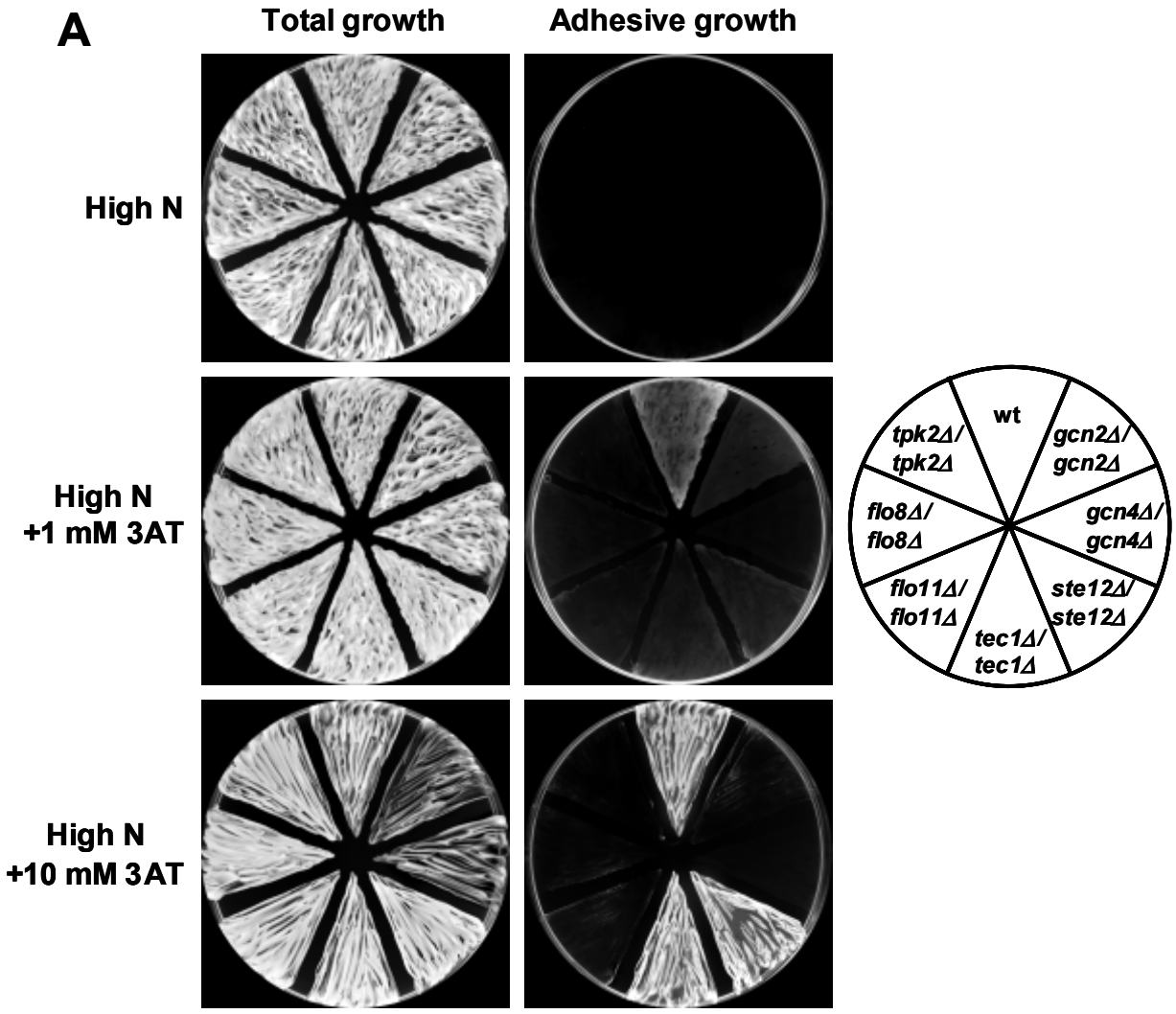
Figure 3. *FLO11* expression and cell adhesion of several haploid mutant strains. (A) The haploid yeast strains RH2648 (wt), RH2649 (*gcn2Δ*), RH2650 (*gcn4Δ*), RH2651 (*gcn2Δ/gcn4Δ*), RH2652 (*flo8Δ*), RH2653 (*flo8Δ/gcn2Δ*) RH2654 (*flo8Δ/gcn4Δ*) and RH2662 (*flo11Δ*), carrying the plasmid B3782 (*FLO11::lacZ* reporter construct) grown under non-starvation (High N) or under amino acid starvation conditions, induced by 1 mM 3AT (High N + 1 mM 3AT) or 10 mM 3AT (High N + 10 mM 3AT). After 24 h incubation (non-starvation plates were incubated 5 d) at 30 °C plates were photographed before (Total growth) and after (Adhesive growth) cells were washed off the agar surface with a stream of water. (B) The same strains as used in (A) were incubated for 8 h in non-starvation (High N) or in amino acid starvation medium, induced by 10 mM 3AT (High N + 10 mM 3AT). After harvesting the ß-galactosidase activity was measured. Bars depict means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15 %.

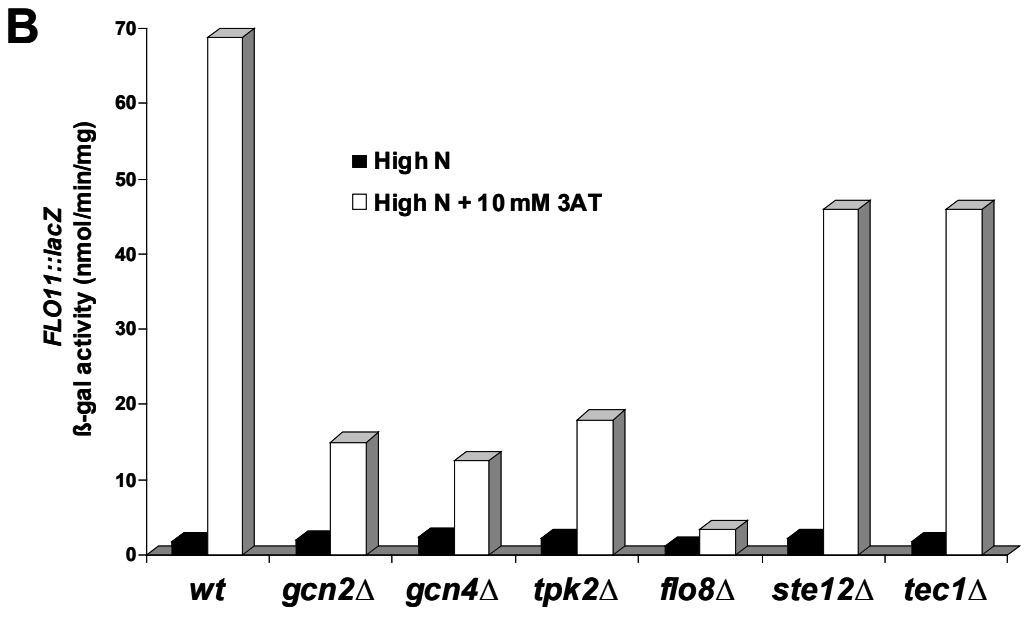
In diploid cells, *GCN4* is required for amino acid starvation-induced adhesive growth and nitrogen starvation-induced pseudohyphal development.

Amino acid starvation-induced adhesive growth, *FLO11-lacZ* expression and *FLO11* transcripts levels were further measured in diploid strains. Diploid *gcn2*Δ*/gcn2*Δ and *gcn4*Δ*/gcn4*Δ mutant strains were indistinguishable from a control strain with regard to adhesive growth behavior, expression of *FLO11-lacZ* and *FLO11* transcript levels under non-starvation conditions (Figure 2 and Figure 4). When starved for amino acids, deletion of either *GCN2* or *GCN4* significantly suppressed adhesive growth, a finding that correlated with a decrease in expression of *FLO11-lacZ* of 4.6-fold in the *gcn2*Δ*/gcn2*Δ strain (15 units) and of 5.5-fold in the *gcn4*Δ*/gcn4*Δ mutant (12.5 units) (Figure 4A and 4B). Concomitantly, *FLO11*-transcripts levels decreased 3-fold in the absence of *GCN2* and 18-fold when *GCN4* was deleted. Thus, amino acid starvation-induced adhesive growth and expression of *FLO11* require *GCN2* and *GCN4* in diploid strains corroborating the data obtained in haploids.

Cell-cell and cell-substrate adhesion are processes essential for the development of pseudohyphal filaments of diploid strains that have been starved for nitrogen (Lo & Dranginis, 1998). Diploid strains lacking *GCN2* or *GCN4* were tested for pseudohyphal development on nitrogen-starvation medium. Only *gcn4*Δ*/gcn4*Δ mutant strains were suppressed for development of pseudohyphae comparable to *flo11*Δ*/flo11*Δ mutant strains (Figure 4C).

In summary, Gcn2p and Gcn4p are required for amino acid starvation-induced adhesive growth in diploids, but for pseudohyphal development, induced by nitrogen starvation, just Gcn4p is necessary.


A
Total growth
Adhesive growth
High N
High N
+1 mM 3AT
High N
+10 mM 3AT
wt
gcn2Δ/
gcn2Δ
gcn4Δ/
gcn4Δ
ste12Δ/
ste12Δ
tec1Δ/
tec1Δ
flo11Δ/
flo11Δ
flo8Δ/
flo8Δ
tpk2Δ/
tpk2Δ


B
FLO11::lacZ
ß-gal activity (nmol/min/mg)
70
60
50
40
30
20
10
0
High N
High N + 10 mM 3AT
wt
gcn2Δ
gcn4Δ
tpk2Δ
flo8Δ
ste12Δ
tec1Δ

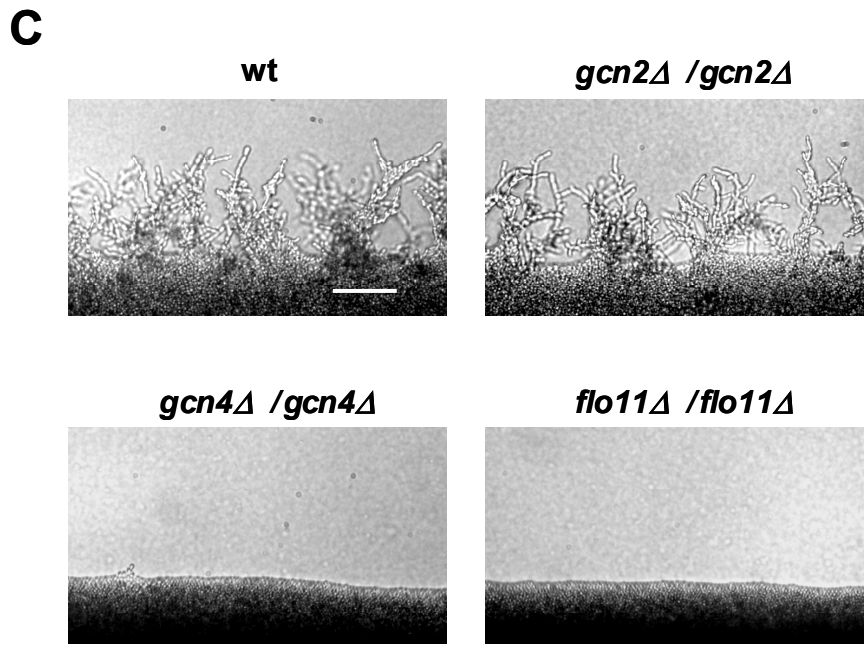


Figure 4. *FLO11* expression and cell adhesion of several diploid mutant strains. (A) The diploid yeast strains RH2656 (wt), RH2657 (*gcn2Δ/gcn2Δ*), RH2658 (*gcn4Δ/gcn4Δ*), RH2659 (*tpk2Δ/tpk2Δ*), RH2660 (*flo8Δ/flo8Δ*), RH2661 (*flo11Δ/flo11Δ*), L5627 (*ste12Δ/ste12Δ*) and HMC267 (*tec1Δ/tec1Δ*), carrying the plasmid B3782 (*FLO11::lacZ* reporter construct) grown under non-starvation (High N) or under amino acid starvation conditions, induced by 1 mM 3AT (High N + 1 mM 3AT) or 10 mM 3AT (High N + 10 mM 3AT). After 24 h incubation (non-starvation plates were incubated 5 d) at 30 °C plates were photographed before (Total growth) and after (Adhesive growth) cells were washed off the agar surface with a stream of water. (B) The same strains as used in (A) were incubated for 8 h in non-starvation (High N) or in amino acid starvation medium, induced by 10 mM 3AT (High N + 10 mM 3AT). After harvesting the ß-galactosidase activity were measured. Bars depict means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15 %. (C) The diploid strains RH2656 (wt), RH2657 (*gcn2Δ/gcn2Δ*), RH2658 (*gcn4Δ/gcn4Δ*) and RH2661 (*flo11Δ/flo11Δ*), carrying the plasmid B3782 were streaked out on SLAD plates for induction of pseudohyphal growth. After 3 d of incubation at 30 °C pictures were taken. Scale bar represents 50 µm.

Amino acid starvation-induced expression of *FLO11* requires the transcription factors Gcn4p and Flo8p, but not Ste12p and Tec1p.

Adhesive growth and expression of *FLO11* are under control of the cAMP pathway and the MAPK pathway (Pan *et al*., 2000; Rupp *et al*., 1999). We tested, whether amino acid starvation-induced adhesive growth and expression of *FLO11* requires Tpk2p or Flo8p, elements of the cAMP-regulated pathway, or the transcription factors Ste12p and Tec1p, elements of the MAPK pathway. Adhesive growth of diploid strains lacking *TPK2* or *FLO8* was suppressed to a degree comparable to a *flo11*Δ*/flo11*Δ control strain under amino acid starvation conditions (Figure 4A). Expression of *FLO11-lacZ* was reduced 3.8-fold in *tpk2*Δ*/tpk2*Δ strains (23 units) and 20-fold in *flo8*Δ*/flo8* mutants (3.4 units). In comparison, deletion of *STE12* or *TEC1* did not suppress adhesive growth in the presence of 10 mM 3AT, and expression of *FLO11-lacZ* was reduced only 1.4-fold in both the *ste12*Δ*/ste12*Δ (46 units) and *tec1*Δ*/tec1*Δ (46 units) mutant strains (Figure 4B). Moreover, expression of neither *STE12* nor *TEC1* is under the control of amino acid starvation or Gcn4p (data not shown). Thus, efficient expression of *FLO11* under amino acid starvation conditions requires *FLO8* and *TPK2*, but not *STE12* and *TEC1*.

3AT-induced adhesive growth and expression of *FLO11-lacZ* was measured in haploid *flo8*Δ *gcn2*Δ and *flo8*Δ *gcn4*Δ double mutant strains and compared to *gcn2*Δ, *gcn4*Δ and *flo8*Δ single mutants, to distinguish between a parallel and a linear configuration of the general control system and the transcription factor Flo8p. Under amino acid starvation conditions, both adhesive growth and expression of *FLO11-lacZ* is lower in the *flo8*Δ *gcn2*Δ (4 units) and *flo8*Δ *gcn4*Δ (2.9) double mutants than in the *gcn2*Δ (71.5 units), *gcn4*Δ (67.3 units) or *flo8*Δ (13 units) single mutants (Figure 3). The additive effects of the *gcn2* and *flo8*Δ or *gcn4*Δ and *flo8*Δ mutations argue for independent functions of the general control system and Flo8p. This conclusion is supported by the fact that transcript levels of *FLO8* are not affected by amino acid starvation or by mutations in *GCN2* or *GCN4* (Figure 2). Expression of *FLO11-lacZ* is completely blocked in the *flo8*Δ *gcn4*Δ mutant strain (378-fold decrease) indicating that Gcn4p and Flo8p are the main regulators that couple amino acid starvation to the expression of *FLO11*.

In summary, amino acid starvation-induced expression of *FLO11* requires the combined action of the transcription factors Gcn4p and Flo8p, but does not depend on Ste12p and Tec1p.

Identification of *FLO11* promoter elements mediating regulation by amino acid starvation.

A set of 15 *flo11-lacZ* promoter deletion constructs spanning the region between the 3000 bp upstream of the *FLO11* initiation codon was used (Rupp *et al*., 1999), to identify *FLO11* promoter elements that confer regulation of *FLO11* expression in response to amino acid starvation. Expression of this set of *flo11-lacZ* reporter constructs, each containing an individual 200 bp deletion, was assayed in haploid and diploid strains under both non-starvation and amino acid starvation conditions and compared to the intact *FLO11-lacZ* reporter (Table 3). A deletion was assigned to contain a UAS (upstream activation site) when leading to at least 50 % reduced expression of *FLO11-lacZ*, and as a URS (upstream repression site) when causing at least 3-fold enhanced expression (Figure 5A).

The *flo11-1*, *flo11-2*, *flo11-3*, *flo11-5* and *flo11-6* deletions define at least five UAS elements under both non-starvation and amino acid starvation conditions in haploids and diploids. In diploid strains, *flo11-15* acts as a 3AT-specific UAS element, because its deletion (bp -2800 to -3000) led to a 4.3-fold reduced expression of *FLO11* in the presence of 3AT, but only a 1.5-fold reduction under non-starvation conditions. UAS sites defined by *flo11-5* and *flo11-6* might confer amino acid starvation signals to expression of *FLO11*, because their deletion drastically blocked activation by 3AT. However, expression of *flo11-5* and *flo11-6* is drastically reduced also under non-starvation conditions pointing to more general function of the UAS elements within these deletions. The UAS sites in *flo11-1*, *flo11-2* and *flo11-3* are not likely to be involved in amino acid starvation-induced activation, because their absence does not suppress induction of *FLO11* expression by 3AT.

Table 3. Relative ß-galactosidase activity of 15 *FLO11::lacZ* reporter constructs, each containing a different 200 bp deletion in the 3 kb segment in the 5'region upstream of the putative ATG that initiates translation

	*MAT***a**			*MAT***a**/α		
	non-starv	**3AT**	**3AT/ non-starv**	**non-starv**	**3AT**	**3AT/ non-starv**
flo11-1	<0.1	<0.1	**17.8**	<0.1	<0.1	**8.0**
flo11-2	0.3	0.3	**20.7**	0.4	0.1	**11.1**
flo11-3	0.3	0.3	**21.9**	0.4	0.1	**10.6**
flo11-4	3.8	1.6	**8.6**	1.6	2.9	**72.9**
flo11-5	0.3	<0.1	**0.7**	<0.1	<0.1	**5.0**
flo11-6	<0,1	<0.1	**3.6**	0.4	<0.1	**2.7**
flo11-7	7.0	4.3	**12.7**	7.6	5.5	**29.2**
flo11-8	7.2	0.4	**1.1**	77.5	1.3	**0.7**
flo11-9	0.6	0.6	**22.1**	0.6	0.6	**38.2**
flo11-10	1.0	1.0	**20.1**	0.5	0.5	**45.1**
flo11-11	1.2	1.3	**20.9**	0.5	0.8	**67.8**
flo11-12	1.3	1.1	**18.6**	0.6	0.6	**37.5**
flo11-13	0.9	0.9	**20.4**	0.7	0.8	**45.0**
flo11-14	2.1	1.2	**12.2**	1.2	1.2	**38.8**
flo11-15	0.7	0.9	**23.9**	0.6	0.2	**14.6**
FLO11	1.0	1.0	**20.3**	1.0	1.0	**40.5**
units	52.1	1059.1		1.7	68.8	

The haploid strain RH2648 (*MAT***a**) and the diploid strain RH2656 (*MAT***a**/α) carrying several *FLO11::lacZ* reporter constructs were incubated under non-starvation conditions (non-starv) and amino acid starvation conditions (3AT). The actual units (nmol/min/mg) are provided in parentheses for each strain containing the intact *FLO11::lacZ* reporter constructs. In the column 3AT/non-starv, the induction of ß-galactosidase expression during amino acid starvation is shown, by dividing the 3AT through the non-starv data. The data means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15 %.

Three URS elements are defined by the *flo11-4*, *flo11-7* and *flo11-8* deletions. The URS element within *flo11-8* (bp -1400 to -1600) appears to act as a repression site that is inactivated by amino acid starvation, because its deletion in diploids led to a 77-fold induction of *FLO11-lacZ* expression in the absence of 3AT, but only a 1.3-fold increase in the presence of 3AT. In addition, expression of *flo11-8* was no longer inducible by the addition of 3AT. A similar expression pattern for *flo11-8* was observed in diploid strains. Deletion of *flo11-4* revealed a further URS element that in is inactivated by 3AT in haploid cells. The URS site in *flo11-7* appears not to be 3AT-specific, because its deletion led to an at least 4.3-fold induction of *FLO11* expression under all conditions measured. Taken together, the *FLO11* promoter contains at least one 3AT-responsive

URS element in segment *FLO11-8* that confers enhanced expression of *FLO11* in both haploid and diploid cells.

Table 4. ß-galactosidase assays of individual 400 bp *FLO11* promoter elements cloned into the UAS of a *CYC1::lacZ* reporter construct.

	non-starv			3AT			3AT/non-starv		
	wt	***gcn4Δ***	***flo8Δ***	**wt**	***gcn4Δ***	***flo8Δ***	**wt**	***gcn4Δ***	***flo8Δ***
FLO11	***1.7***	***2.4***	***1.2***	***68.8***	***12.5***	***3.4***	40.5	3.1	2.8
no insert	7.1	6.9	7.0	7.7	7.2	7.3	1.1	1.0	1.0
2/1	3.3	2.2	3.0	2.6	3.1	3.0	0.8	1.4	1.0
3/2	41.6	8.3	4.9	47.2	22.2	6.1	1.1	2.7	1.2
4/3	5.4	1.9	4.4	9.8	4.3	6.0	1.8	2.3	1.4
5/4	3.9	4.2	2.6	8.7	3.9	2.7	2.2	0.9	1.0
6/5	234.6	111.5	5.6	1143.0	320.3	28.2	4.9	2.9	5.0
7/6	379.3	181.4	6.6	999.5	332.5	32.5	2.6	1.8	4.9
8/7	10.8	4.0	2.7	14.4	5.8	3.9	1.3	1.5	1.4
9/8	8.6	5.0	21.0	20.6	8.4	13.3	2.4	1.7	0.6
10/9	40.4	31.9	24.6	265.9	62.7	119.6	6.6	2.0	4.9
11/10	43.4	24.4	29.0	109.5	81.4	59.5	2.5	3.3	2.1
12/11	11.3	2.8	6.8	10.3	2.8	6.7	0.9	1.0	1.0
13/12	2.1	1.2	1.5	2.0	1.8	2.2	1.0	1.5	1.5
14/13	1.7	1.1	1.4	1.6	1.9	1.5	1.0	1.7	1.0
15/14	2.1	1.3	2.0	1.7	2.1	2.3	0.8	1.6	1.2

The diploid strains RH2656 (wt), RH2658 (*gcn4Δ*) and RH2660 (*flo8Δ*) carrying several *FLO11::lacZ* reporter constructs or pLG669Z (no insert), were incubated under non-starvation conditions (non-starv) and amino acid starvation conditions induced by adding 10 mM 3AT (3AT). The received ß-galactosidase units (nmol/min/mg) are presented in the table. In the columns 3AT/non-starv, the induction of ß-galactosidase expression during amino acid starvation is shown, by dividing the 3AT through the non-starv data of each strain. The data means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15 %.

UAS elements that mediate regulation by amino acid starvation were identified in a second approach using a series of 14 reporter constructs containing individual 400 bp *FLO11* promoter fragments that overlap by 200 bp and are cloned in front of a *CYC1::lacZ* fusion gene (Rupp *et al*., 1999). This series of reporter constructs identified UAS elements in the segments *FLO11-3/2* (bp -600 to -200), *FLO11-6/5* and *FLO11-7/6* that overlap between bp -1200 and -1000, and *FLO11-10/9* and *FLO11-11/10* that overlap between bp -2000 and -1800 (Table 4; Figure 5B). These *FLO11* promoter elements increase expression of *CYC1::lacZ* by a factor of more than 2 under both non-starvation and amino acid starvation conditions as compared with the reporter without

any insert. The activity of *FLO11-6/5*, *FLO11-7/6*, *FLO11-10/9* and *FLO11-11/10* is induced more than 2-fold by addition of 3AT suggesting that elements *FLO11-6* and *FLO11-10* contain UAS elements that mediate activation in response to amino acid starvation. An additional UAS elements was identified by *FLO11-9/8*. However, *FLO11-9/8* conferred UAS activity only in the presence of 3AT pointing to a specific role of segments *FLO11-8* and *FLO11-9* in coupling amino acid starvation to expression of *FLO11*. Taken together, at least three 3AT-responsive UAS elements were identified in the *FLO11* promoter in the segments *FLO11-6*, *FLO11-9/8* and *FLO11-10* (Figure 6).

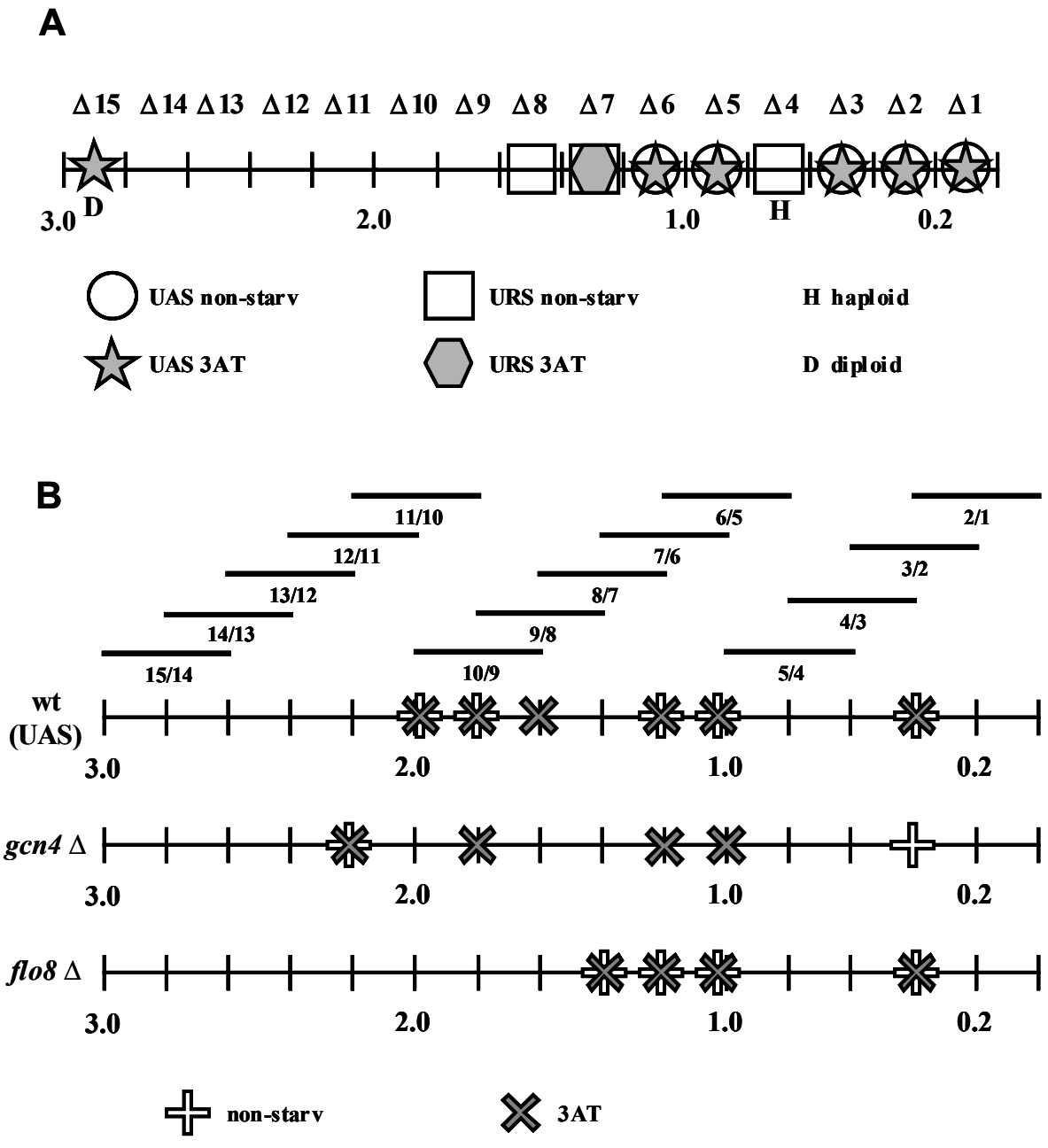


Figure 5. Sequence elements involved in regulation of *FLO11*.
(A) The ß-galactosidase activities of 15 individual 200 bp deletions of the *FLO11* promoter region were compared with the full-length 3 kbp promoter under non-starvation (non-starv) and amino acid starvation conditions (3AT). A ratio of ß-galactosidase activity (deletion/3 kbp promoter) of 3 or higher led to that deletion's association with a URS (URS elements under non-starvation conditions are marked by a square and under starvation conditions by a hexagon). At least 50 % lower expression than the wild type reporter is represented as a UAS (UAS elements under non-starvation conditions are marked by a circle and under starvation conditions by a star). Elements, which occur only in the haploid strain are marked by a H and elements only occur in the diploid strain are marked by a D. (B) The ß-galactosidase activity of 15 isolated 400 bp elements of the *FLO11* promoter region, that activate a *CYC1::lacZ* reporter, was determined in wild type cells or in cells deleted for the transcription factors *GCN4* or *FLO8* under non-starvation or amino acid starvation conditions. The symbols (white cross = non-starvation conditions, gray cross = starvation conditions) are placed on a line in apposition that indicates, which of the 400 bp fragments stimulated ß-galactosidase activity. Each line represents the *FLO11* promoter in a different genetic background. The first row [denoted wt (UAS)] denotes sequence elements showing a > 3-fold elevation of the reporter over a plasmid without an insert as measured in wild type cells. The next two lines, *gcn4Δ* and *flo8Δ*, represented sequence elements showing a > 3-fold reduction of the ß-galactosidase activity in the mutant (e.g. *gcn4Δ*) as compared with the activity of the element in the wild type cells.

***FLO11* promoter elements mediating regulation by Gcn4p and Flo8p in response to amino acid starvation.**

Activation of *FLO11-lacZ* expression by 3AT is completely blocked when both Gcn4p and Flo8p are absent (Figure 4). To identify the regions of the *FLO11* promoter that are targeted by Gcn4p and Flo8p, the set of 400 bp reporter constructs was transformed into strains deleted for *GCN4* or *FLO8*. Deletion of *GCN4* led to a more than 3-fold reduction in the expression of *FLO11-3/2* and *FLO11-12/11* under non-starvation conditions and of *FLO11-6/5*, *FLO11-7/6*, *FLO11-10/9* and *FLO11-12/11* in cells starved for amino acids (Table 4; Figure 5B). These results suggest that Gcn4p controls expression of *FLO11* by two distinct mechanisms that depend on distinct promoter elements. First, Gcn4p is involved in activation of *FLO11* expression in response to amino acid starvation, a mechanism that depends on *FLO11-6* and *FLO11-10/9*, elements that are responsive to 3AT. In addition, Gcn4p regulates basal expression of *FLO11*, a regulatory mechanism that involves *FLO11-3/2* and *FLO11-12/11*, segments that do not confer induction by 3AT.

Under non-starvation conditions, deletion of *FLO8* had significant effects on expression of *FLO11-3/2*, *FLO11-6/5*, *FLO11-7/6* and *FLO11-8/7*, regions of the *FLO11* promoter that were previously identified to be under control of Flo8p (Rupp *et*

al., 1999). Under amino acid starvation, deletion of *FLO8* significantly affected expression of the same four elements, *FLO11-3/2*, *FLO11-6/5*, *FLO11-7/6* and *FLO11-8/7*. However, Flo8p does not appear to be involved in mediating amino acid starvation signals to these elements, because *FLO11-3/2* and *FLO11-8/7* are not inducible by 3AT, and absence of *FLO8* did not suppress activation of *FLO11-6/5* and *FLO11-7/6* by amino acid starvation.

Discussion

Cell-cell and cell-substrate adhesion are morphogenetic events that are required for several developmental processes of bakers' yeast including mating, invasive growth, biofilm formation and filamentation (Erdman *et al*., 1998; Guo *et al*., 2000; Mösch, 2000; Reynolds & Fink, 2001). Each of these events depends on distinct intrinsic and extrinsic signals, corresponding signaling pathways and specific cell surface proteins (Guo *et al*., 2000). The cell surface flocculin Flo11p is required for haploid adhesive growth in response to glucose starvation and for diploid filamentous growth in response to nitrogen starvation (Cullen & Sprague, 2000; Lo & Dranginis, 1998; Madhani, 2000). Here, we found that starvation for amino acids activates adhesive growth and expression of *FLO11* in the presence of glucose and ammonium. Activation depends on Flo11p, indicating that the phenotype is mainly established by activation of *FLO11* gene expression. We have identified at least three distinct types of regulatory elements in the *FLO11* promoter that are involved in 3AT-induced regulation. (i) Two UAS elements that confer regulation by amino acid starvation, termed UAS^R, are found in segments *FLO11-6* and *FLO11-10* and depend on Gcn4p for regulation by 3AT (see discussion below). (ii) One UAS^R element that confers Gcn4p-independent activation by 3AT is present in segment *FLO11-9*. (iii) One URS element that is inactivated by amino acid starvation is present in segment *FLO11-8* (Figure 6).

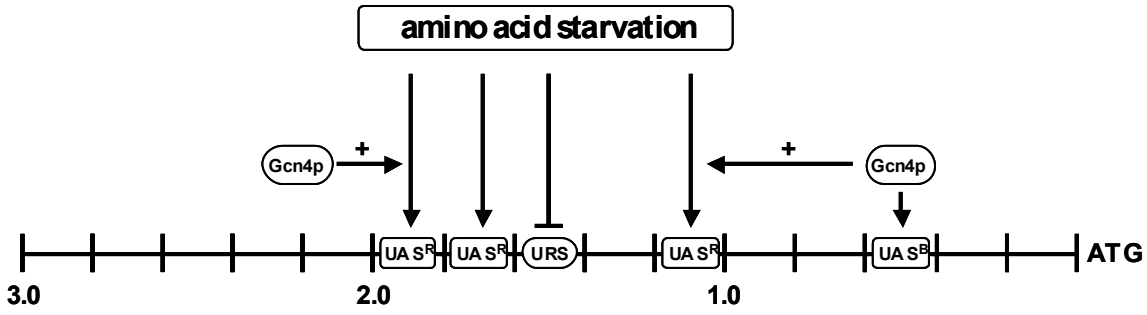


Figure 6. Model for the regulation of *FLO11* under amino acid starvation conditions.
The model shows the 3 kb promoter of *FLO11* containing the identified UAS and URS elements, which were Gcn4p and/or amino acid starvation dependent. UAS elements that confer regulation by amino acid starvation, termed UAS^R, and UAS^B, if it is also a UAS element under non-starvation conditions.

What factors transmit the amino acid starvation signal to *FLO11*? Our study has not uncovered the regulatory proteins that directly bind to the *FLO11* promoter. However, we have identified the cAMP pathway to be required for activation of *FLO11*. How Tpk2p affects stimulation of *FLO11* expression in response to the amino acid starvation signal remains unclear. The transcription factor Flo8p is required for cAMP-mediated *FLO11* transcription (Rupp *et al*., 1999). We found that Flo8p is also required for 3AT-induced expression of *FLO11*. However, Flo8p does not appear to be involved in mediating the amino acid starvation signal, because absence of *FLO8* did not suppress activation of any of the UAS^R elements in response to 3AT. Our study excludes involvement of the MAPK cascade, the second major signaling pathway previously known to affect *FLO11* expression (Rupp *et al*., 1999), from mediating the amino acid starvation signal to *FLO11*, because neither Ste12p nor Tec1p are required for activation by 3AT.

We identified Gcn2p and Gcn4p, elements of the general control system for amino acid biosynthesis, to be involved in adhesive growth and regulation of *FLO11* expression. Gcn2p and Gcn4p regulate adhesion mainly by control of *FLO11* expression, because adhesive growth defects caused by deletion of *GCN2* and *GCN4* equal that observed by deletion of *FLO11*. The general control system was previously unknown to regulate adhesive growth. In contrast, its function in coupling amino acid starvation to enhanced expression of biosynthetic genes is known in great molecular detail (Hinnebusch, 1997). In response to amino acid starvation, the sensor kinase Gcn2p phosphorylates the translation elongation factor eIF-2α leading to efficient translation of *GCN4* mRNA. In turn, Gcn4p activates transcription of amino acid biosynthetic genes by direct promoter binding at sequence-specific GCRE sites. How are Gcn2p and Gcn4p involved in coupling amino acid starvation to enhanced expression of *FLO11*? Several observations suggest that expression of *FLO11* does not involve direct binding of Gcn4p to the *FLO11* promoter and that an increase in protein levels of Gcn4p *per se* is not sufficient for enhanced *FLO11* transcription: (i) Sequence analysis of the *FLO11* promoter does not predict any GCRE sites. (ii) Gcn4p protein purified from *E. coli* does not bind to any region of the *FLO11* promoter when tested *in vitro* (our unpublished results). (iii) High-level expression of Gcn4p in non-starved cells is not sufficient to induce enhanced expression of *FLO11*. However, Gcn4p controls the activity of three UAS elements identified in the *FLO11* promoter, even though it does

not regulate *FLO11* expression by direct promoter binding at GCREs. Gcn4p is required for the activity of two of the UAS elements that confer activation by amino acid starvation, termed UAS^R, in the segments *FLO11-6* and *FLO11-10*, and one UAS element that does not confer regulation by 3AT, termed UAS^B, in the segment *FLO11-3* (Figure 6). The following model for the regulation of *FLO11* expression by Gcn4p accounts for these findings. Under non-starvation conditions, basal levels of Gcn4p are required for expression of factors that regulate the UAS^B element in segment *FLO11*-3 and to a minor extent for factors that regulate the UAS^R elements in *FLO11-6* and *FLO11-10*. In response to amino acid starvation, high levels of Gcn4p levels are not sufficient for enhanced *FLO11* transcription, but Gcn4p is required for expression of the factors that mediate the signal to the UAS^R elements (Figure 6). In this scenario, the role of Gcn2p is to assure basal expression of Gcn4p under both non-starvation and amino acid starvation conditions, because absence of Gcn2p is known to cause a drop in intracellular levels of Gcn4p (Hinnebusch, 1997).

Under nitrogen starvation conditions, the expression of *GCN4* is strongly repressed, resulting in a very low intracellular Gcn4p level (Grundmann *et al*., 2001), which is sufficient but also necessary for pseudohyphal development (Figure 4C). Although Gcn2p is necessary for adhesive growth under amino acid starvation conditions, it is not required for pseudohyphal growth during nitrogen limitation. Upon nitrogen starvation the *GCN4* expression is lower than the basal expression under non-starvation conditions, so that the influence of Gcn2p on *GCN4* expression is so low, that apparently the concentration of *GCN4* is not important but rather the fact that Gcn4p is present. This is supported by the observation that an overexpression of *GCN4* did not lead to an improved pseudohyphal growth (data not shown).

Our study suggests that the amino acid starvation signal is mediated to *FLO11* by a signaling pathway that is distinct from the general control system. Functionality of this pathway, however, requires Gcn4p. The fact that basal levels of Gcn4p are required for signaling in response to nutrient limitation is further supported by our finding that pseudohyphal development requires Gcn4p. In contrast to amino acid starvation, nitrogen starvation leads to a decrease in intracellular Gcn4p pools, although basal levels remain detectable (Grundmann *et al*., 2001). Thus, basal amounts of Gcn4p are not only required for adhesive growth in response to starvation for amino acids, but as well for pseudohyphal development in response to nitrogen starvation. What signaling

genes might be under control of Gcn4p? Computer analysis of the entire *S. cerevisiae* genome has identified more than 80 target genes for Gcn4p, based on the presence of at least three GCRE sites in their promoter region (Schuldiner *et al*., 1998). These genes include *TOR1*, *NPR1* and *URE2*, elements of the TOR signaling pathway known to control cell growth and development in response to nutrients (Cardenas *et al*., 1999). Whether expression of *TOR1*, *NPR1* or *URE2* is regulated by Gcn4p, and whether the TOR pathway couples amino acid starvation to enhanced expression of *FLO11* and adhesive growth, has not yet been determined. The plasma membrane Ssy1p-Prt3p-Ssy5p sensor of amino acids is a further system that mediates nutritional signals in yeast (Forsberg & Ljungdahl, 2001). Deletion of *SSY1* or *PTR3* enhances adhesive growth in haploid strains (Forsberg & Ljungdahl, 2001). The possibility that the Ssy1p-Prt3p-Ssy5p system is involved in induction of *FLO11* gene expression and adhesive growth in response to amino acid starvation remains to be investigated.

Here, we found that starvation for amino acids activates adhesive growth and expression of *FLO11* in the presence of glucose and ammonium. These growth conditions reflect the nutritional situation of yeast cells that grow on fruits, a natural habitat of *S. cerevisiae*. Fruits are rich in carbon sources such as saccharose or glucose and contain a variety of nitrogen sources at ample concentrations (Bisson, 1991). The content of different amino acids, however, is highly unbalanced in fruits. In grapes, an important natural substrate for *S. cerevisiae,* proline and arginine are the predominant amino acids, and their concentration often exceeds that found for histidine by 10 to 100 times (Huang & Ough, 1989). External amino acid imbalance is one of the signals that activate the general control system in *S. cerevisiae* (Niederberger *et al*., 1981). In conclusion, our study suggests that starvation for amino acids rather than a general lack of carbon or nitrogen sources is the signal that activates cell-cell and cell-surface adhesion of yeast living on the natural habitat.

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Chapter IV

Genome-wide transcription profile analysis of Gcn4p-dependent gene transcription in *Saccharomyces cerevisiae*

Summary

In the bakers' yeast *Saccharomyces cerevisiae*, more than 50 genes involved in amino acid, as well as tRNA and nucleotide biosynthesis are transcriptionally activated by Gcn4p in response to amino acid starvation conditions. A genome-wide transcription profile analysis was performed in order to identify further genes regulated by Gcn4p under these conditions. We detected that upon amino acid starvation, the expression of about 200 further genes was Gcn4p-dependent elevated. Most of those genes do not possess a Gcn4p binding sites in its promoter region, implicating an indirect effect of Gcn4p on transcription activation. In addition to the previously identified amino acid metabolic genes we detected several genes involved in carbon, phosphate and fatty acid metabolism to be regulated by Gcn4p. As for amino acid biosynthesis, precursors generated in the other metabolisms are absolutely required, this seems to be an efficient mechanism of regulation. Apart from this, Gcn4p was found to regulate transcription of genes involved in cellular structure, cell adhesion and differentiation. Thus, in *S. cerevisiae* the transcriptional activator Gcn4p might play a more general role in gene expression than previously anticipated.

Introduction

In yeast, it is well established that regulation of gene expression occurs primarily via transcriptional control through interactions between promoter and transcriptional regulators (Guarente, 1992; Keaveney & Struhl, 1998). Transcription abundance, a function of transcript synthesis and degradation, provides an estimate of the gene product titer. Thus, accurate measurement of mRNA levels could reveal expression alterations caused by environmental challenges. In the past, Northern hybridizations and promoter-reporter fusion constructs have allowed simultaneous measurement of small amounts of mRNA species. Now, DNA array analysis facilitates assessment of the regulation of a large number of genes in response to a given stimulus or genetic alteration (Jia *et al*., 2000; Sudarsanam *et al*., 2000). The use of this technique for genome-wide transcription profile analysis should reveal further genes regulated in a Gcn4p-dependent manner.

Under amino acid starvation conditions, in *Saccharomyces cerevisiae* a system is induced, which activates more than 50 genes involved in several different amino acid biosynthetic pathways, as well as tRNA- and purine biosynthesis (Hinnebusch, 1992). This system is called “general control of amino acid biosynthesis” or in short “general control”. The general control is activated if just one amino acid is missing or if an amino acid imbalance prevails (Hinnebusch, 1992). The sensor kinase of the system, Gcn2p, detects the amount of uncharged tRNAs in the cell (Hinnebusch, 1997), which occur, when cells are starved for one or several amino acids. Gcn2p then phosphorylates eIF-2, resulting in a delayed reactivation of the small subunit of the ribosome during translation. The unusual arrangement of the *GCN4* mRNA, having four small upstream open reading frames (uORFs) in front of the *GCN4* structural gene, leads to an improved Gcn4p expression, if reinitiation of the ribosome’s 40S subunit is retarded. Gcn4p, the transcription factor of the general control, then activates the transcription of its target genes, most of them involved in amino acid biosynthesis.

GCN4 encodes a polypeptide comprising 281 amino acids, which is the prototype of the family of alkaline leucine zipper transcription factors (Hinnebusch, 1984; Thireos *et al*., 1984). The sixty C-terminal amino acids are sufficient for dimerization and DNA binding (Hope & Struhl, 1986). Gcn4p specifically binds as homodimer on a 9 bp palindromic nucleotide sequence (5’-ATGA(C/G)TCAT-3’) called Gcn4-Protein

Responsive Element (GCRE) (Arndt & Fink, 1986; Hope & Struhl, 1987; Oliphant *et al*., 1989). Recent investigations of Hollenbeck & Oakley (2000) revealed, that *in vitro* a homodimer of Gcn4p can also bind to a GCRE half site with high affinity. 81 genes of *S. cerevisiae* have at least three of those GCRE sites in the promoter region (Schuldiner *et al*., 1998). Nevertheless, it was also found that Gcn4p can activate the target gene transcription in a GCRE site independent manner (Grundmann *et al*., 2001).

As *S. cerevisiae* is able to synthesize all 20 amino acids *de novo*, it does not suffer amino acid deficiency under laboratory conditions The basic transcription rate of the amino acid biosynthesis genes is high enough to supply the cell with all amino acids even during growth on minimal medium. Therefore, analogues of amino acid precursors are used to initiate amino acid limitation artificially. The most frequently substance applied is 3-amino-triazole (3AT), an analogue in histidine biosynthesis (Dever, 1997).

In this study we found that more than 200 genes were transcriptionally activated in a Gcn4p-dependent manner under amino acid starvation conditions. Besides several genes of amino acid biosynthesis, which were already described to be Gcn4p-dependent activated, genes involved in almost the complete cellular metabolism could be identified, such as carbon, phosphate and fatty acid metabolic genes. Additionally, a Gcn4p-dependent activation of genes encoding proteins determining cell structure, cell adhesion and chromatin structure was detected.

Materials & Methods

Yeast strains and growth conditions.

The two isogenic strains RH2656 (*MAT***a**/α *ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/HIS3*) and RH2658 (*MAT***a**/α *ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/HIS3 gcn4Δ::LEU2/gcn4Δ::LEU2*) used in this study are derivatives of the *Saccharomyces cerevisiae* strain background Σ*1278b*. The plasmid pRS316 was introduced in either strain using the lithium-acetate yeast transformation method (Ito *et al*., 1983). Strains were cultivated in liquid synthetic minimal medium with added arginine (YNB) at 30°C overnight, diluted and cultivated in YNB containing 10 mM 3-amino-triazole (3AT) for 8 hours before isolation of total RNA was performed. Solid media were prepared by using 2 % (w/v) agar.

Miniarray filter membrane hybridization.

Yeast total RNA was isolated following the protocol described by Cross and Tinkelenberg (Cross & Tinkelenberg, 1991). The RNA concentration was determined by using an ultraspec 4000 photometer (Amersham Pharmacia Biotech, Freiburg, Germany), scanning the optical intensity between 240 nm and 340 nm. One microgram of total RNA (in water) was added to 2 µl of oligo dT (1 µg/ml 10-20 mer mixture, Research Genetics, Groningen, Netherlands) to give a final volume of 10 µl. After heat denaturation at 70 °C for 10 min the tubes containing the RNA and the primer were placed on ice. The labeling reaction contained 6 µl 5x first strain buffer (Life Technologies Inc., Rockville, USA), 1 µl 0.1 M dithiothreitol, 1.5 µl 20 mM dNTP mix (dCTP, dGTP and dTTP each 20 mM, MBI Fermentas, St. Leon-Rot, Germany), 1.5 µl Superscript II Reverse Transcriptase (200 units; Life Technologies Inc., Rockville, USA) and 10 µl [^{33}P]-dATP (100 µCi, 3000 Ci/nmol; Hartmann Analytics, Braunschweig, Germany). The labeling reaction (total 30 µl) was incubated at 37 °C for 90 min. The radiolabeled cDNA was purified by using a Quick Spin Column Sephadex G50 Fine (Roche, Mannheim, Germany).

Miniarray GeneFilters (GF100, Research Genetics, Groningen, Netherlands) were prehybridized with 10 ml of MicroHyb solution (Research Genetics, Groningen, Netherlands) containing 1 µg/ml poly dA (Research Genetics, Groningen, Netherlands) for at least 3 h. The purified cDNA probe was denatured at 95 °C for 5 min and added

to the prehybridization mixture. After overnight hybridization at 42 °C the filters were washed twice in 2x SSC (0.30 M NaCl, 0.030 M sodium citrate) with 1 % (w/v) SDS at 50 °C for 20 min, and finally in 0.5x SSC with 1 % (w/v) SDS at room temperature for 15 min. Filters were kept humid to facilitate stripping between hybridizations. Stripping was accomplished by submering filters in 0.5 % (w/v) SDS preheated to 100 °C, filters were slowly shaken in this solution for 20 min. More than 95 % of the signal was removed using this procedure.

The hybridization signals, obtained after 1 to 2 days of exposure, were read by a Storm 860 Phosphorimager (Amersham Pharmacia Biotech, Freiburg, Germany) and the data were transferred to the Pathway Software 2.01 (Research Genetics, Groningen, Netherlands) for analyzing the genes' transcriptional levels. The data were then exported to Microsoft Excel by which the final evaluation was done.

Results

Under amino acid starvation conditions, Gcn4p is required for transcriptional activation of more than 200 genes.

A genome-wide transcription profile analysis of a wild type and a *gcn4Δ* mutant strain was performed, in order to identify further genes whose expression requires Gcn4p. As Gcn4p is efficiently expressed only under amino acid starvation conditions, the analysis was performed in the presence of the amino acid analogue 3-amino-triazole (3AT).

Two independent isolations of total RNA were prepared form either strain incubated in the presence of 10 mM 3AT for 8 h and used for hybridization of two independent miniarray filter sets. Each filter set was hybridized in succession with four different cDNA pools obtained by reverse-transcription of the prepared RNAs. In between the filters were imaged and stripped. In the first step, the filters were hybridized with the first cDNA pool of the wild type strain, before the first cDNA pool of the *gcn4Δ* strain was used. After that, the second cDNA pools were used in the same order.

In order to assure a comparison of the hybridization signal intensities of the different hybridized filters, the filters contained total DNA spots in every ninth line, which should in principle result in almost constant signal intensity. For calibration, the signal intensities of all those spots were added and divided through the total number of those spots on the filter. This average value was used as denominator for the division of the signal intensities of all other spots on the same filter.

After this calibration, standard deviations were calculated for the values of the four independent hybridization signals of each gene (two independent cDNA pools hybridized on two different filters). Only genes having a standard deviation of less than 30 % were chosen for further analysis. This step should exclude analysis of gene spots that had signal intensities equivalent to the background intensity, which would lead to the determination of a possible artificial activation rate. This evaluation excluded 2557 genes from further analysis that means, 3587 of the total 6144 yeast genes were left over for evaluation, corresponding to 60 %. These raw data were evaluated in more detail by dividing the average of the four wild type hybridization signal intensities of each spot through the average of the four *gcn4Δ* signal intensities of the corresponding spot, which resulted in the wild type to *gcn4Δ* ratio (ratio wt/*gcn4Δ*). A wt/*gcn4Δ* ratio higher

than 1.0 represented an activation of gene transcription by Gcn4p, a ratio less than 1.0 a repression of transcription by Gcn4p. The factor 2.0 was chosen as minimal value of activation and 0.4 as minimal value of repression (corresponding to a 2.5-fold repression). Further analysis of the data revealed that the expression of 706 genes was positively or negatively effected by a factor of at least 2.0 in the absence of Gcn4p. 225 genes (6.3 %) had a wt/*gcn4Δ* ratio of 2.0 or even more (Table 1), while 481 genes (13.4 %) had a wild type to *gcn4Δ* ratio (ratio wt/*gcn4Δ*) of less than 0.4 (Table 2).

Gcn4p regulates the transcription of genes involved in amino acid metabolism as well as nucleotide, carbon, phosphate and fatty acid metabolism.

Before the final evaluation of the data was performed, the deletion of *GCN4* was examined by the hybridization signals on the filter. Indeed, with the cDNA from the *gcn4Δ* strain no *GCN4* hybridization signal could be obtained (Figure 1).

Our transcriptome profile analysis identified 24 genes of amino acid metabolism and 4 genes of nucleotide metabolism (Table 1), but just 11 of them (*ADE1, ARG1, ARG3, ARO4, GCV3, HIS4, HOM2, HOM3, LEU3, MET17* and *SHM2*) were already known to be transcriptionally activated by Gcn4p, according to YPD database (Costanzo *et al*., 2001). Thus, our screen revealed 17 further genes, previously unknown to be regulated in a Gcn4p-dependent manner. However, we did not found at least 20 genes described to be Gcn4p-dependent transcriptionally activated. One reason might be the low induction rate of many general control regulated genes. Apart from amino acid biosynthetic genes we found that also 8 genes involved in amino acid degradation (*ASP3B, ASP3D, BNA1, GAD1, GAT1, GCV1, GCV3* and YLR231C) required Gcn4p for expression.

Additionally to amino acid and nucleotide metabolic genes, 10 genes of carbon metabolism (*ADH4, ALD4, ALD6, CAT5, GLG1, GLK1, GND1, GPM1, MTH1* and *PDX1*), 1 gene of phosphate metabolism (*PHO85*) and 3 genes of fatty acid metabolism (*OPI3, PLB2* and *PLC1*) were Gcn4p-dependent activated (Table 1). This suggested that almost the complete cellular metabolism was influenced by Gcn4p during amino acid starvation conditions. In summary, further Gcn4p-controlled genes were identified which are involved in amino acid and nucleotide metabolism. Furthermore, our results indicate that Gcn4p regulates genes of other metabolic pathways, such as carbon, fatty acid and phosphate metabolism as well.

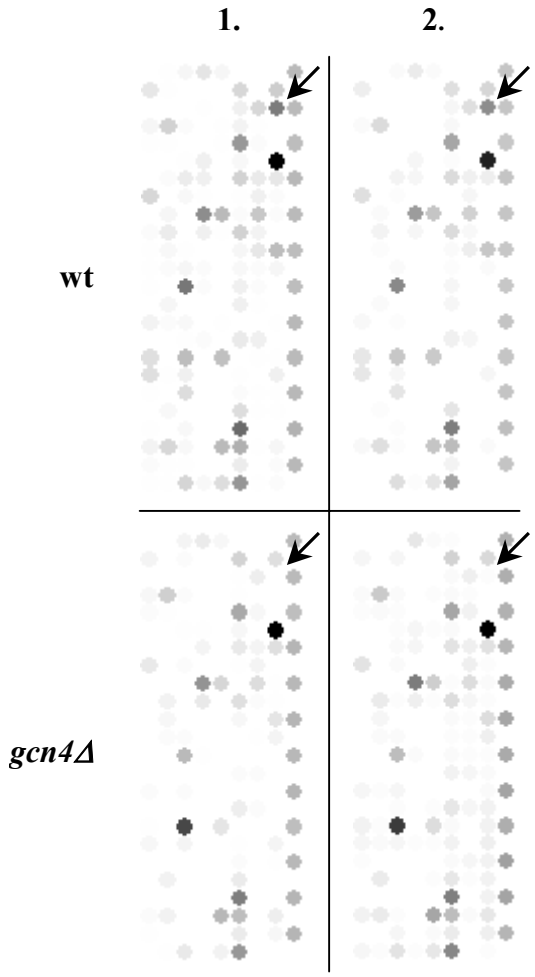

Figure 1. Miniarray analysis of *GCN4* gene expression in a wild type (wt) and a *gcn4Δ* strain (*gcn4Δ*). Corresponding sections from one filter hybridized with two radioactive labeled wild type cDNA (1. and 2.) and two *gcn4Δ* cDNA probes harvested after 8 h of grown on 10 mM 3AT containing YNB medium. Arrows indicate the position corresponding to *GCN4*.

Gcn4p is required for expression of genes involved in pseudohyphal development and adhesion.

Amino acid starvation activates adhesion and expression of *FLO11* depending on Gcn4p (Grundmann *et al*., 2001). This fact caused us to search for further genes known to be involved in pseudohyphal development or cell adhesion. In addition to the known *FLO11* gene one further gene was identified, *DDR48*, which is already known to be involved in flocculation (Tonouchi *et al*., 1994) and which was activated under the applied conditions. *DDR48* was repressible by Sfl1p and a deletion of it resulted in a

less flocculent phenotype (Tonouchi *et al*., 1994). Furthermore, *DDR48* is Ste12p- and Tec1p-dependent activated, which indicated that it might be regulated in a manner similar to *FLO11*. On the other hand, *DDR48* deletion strains did not have an obvious defect in haploid invasive growth or pseudohyphal development (Madhani *et al*., 1999).

The screen further revealed 3 genes, *ASH1* (Chandarlapaty & Errede, 1998), *BMH1* (Roberts *et al*., 1997) and *TPK2* (Pan & Heitman, 1999), which are necessary for pseudohyphal development during nitrogen starvation. Additionally *PHD1* was found, which triggers pseudohyphal growth independently of nitrogen starvation conditions (Gimeno & Fink, 1994). The cyclin *CLN1* and its regulator *CLN3* (Levine *et al*., 1996) were also induced. *CLN1* is required for pseudohyphal as well as haploid invasive growth (Madhani *et al*., 1999; Oehlen & Cross, 1998). In conclusion, five genes could be detected, which are known to be involved in pseudohyphal growth and which were Gcn4p-dependent activated under amino acid starvation conditions.

Furthermore, inhibitors of pseudohyphal development were identified by their reduced expression levels. Rupp and co-workers (Rupp *et al*., 1999) already suggested that *FLO11* expression is strongly blocked under log-phase conditions. In our screen three known inhibitors of *FLO11* expression exhibited a reduced transcription level by a factor of two at least. Expression of *SFL1*, which can be efficiently repressed by the 2-fold activated *TPK2*, was 2-fold reduced, while the transcription of *HOG1* and *BEM2* was even repressed by a factor of 2.5 and 3.3, respectively (Table 2).

Gcn4p regulates genes involved in cell wall maintenance and cell structure.

15 genes were identified, which are involved in cell wall maintenance or cell structure (Table 1). For 6 of them the biochemical function was already described: 2 genes encode structural proteins, *CIS3* and *CWP2*, while 4 genes encode for modifying proteins like hydrolases (*CTS1* and *SCW11*) or transferases (*PSA1* and *SPT14*). *SPT14* for instance, is a protein required for GPI anchor synthesis. GPI anchors play key roles in the cell wall assembly. *CTS1* encodes an endochitinase that is required for separation of mother and daughter cells following cytokinesis. This implicates that the structure of the cell wall and perhaps the cell morphology might be influenced Gcn4p-dependently under amino acid starvation conditions.

Gcn4p is required for expression of genes involved in chromatin structure.

The importance of chromatin structure for the Gcn4p-dependent activation of target genes was already recognized by Dudley and co-workers (Dudley *et al*., 1999). The fundamental unit of chromatin structure is the nucleosome, which contains 146 bp of DNA wrapped about twice around an octamer of histone proteins (two each of H2A, H2B, H3 and H4). The histone octamer is organized as a central hetero-tetramer of the histones H3 and H4, flanked by two heterodimeres of histones H2A and H2B (Arents *et al*., 1991). In the screen we detected a Gcn4p-dependent transcriptional activation of 4 genes encoding subunits of the histone octamer H4, H2A and H2B. For the two histone octamers H2A and H2B two divergently transcribed gene pairs exist, *HTA1-HTB1* and *HTA2-HTB2* (Norris & Osley, 1987). Both sets play different roles in the response to nutrient starvation. Just the transcription of *HTA2-HTB2* was observed to be Gcn4p-dependent activated. Furthermore, both genes, *HHF1* and *HHF2*, encoding the histone protein H4, were also activated, supporting an influence of Gcn4p on chromatin structure. That means Gcn4p might activate the transcription of several target genes by changing the chromatin structure.

The transcription of *ADA1* was regulated by Gcn4p as well. Ada1p is a component of the histone modifying SAGA complex (Sterner *et al*., 1999) and acts as a co-activator of Gln3p in nitrogen-regulated transcription (Soussi-Boudekou & Andre, 1999). If it turns out, that *ADA1* is transcriptionally activated, it should be possible to find target genes, which are activated depending on Ada1p and Gln3p. Indeed, in the miniarray we could detect an activation of two of the four genes identified by Soussi-Boudekou and Andre (Soussi-Boudekou & Andre, 1999), *PUT4* and *GDH1*, Ada1p-depending activated.

In summary, activation of genes, which encode proteins determining chromatin structure (histones) as well as a gene necessary for the regulation of chromatin structure were identified to be Gcn4p-dependent activated. This implicates a potential influence of Gcn4p on chromatin structure.

Discussion

Under amino acid starvation conditions, more than 50 genes involved in amino acid and nucleotide metabolism are known to be Gcn4p-dependent activated. By performing transcriptome analysis of a yeast wild type in contrast to a *gcn4Δ* strain we were able to confirm this fact just for 11 genes. The question was, why we were not able to detect an activation of the other, more than 30 genes, which were known to be Gcn4p-dependent regulated. One reason might be that under amino acid limitation the general control is usually activated by a factor of 2.0 to 3.0 in Northern hybridizations (Hinnebusch, 1992). In agreement with other groups we noticed that the factor of activation detected in miniarrays was usually lower than that determined by Northern hybridization experiments (unpublished data). This might be the reason, why several genes were excluded from evaluation by setting the minimal activation factor to 2.0, so happened e.g. for *HIS3* and *ARO3* showing a Gcn4p-dependent activation by a factor of 1.9 and 1.5, respectively. An additional reason for this phenomenon could the low transcription rate of several genes, which made a correct analysis quite complicated. Low variations in the background intensity already resulted in drastic deviations of the calculated expression value. The determination of negative expression rates after subtracting the intensity of the filter background from the intensity of the gene raw data further supported this assumption. For this reason most of the genes (2557) had a standard deviation of more than 30 % and were therefore not used for the final evaluation. These 30 % included many of the known Gcn4p-dependent regulated genes as well.

Although Gcn4p is the transcription factor of amino acid biosynthesis, 8 genes encoding proteins of amino acid degradation were identified, too. This would make sense for the cell, as under amino acid limitation, yeast cells are unable to induce the expression of just one specific amino acid biosynthetic pathway (general control of amino acid biosynthesis), and thus, the activation of amino acid degradation pathways would assures that enough amino acid precursors are available for biosynthesis of the respective amino acid. So this means that indeed, the biosynthesis of all amino acids is activated by Gcn4p during amino acid limitation, but that a part of those amino acids is degraded again to supply sufficient precursors for the biosynthesis of the limiting amino acid. This implicates that the simultaneous activation of genes involved in biosynthesis

and degradation of amino acids allows the cell to survive during starvation for only one or a few amino acids.

In addition to the classical Gcn4p-dependent activated amino acid metabolic genes, 20 genes involved in other metabolisms of the cell were identified. A comparison of the number of genes involved in the different metabolisms and activated by Gcn4p revealed, that metabolic pathways which are more essential for amino acid biosynthesis, like e.g. carbon metabolism (10 genes), were stronger represented than minor important metabolic pathways like phosphate metabolism (1 gene). As for amino acid biosynthesis precursors generated in the other metabolisms are absolutely required, this is a significant mechanism of regulation. One example for a Gcn4p-regulated metabolic gene is *ADL6*, which is involved in carbon metabolism. The corresponding protein plays a role in the production of cytosolic acetyl-CoA (Meaden *et al*., 1997), which is a very central intermediate in metabolism. Acetyl-CoA is produced during sugar degradation and is therefore involved in carbon metabolism. Furthermore, amino acid precursors are recruited from sugar degradation products, resulting in a dependency of the amino acid metabolism on carbon metabolism. Last but not least, acetyl-CoA is the starter unit of fatty acid biosynthesis as well as a product of fatty acid degradation (ß-oxidation of fatty acids). This implicates that all processes producing or consuming acetyl-CoA are linked to each other and therefore have to be regulated coordinatively.

FLO11 was already known to be involved in morphological differentiation of yeast cells during nitrogen starvation (Lo & Dranginis, 1998) as well as under amino acid starvation conditions (Grundmann *et al*., 2001). The induction of two activators, *TPK2* (Pan & Heitman, 1999) and *PHD1* (Gimeno & Fink, 1994), and the inhibition of three repressors of *FLO11* transcription, *SFL1*, *HOG1* and *BEM2* (Madhani & Fink, 1997; Palecek *et al*., 2000; Robertson & Fink, 1998), were detected during nitrogen starvation. This implicates that signal transduction systems and target genes activated during nitrogen starvation trigger the switch to flocculation under amino acid starvation as well.

In our screen 225 genes were found to be Gcn4p-dependent regulated under amino acid starvation conditions. A Gcn4p-dependent transcriptional activation is already published for just 15 of them (*ADE1, ARG1, ARG3, ARO4, GCV3, HIS4, HOM2, HOM3, LEU3, MET17, SHM2,* YBR147W, YER175C, YGL117W and YHR029C). 3 further genes (*BIO5, MTH1* and *THI7*) contain at least 3 GCRE sites in the promoter

region (Schuldiner *et al*., 1998). This raised the question, in which way Gcn4p regulated the transcription of the remaining 207 genes, which do not have an obvious Gcn4p binding site in its promoter. One possible explanation is that Gcn4p is able to bind onto sequences, which are unknown until now. Furthermore, Gcn4p could activate the transcription of additional transcription factors by activation via GCRE sites, which in their turn activate the transcription of the target genes. This type of activation is e.g. used by the transcription factor Leu3p, which is activated by Gcn4p and able to increase the transcription of *GDH1* (according YPD database), which was also found in our screen (Table 1). Nevertheless, the identification of such a large amount of Gcn4p-dependent activated genes implicated an involvement of a more general transcriptional activation system, for instance modifications of chromatin structure. Indeed, we detected 4 genes, which were Gcn4p-dependent activated and which encoded for proteins of the histone complex and 1 gene, which is involved in modifycations of histones. If this Gcn4p-dependent activation via remodeling of chromatin structure should happen, it would explain, why transcription of *FLO11* is Gcn4p-dependent activated, although no GCRE sites are present in its promoter region (Grundmann *et al*., 2001).

In parallel to our transcriptome analysis using filter miniarray hybridizations, Dr. Muffler (BASF-LYNX Bioscience AG, Germany) performed a glass slide microarray hybridization by applying identical RNA samples prepared in our lab (unpublished data). 290 genes were identified, which at least had a 2-fold Gcn4p-dependent activation of transcription that correlated well with the number of activated genes we detected (225). But the investigation of both data sets in more detail revealed that just 27 genes were transcriptionally activated in both screens (Table 1). As in both assays the RNA was isolated from identical strains grown under equal culture conditions, this is a really low conformity. Nevertheless, the data sets seem to complete each other. For example, in the miniarray hybridization we found 10 amino acid metabolic genes described to be Gcn4p-dependent regulated (Table 1), whereas the microarray of BASF-LYNX revealed 20 amino acid metabolic genes, but only 4 genes were identified in both hybridizations. So in conclusion, altogether 26 genes of at least 40 genes, known to be Gcn4p-dependent activated, were detected in both assays. This result implicates that transcriptome analysis carried out with one hybridization method reveals only a fraction of the total number of genes, which are influenced by Gcn4p,

suggesting that the obtained data of either method have to be confirmed by applying a third, independent hybridization method.

Overall, both hybridization data sets clearly demonstrate that Gcn4p does not only activate amino acid metabolic genes, but rather influences genes involved in several different cellular processes.

Table 1. Gcn4p-dependent activated genes identified by the evaluation of a comparative transcriptome analysis of a wild type and a *gcn4Δ* strain grown under amino acid starvation conditions.

Classification of the genes into the different functional groups was done according to the cellular role mentioned in the YPD database (Costanzo *et al*., 2001). The "ratio wt/*gcn4Δ*" is the ratio of the average intensity of the wild type hybridization signals divided through the average intensity of the corresponding *gcn4Δ* hybridization signals. If gene expression was detected in the microarray hybridization, the ratio was set into brackets. "Description" of the genes' function was done according to the YPD database for miniarray hybridization and according to MIPS database (Mewes *et al*., 1999) for microarray hybridization data.

[A] Microarray data are unpublished results of Dr. Muffler (BASF-LYNX Bioscience AG, Germany). For the hybridization mRNA was prepared from the same strains under identical conditions as used for miniarray hybridization, but in contrast to miniarray hybridization, where total RNA was applied for cDNA synthesis, poly-A purified mRNA was used.

[B] GCRE sites according to Schuldiner et al. (Schuldiner *et al*., 1998).

[C] Genes already described in YPD database (Costanzo *et al*., 2001) to be Gcn4p-dependent regulated.

Amino acid metabolism

GENE/ ORF	Description	Ratio wt/*gcn4Δ*	Identified by miniarray hybridization	Identified by microarray hybridization (BASF-LYNX)[A]	At least 3 GCRE sites[B]	Known to be Gcn4p-dependent regulated[C]
Amino acid biosynthesis						
ARG1	Argininosuccinate synthetase (citrulline-aspartate ligase); catalyzes the penultimate step in arginine synthesis	8.4 (26.21)	+	+		+
ARG3	Ornithine carbamyltransferase, catalyzes the sixth step in the arginine biosynthesis pathway	3.3 (6.24)	+	+		+
ARG4	Arginosuccinate lyase	(43.20)		+		+
ARG8	Acetylornithine aminotransferase	(2.33)		+		+
ARG81	Component of the ARGR regulatory complex	2.7	+			
ARO2	Chorismate synthase	(3.26)		+		
ARO4	2-Dehydro-3-deoxyphosphoheptonate aldolase (3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; DAHP synthase), tyrosine-inhibited	2.0	+		+	+
ARO8	Aromatic amino acid aminotransferase I	(2.84)		+		
ARO9	Aromatic amino acid aminotransferase II	(9.87)		+		
ARO10	Putative indole-3-pyruvate decarboxylase	3.0	+			
ASN1	Asparagine synthetase	(2.76)		+		+
ASN2	Asparagine synthetase	(2.15)		+		+

CAR1	Arginase	(2.21)		+		
ECM40	Acetylornithine acetyltransferase	(12.60)		+		+
GCN4	Transcription factor of the basic leucine zipper (bzip) family, regulates general control in response to amino acid or purine starvation	97.6 (40.93)	+	+		
GDH1	Glutamate dehydrogenase (NADP+), combines ammonia and alpha-ketoglutarate to form glutamate	2.4	+			
GLN1	Glutamate-ammonia ligase	(2.93)		+	+	+
HIS1	ATP phosphoribosyltransferase	(2.74)		+		+
HIS3	Imidazoleglycerol-phosphate dehydratase	(2.63)		+	+	+
HIS4	Phosphoribosyl-AMP cyclohydrolase / phosphoribosyl-ATP pyrophosphohydrolase / histidinol dehydrogenase, second, third, and tenth steps of histidine biosynthesis pathway	2.6 (6.12)	+	+	+	+
HOM2	Aspartate-semialdehyde dehydrogenase (L-aspartate-beta-semialdehyde)	2.5	+			+
HOM3	Aspartate kinase (L-aspartate-4-P-transferase); first step in the common pathway for methionine and threonine biosynthesis	2.6 (2.77)	+	+		+
ILV2	Acetolactate synthase	(2.31)		+		+
ILV3	Dihydroxy-acid dehydratase	(4.04)		+		
LEU1	3-Isopropylmalate dehydratase	(2.93)		+		
LEU3	Transcription factor regulating genes of branched chain amino acid biosynthesis pathways, acts as both a repressor and an inducer	2.0	+			+
LEU4	2-Isopropylmalate synthase	(4.19)		+	+	+
LEU9	Alpha-isopropylmalate synthase II	2.4 (3.35)	+	+		
LPD1	Dihydrolipoamide dehydrogenase precursor	(3.66)		+		+
LYS9	Saccharopine dehydrogenase (NADP+, L-glutamate forming)	(2.55)		+		
LYS20	Homocitrate synthase isoenzyme, involved in lysine biosynthesis	3.7 (4.34)	+	+		
LYS21	Homocitrate synthase isoenzyme, involved in lysine metabolism	2.8 (4.97)	+	+		
MET6	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	(4.57)		+		
MET13	Putative methylene tetrahydrofolate reductase	(2.94)		+		
MET17	O-acetylhomoserine sulfhydrylase (OAH shlase); converts O-acetylhomoserine into homocysteine	2.1	+			+
MET22	Protein ser/thr phosphatase	(3.67)		+		
SER1	Phosphoserine transaminase	(2.37)		+		+
SER3	3-Phosphoglycerate dehydrogenase	(2.26)		+		
SHM2	Serine hydroxymethyltransferase (glycine hydroxymethyltransferase), cytosolic isoform, catalyzes the transfer of the hydroxymethyl group of serine to tetrahydrofolate to form 5,10-methylenetetrahydrofolate and glycine	2.0	+		+	+
TRP1	Phosphoribosylanthranilate isomerase, catalyzes the third step in the tryptophan biosynthesis pathway	3.4 (9.31)	+	+		
TRP2	Anthranilate synthase component I	(3.03)		+		+
TRP4	Anthranilate phosphoribosyltransferase	(4.10)		+	+	+

TRP5	Tryptophan synthase	(4.71)		+		+
YER069W	ARG5,6 acetylglutamate kinase and acetylglutamyl-phosphate reductase	(10.67)		+	+	+
YJR130C	Similarity to O-succinylhomoserine (thiol)-lyase	(5.65)		+		
YKL218C	Strong similarity to *E.coli* and *H.influenzae* threonine dehydratases	(7.56)		+	+	
Amino acid catabolism						
ASP3B	L-asparaginase II, extracellular	2.2	+			
ASP3D	L-asparaginase II, extracellular	3.4	+			
BNA1	3-Hydroxyanthranilate 3,4-dioxygenase, involved in biosynthesis of nicotinic acid from tryptophan	3.9	+			
GAD1	Glutamate decarboxylase	2.2	+			
GAT1	GATA zinc finger transcription factor that plays a supplemental role to Gln3p, activating genes needed to use non-preferred nitrogen sources	2.9 (22.71)	+	+		
GCV1	Glycine decarboxylase T subunit (glycine cleavage T protein), functions in the pathway for glycine degradation	3.1	+			
GCV3	Glycine decarboxylase hydrogen carrier protein H subunit (glycine cleavage H protein), functions in the pathway for glycine degradation	2.2	+		+	+
UGA1	4-Aminobutyrate aminotransferase (GABA transaminase)	(3.48)		+		
YLR231C	Protein with weak similarity to rat kynureninase (PIR)	3.2 (3.71)	+	+		

Nucleotide metabolism

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
ADE1	Phosphoribosylamidoimidazole-succinocarboxamide synthase (SAICAR synthetase), catalyzes the seventh step in de novo purine biosynthesis pathway	2.2	+		+	+
ADE12	Adenylosuccinate synthetase	(2.72)		+	+	
ADE13	Adenylosuccinate lyase	(2.30)		+		
ADE16	5-Aminoimidazole-4-carboxamide ribotide transformylase	(2.32)		+		
ADE17	5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase/IMP cyclohydrolase	2.0 (3.05)	+	+		
CDC21	Thymidylate synthase, catalyzes the reductive methylation of dump by 5,10-methylene-5,6,7,8-tetrahydrofolate to produce dtmp and 7,8-dihydrofolate	2.7	+			
MTD1	NAD-dependent 5,10-methylenetetrahydrafolate dehydrogenase	2.1 (3.20)	+	+	+	
RNR2	Ribonucleoside-diphosphate reductase, small subunit	(2.25)		+		
URA8	CTP synthase 2	(2.27)		+		
URA10	Orotate phosphoribosyltransferase2	(2.93)		+	+	

YLR245C	Strong similarity to *B.subtilis* cytidine deaminase	(3.29)		+		

Carbon metabolism

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
ADH4	Alcohol dehydrogenase IV	3.1	+			
ALD4	Mitochondrial aldehyde dehydrogenase	2.5	+			
ALD6	Cytosolic acetaldehyde dehydrogenase	2.9	+			
CAT5	Protein required for derepression of gluconeogenic enzymes and for coenzyme Q (ubiquinone) biosynthesis	2.1	+			
CIT1	Citrate (si)-synthase, mitochondrial	(3.30)		+		
ENO1	Enolase I (2-phosphoglycerate dehydratase)	(3.93)		+		
ENO2	Enolase II (2-phosphoglycerate dehydratase)	(3.87)		+		
ERR1	Enolase related protein	(3.88)		+		
ERR2	Enolase related protein	(4.49)		+		
FBA1	Fructose-bisphosphate aldolase	(3.25)		+	+	
GLG1	Self-glucosylating initiator of glycogen synthesis	2.5	+			
GLK1	Glucokinase, specific for aldohexoses	2.2	+			
GLO1	Glyoxalase I	(2.68)		+		
GLO2	Glyoxalase II (hydroxyacylglutathione hydrolase)	(2.43)		+		
GND1	6-Phosphogluconate dehydrogenase, decarboxylating, converts 6-phosphogluconate + NADP to ribulose-5-phosphate + NADPH + CO_2	2.6 (3.93)	+	+		
GND2	Phosphogluconate dehydrogenase	(4.10)		+		
GPM1	Phosphoglycerate mutase that converts 2-phosphoglycerate to 3-phosphoglycerate in glycolysis	2.0 (4.43)	+	+		
GPM2	Phosphoglycerate mutase	(100.00)		+		
HXK1	Hexokinase I	(13.79)		+		
HXK2	Hexokinase II	(2.25)		+		
IDP1	Isocitrate dehydrogenase (NADP+), mitochondrial	(3.99)		+	+	
LPD1	Dihydrolipoamide dehydrogenase precursor	(3.66)		+		
LSC2	Succinate-coa ligase beta subunit	(3.24)		+		
MTH1	Repressor of hexose transport genes	6.4	+			
OSM1	Osmotic growth protein	(3.08)		+		
PDX1	Pyruvate dehydrogenase complex protein X; binding protein for Lpd1p	2.6	+			

PFK1	6-Phosphofructokinase, alpha subunit	(2.36)		+		
PGK1	Phosphoglycerate kinase	(3.27)		+		
SDH1	Succinate dehydrogenase flavoprotein precursor	(2.83)		+		
TAL1	Transaldolase	(5.10)		+		
TDH1	Glyceraldehyde-3-phosphate dehydrogenase 1	(3.94)		+		
TDH2	Glyceraldehyde-3-phosphate dehydrogenase 2	(3.99)		+		
TDH3	Glyceraldehyde-3-phosphate dehydrogenase 3	(4.08)		+		
TPI1	Triose-phosphate isomerase	(2.65)		+		
YGR043C	Strong similarity to transaldolase	(2.91)		+		
YJL200C	Strong similarity to aconitate hydratase	(4.03)		+		
YLR345W	Similarity to PFK26P and other 6-phosphofructo-2-kinases	(2.40)		+		
YMR323W	Strong similarity to phosphopyruvate hydratases	(3.73)		+		
ZWF1	Glucose-6-phosphate dehydrogenase	(3.38)		+		

Other metabolism

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
Fatty acid metabolism						
BET4	Geranylgeranyl transferase, alpha chain	(2.47)		+		
CEM1	Beta-keto-acyl-ACP synthase, mitochondrial	(2.36)		+		
CHO2	Phosphatidylethanolamine N-methyltransferase	(13.87)		+		
HAC1	Transcription factor	(2.55)		+		
OPI3	Phospholipid-N-methyltransferase, carries out the second and third methylation steps of the phosphatidylcholine biosynthesis pathway	8.4	+			
PLB2	Phospholipase B2 (lysophospholipase), releases fatty acids from lysophospholipids	2.7	+			
PLC1	Phosphoinositide-specific phospholipase C (1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase 1), produces diacylglycerol and inositol 1,4,5-trisphosphate	2.5	+			
Phosphate metabolism						
PHO85	Cyclin-dependent protein kinase that interacts with cyclin Pho80p to regulate phosphate pathway, also interacts with other Pho80p-like cyclins	2.3	+			
PPA2	Inorganic pyrophosphatase, mitochondrial	(2.30)		+		

Others

AAD10	Putative aryl alcohol dehydrogenase, may participates in late steps of degradation of aromatic compounds that arise from the degradation of lignocellulose	10.0	+	
BIO2	Biotin synthetase	(17.72)		+
BIO3	DAPA aminotransferase	(8.15)		+
BIO4	Putative dethiobiotin synthetase	(5.07)		+
DAL80	GATA-type zinc finger transcriptional repressor for allantoin and 4-aminobutyric acid (GABA) catabolic genes	3.1	+	
DBI56	Ubiquinone biosynthesis, methyltransferase	(3.09)		+
FET4	Low affinity Fe(II) iron transport protein	(2.63)		+
FOL2	GTP cyclohydrolase I	(2.43)		+
MET13	Putative methylene tetrahydrofolate reductase	(2.94)		+
MMT2	Mitochondrial iron transporter	(5.34)		+
PDX3	Pyridoxamine-phosphate oxidase	(3.12)		+
PET18	Protein involved in maintenance of mitochondrial DNA	2.3	+	
PNC1	Pyrazinamidase and nicotinamidase	2.9	+	
SER1	Phosphoserine transaminase	(2.37)		+
SNO1	Putative pyridoxine (vitamin B6) biosynthetic enzyme with similarity to glutamine aminotransferases, has strong similarity to Sno1p and Sno3p	5.7	+	
SPE3	Putrescine aminopropyltransferase (spermidine synthase)	(5.43)		+
THI6	Thiamin-phosphate pyrophosphorylase and hydroxyethylthiazole kinase	(5.30)		+
THI20	Bipartite protein consisting of N-terminal hydroxymethylpyrimidine phosphate (HMP-P) kinase domain, needed for thiamine biosynthesis, fused to C-terminal Pet18p-like domain of indeterminant function	5.1	+	
VMA8	H+-atpsynthase V1 domain 32 KD subunit, vacuolar	(2.76)		+
YBR006W	Strong similarity to *E.coli* succinate semialdehyde dehydrogenase	(2.32)		+
YFR047C	Strong similarity to human quinolinate phosphoribosyltransferase	(2.69)		+
YGL096W	Similarity to copper homeostasis protein CUP9P	(4.01)		+
YLR231C	Strong similarity to rat kynureninase	(3.71)		+
YMR113W	Similarity to folylpolyglutamate synthetases and strong similarity to YKL132c	(4.53)		+

Transporter

GENE/ ORF	Description	Ratio wt/*gcn4Δ*	Identified by miniarray hybridization	Identified by microarray hybridization (BASF-LYNX)[A]	At least 3 GCRE sites[B]	Known to be Gcn4p-dependent regulated[C]
AKR2	Protein involved in constitutive endocytosis of Ste3p	2.1	+			
BAP3	Valine transporter	(4.14)		+		
BIO5	Transmembrane regulator of KAPA/DAPA transport, involved in biotin biosynthesis	2.6 (12.26)	+	+	+	
BPH1	Probable acetic acid export pump, contains possible WD (WD-40) repeats in the C-terminal region	2.0	+			
CAF16	ATP-binding cassette transporter family member	(2.76)		+		
DIP5	Dicarboxylic amino acid permease	(2.50)		+		
FET4	Low affinity Fe(II) iron transport protein	(2.63)		+		
GAP1	General amino acid permease	(4.76)		+		
HXT4	Moderate- to low-affinity glucose transporter	(2.74)		+		
HXT6	High-affinity hexose transporter	(2.86)		+		
HXT7	High-affinity hexose transporter	(2.87)		+		
HXT8	Hexose transport protein	(3.14)		+		
MEP2	High-affinity low-capacity ammonia permease	(4.97)		+		
MMT2	Mitochondrial iron transporter	(5.34)		+		
MSO1	Secretion protein, multicopy suppressor of SEC1	(6.60)		+		
MSP1	Intra-mitochondrial sorting protein	(2.60)		+		
NCE102	Involved in non-classical protein export pathway	(2.22)		+		
NFS1	Regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution	(3.26)		+		
ODC1	2-Oxodicarboxylate transporter, member of the mitochondrial carrier (MCF) family of membrane transporters	2.2	+			
PUT4	Proline permease required for high-affinity proline transport	2.4	+			
RSE1	Involved in RNA splicing and ER to Golgi transport	(7.18)		+		
STP22	Protein required for vacuolar targeting of temperature-sensitive plasma membrane proteins such as Ste2p and Can1p	2.3	+			
TAT2	High-affinity tryptophan transport protein	(2.35)		+		
THI7	Thiamine transport protein	2.5	+		+	
VHT1	Plasma membrane H+-biotin symporter	(4.67)		+		
VMA8	H+-ATP synthase V1 domain 32 KD subunit, vacuolar	(2.76)		+		
YHM1	Member of the mitochondrial carrier family (MCF)	(4.32)		+		
YIP2	Ypt-interacting protein	(2.34)		+		

YLR004C	Protein with similarity to Dal5p and other members of the allantoate permease family of the major facilitator superfamily (MFS)	6.3 (32.62)	+	+		
YMC1	Mitochondrial carrier protein (MCF)	(2.91)		+		
YMR162C	Similarity to ATPases	(14.51)		+		
YNL321W	Member of the calcium permease family of membrane transporters	2.3	+			
YNR070W	Probable multidrug resistance protein of the ATP-binding cassette (ABC) family, has similarity to Snq2p, Pdr5p and *Candida albicans* Cdr1p	4.1	+			
YOR192C	Probable low-affinity thiamine transporter	3.7	+			
YOR222W	Similarity to ADP/ATP carrier proteins	(3.31)		+		
YOR382W	Possible transporter	3.0	+			
YOR383C	Possible transporter	2.3	+			

Energy generation

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
AAD6	Strong similarity to aryl-alcohol dehydrogenases	(2.20)		+		
ADH1	Alcohol dehydrogenase I	(2.53)		+		
ADH2	Alcohol dehydrogenase II	(2.45)		+		
ALD5	Aldehyde dehydrogenase (NAD+), mitochondrial	(5.40)		+		
ATP15	Protein required for derepression of gluconeogenic enzymes and for coenzyme Q (ubiquinone) biosynthesis	2.1	+			
CEM1	Beta-keto-acyl-ACP synthase, mitochondrial	(2.36)		+		
COX5B	Cytochrome-c oxidase chain Vb	(4.56)		+		
COX14	Protein required for assembly of cytochrome oxidase	2.1	+			
FRE2	Protein that may be associated with ferric reductase activity	2.5	+			
FUN49	Protein with similarity to alcohol/sorbitol dehydrogenase, member of the zinc-containing alcohol dehydrogenase family	2.0 (5.14)	+	+		
IMG1	Putative mitochondrial ribosomal protein, essential for respiration and integrity of the mitochondrial genome	2.6	+			
MRPL19	Mitochondrial ribosomal protein of the large subunit	5.4	+			
PDC1	Pyruvate decarboxylase, isozyme 1	(3.18)		+		
PDC5	Pyruvate decarboxylase, isozyme 2	(4.13)		+		
PDC6	Pyruvate decarboxylase 3	(3.01)		+		
PPA2	Inorganic pyrophosphatase, mitochondrial	(2.30)		+		

RSM19	Putative mitochondrial ribosomal protein of the small subunit, has similarity to prokaryotic ribosomal protein S19	3.3	+			
SOM1	Possible subunit of Imp peptidase complex, required for mitochondrial inner peptidase function	3.5	+			
STF1	ATPase stabilizing factor, 10 kda	(7.31)		+		
STF2	ATPase stabilizing factor	(5.78)		+		
YAH1	Protein with similarity to adrenodoxin and ferrodoxin	2.5	+			
YHM1	Protein of the mitochondrial carrier (MCF) family that when overexpressed suppresses loss of Abf2p	2.6	+			
YJR155W	Strong similarity to aryl-alcohol dehydrogenase	(4.14)		+		
YLR294C	Protein of unknown function, possibly involved in respiration	2.4	+			
YLR327C	Protein with strong similarity to Stf2p	11.2	+			
YMS18	Mitochondrial ribosomal protein of the small subunit (yms18), has similarity to bacterial and chloroplast ribosomal protein S11	2.3	+			

Cell stress

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
AHP1	Alkyl hydroperoxide reductase	2.3	+			
CPH1	Cyclophilin (peptidylprolyl isomerase)	(2.29)		+		
DAK1	Dihydroxyacetone kinase, induced in high salt	(3.26)		+		
GTT1	Glutathione S-transferase	(2.64)		+		
HOR7	Hyperosmolarity-responsive protein	(5.28)		+		
HSP104	Heat shock protein	(3.31)		+		
HYR1	Glutathione peroxidase	(2.75)		+		
LAP3	Member of the GAL regulon	(2.53)		+		
MET22	Protein ser/thr phosphatase	(3.67)		+		
MRH1	Membrane protein related to Hsp30p	(2.64)		+		
OSM1	Osmotic growth protein	(3.08)		+		
PRX1	Mitochondrial thiol peroxidase	2.5	+			
RAD50	DNA repair protein	(20.54)		+		
REV3	DNA polymerase zeta, involved in mutagenic translesion DNA repair synthesis	4.3	+			
SSU1	Sulfite sensitivity protein	(16.61)		+		
TRX2	Thioredoxin II	(2.39)		+		
TRX3	Mitochondrial thioredoxin	(3.40)		+		

YBL064C	Strong similarity to thiol-specific antioxidant enzyme	(4.25)		+		
YKL033W-a	Strong similarity to holacid-halidohydrolase	(3.93)		+		
YLR109W	Type II thioredoxin peroxidase	(4.80)		+		
YNL234W	Hemoprotein with similarity to mammalian globins	2.5	+			
YOL158C	Similarity to subtelomeric encoded proteins	(2.57)		+		
ZWF1	Glucose-6-phosphate dehydrogenase	(3.38)		+		

Meiosis

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
AUT2	Protein that mediates attachment of autophagosomes to microtubules, also required for sporulation in combination with Aut7p	2.3	+			
BBP1	Protein of the spindle pole body that binds to Bfr1p	14.2	+			
MCD4	Sporulation protein	(3.99)		+		
RDH54	Protein required for mitotic diploid-specific recombination and repair and for meiosis	2.1	+			
SLZ1	Sporulation-specific protein	(6.39)		+		
SST2	Protein involved in desensitization to alpha-factor pheromone	3.0	+			

Mitosis

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
CDC37	Cell division control protein	(2.30)		+		
CIN1	Protein involved in chromosome segregation, required for microtubule stability	2.8	+			
CTF13	Component (subunit c) of Cbf3 kinetochore complex, contains an F-box domain	7.9	+			
DSK2	Protein required with Rad23p for duplication of the spindle pole body; has similarity to ubiquitin	3.5	+			
IPL1	Serine/threonine protein kinase of the mitotic spindle, involved in chromosome segregation	2.1	+			
SNZ1	Stationary phase protein	(54.64)		+		

Cell cycle control

GENE/ ORF	Description	Ratio wt/*gcn4Δ*	Identified by miniarray hybridization	Identified by microarray hybridization (BASF-LYNX)[A]	At least 3 GCRE sites[B]	Known to be Gcn4p-dependent regulated[C]
CLB1	Cyclin, G2/M-specific	(3.30)		+		
CLN1	G1/S-specific cyclin that interacts with Cdc28p protein kinase to control events at START	2.5	+			
CLN3	G1/S-specific cyclin that interacts with Cdc28p protein kinase to control events at START	2.2	+			
NBP1	Essential protein required for G2/M transition	2.5	+			
PCL5	Cyclin that associates with Pho85p	5.4	+			
PCL8	Cyclin like protein interacting with Pho85p	(2.20)		+		
PCL9	Cyclin that associates with Pho85p	3.0	+			
SIW14	Protein tyrosine phosphatase (ptpase) involved in nutritional control of the cell cycle	3.6	+			
YOL158C	Similarity to subtelomeric encoded proteins	(2.57)		+		

Protein synthesis, modification and degradation

GENE/ ORF	Description	Ratio wt/*gcn4Δ*	Identified by miniarray hybridization	Identified by microarray hybridization (BASF-LYNX)[A]	At least 3 GCRE sites[B]	Known to be Gcn4p-dependent regulated[C]
BET4	Geranylgeranyl transferase, alpha chain	(2.47)		+		
CPH1	Cyclophilin (peptidylprolyl isomerase)	(2.29)		+		
DPS1	Aspartyl-tRNA synthetase, cytosolic	(2.28)		+		
LAP3	Member of the GAL regulon	(2.53)		+		
LAP4	Aminopeptidase ysci precursor, vacuolar	(6.05)		+		
MAK3	Protein N-acetyltransferase, acetylates N-terminus of L-A virus GAG protein	11.9	+			
MET13	Putative methylene tetrahydrofolate reductase	(2.94)		+		
MNN1	Alpha-1,3-mannosyltransferase	(3.28)		+		
MNN4	Regulates the mannosylphosphorylation	(2.86)		+		
MRP8	Ribosomal protein, mitochondrial	(4.19)		+		
MSP1	Intra-mitochondrial sorting protein	(2.60)		+		
NYV1	V-SNARE, vacuolar	(2.47)		+		

PBI2	Protease B (yscb or Prb1p) inhibitor 2 (I2B), has activity related to vacuolar fusion that is not related to protease activity	11.4	+			
PIM1	ATP-dependent protease, mitochondrial	(2.32)		+	+	
RPL23B	Ribosomal protein L23.e	(8.48)		+		
RPN8	26S proteasome regulatory subunit	(2.20)		+		
RPN13	Putative proteasomal subunit	2.5	+			
RPS30B	Strong similarity to human ubiquitin-like protein/ribosomal protein S30	(6.05)		+		
SHR5	Protein involved in ras function, mutants can block attachment and palmitoylation of ras proteins	3.0	+			
SPC2	Signal peptidase 18 kda subunit	(3.74)		+		
SPT4	Transcription elongation protein	(3.10)		+		
TRX2	Thioredoxin II	(2.39)		+		
UMP1	Proteasome maturation factor; chaperone involved in proteasome assembly	2.5	+			
YCR099C	Strong similarity to Pep1p, Vth1p and Vth22p	(2.51)		+		
YCR100C	Strong similarity to Pep1p, Vth1p and Vth22p	(2.66)		+		
YLR327C	Strong similarity to Stf2p	(5.93)		+		
YMR31	Ribosomal protein, mitochondrial	(2.44)		+		
YNR069C	Similarity to central part of Bul1p	(3.01)		+		

RNA processing and modification or splicing

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
BRR2	RNA helicase-related protein required for pre-mRNA splicing	2.1	+			
LEA1	Component of the U2 snRNP complex similar to human U2A' protein, involved in the initial steps of pre-mRNA splicing	3.2	+			
MRS2	Protein involved in splicing of mitochondrial group II introns; also involved in a mitochondrial function unrelated to splicing	2.3	+			
NAM2	Leucyl-tRNA synthetase, mitochondrial, dominant alleles suppress mutations in the bi4 maturase	2.9	+			
NAM7	Protein involved with Nmd2p and Upf3p in decay of mrna containing nonsense codons	2.8	+			
PRP9	Pre-mRNA splicing factor (snRNA-associated protein), required for addition of U2 snRNA to prespliceosomes, has two C2H2-type zinc fingers	2.0	+			
PRP22	Pre-mRNA splicing factor of DEAH box family, required for release of mature mRNA from the spliceosome	2.1	+			
RNR2	Ribonucleoside-diphosphate reductase, small subunit	(2.25)		+		

RSE1	Involved in RNA splicing and ER to Golgi transport	(7.18)		+		
SGS1	DNA helicase of DEAD/DEAH family, has similarity to the human Werner's and Bloom's syndromes genes and to *E. coli* recQ 3'-5' DNA helicase	4.5	+			
STP4	Protein with strong similarity to Stp1p, which is involved in tRNA splicing and branched-chain amino acid uptake	4.0	+			

Chromatin and chromosome structure, transcription

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
ADA1	Component of the ADA complex, interacts with Taf61p to form a histone-like paired complex	3.8	+			
CAF17	CCR4 associated factor	(3.43)		+		
HAC1	Transcription factor	(2.55)		+		
HAP1	Transcription factor	(4.02)		+		
HHF1	Histone H4, identical to Hhf2p	2.3	+			
HHF2	Histone H4, identical to Hhf1p	2.9 (2.40)	+	+		
HST3	Protein with similarity to Sir2p	2.2	+			
HTA2	Histone H2A, nearly identical to Hta1p	6.1	+			
HTA3	Histone-related protein that can suppress histone H4 point mutation	2.6	+			
HTB2	Histone H2B, nearly identical to Htb1p	2.6	+			
PET18	Similarity to regulatory protein	(2.89)		+		
SOL2	Multicopy suppressor of LOS1-1	(2.26)		+		
SOL3	Weak multicopy suppressor of LOS1-1	(2.48)		+		
SPT4	Transcription elongation protein	(3.10)		+		
SSN8	Cyclin C homolog, component of RNA polymerase holoenzyme complex and Kornberg's mediator (SRB) subcomplex	5.4	+			
TEL2	Protein involved in controlling telomere length and telomere position effect	2.0	+			
YLR266C	Protein with similarity to transcription factors, has Zn[2]-Cys[6] fungal-type binuclear cluster domain in the N-terminal region	3.1	+			
YOL055C	Similarity to *B. subtilis* transcriptional activator tena	(6.90)		+		

Signal transduction and differentiation

GENE/ ORF	Description	Ratio wt/*gcn4Δ*	Identified by miniarray hybridization	Identified by microarray hybridization (BASF-LYNX)[A]	At least 3 GCRE sites[B]	Known to be Gcn4p-dependent regulated[C]
AFR1	Involved in morphogenesis of the mating projection	(2.96)		+		
ARF2	GTP-binding protein of the ARF family	(2.63)		+		
ASH1	GATA-type transcription factor, negative regulator of HO expression localized preferentially in daughter cells	2.1	+			
BMH1	Protein involved in signal transduction during filamentous growth, homolog of mammalian 14-3-3 protein	2.5 (2.18)	+	+		
CMK1	Ca2+/calmodulin-dependent ser/thr protein kinase type I	(3.67)		+		
FRM2	Protein involved in the integration of lipid signaling pathways with cellular homeostatis	2.8	+			
FRQ1	Regulator of phosphatidylinositol-4-OH kinase protein	(2.23)		+		
GDI1	GDP dissociation inhibitor	(2.40)		+		
GLC8	Modulator of protein serine/threonine phosphatase Glc7p, involved in control of vacuole fusion	2.6	+			
GSP2	Ran, a GTP-binding protein member of the ras superfamily involved in trafficking through nuclear pores	2.2	+			
PHD1	Transcription factor involved in regulation of filamentous growth	3.6	+			
RPI1	Negative regulator of ras-camp pathway, downregulates normal but not mutant ras function	3.3	+			
SPO16	Early meiotic protein required for efficient spore formation	2.1	+			
TPK2	cAMP-dependent protein kinase 2, catalytic chain of protein kinase A	2.2	+			

Cell wall maintenance and cell structure

GENE/ ORF	Description	Ratio wt/*gcn4Δ*	Identified by miniarray hybridization	Identified by microarray hybridization (BASF-LYNX)[A]	At least 3 GCRE sites[B]	Known to be Gcn4p-dependent regulated[C]
ARP1	Centractin	(2.28)		+		
CIS3	Cell wall protein with similarity to members of the Pir1p/Hsp150p/Pir3p family	2.5	+			
COF1	Cofilin, actin binding and severing protein	(3.28)		+		
CTS1	Endochitinase	3.0	+			
CWP2	Mannoprotein of the cell wall, member of the PAU1 family	2.7	+			

DDR48	Stress protein induced by heat shock, DNA damage, or osmotic stress	3.0 (5.25)	+	+		
ECM4	Protein possibly involved in cell wall structure or biosynthesis	2.3	+			
ECM17	Involved in cell wall biogenesis and architecture	(3.16)		+		
ECM30	Involved in cell wall biogenesis and architecture	(3.44)		+		
EXG1	Exo-beta-1,3-glucanase (I/II), major isoform	(2.23)		+		
FLO11	Cell surface flocculin, required for invasive and pseudohyphal growth	6.5	+			
GAS1	Glycophospholipid-anchored surface glycoprotein	(2.88)		+		
PSA1	Mannose-1-phosphate guanyltransferase; GDP-mannose pyrophosphorylase	2.4	+			
SCW11	Putative cell wall protein with similarity to Scw10p	2.2	+			
SPO19	Sporulation specific protein, putative glycophosphatidylinositol (GPI)-anchored spore wall protein	9.6	+			
SPT14	N-acetylglucosaminyltransferase, required for GPI anchor synthesis	2.0	+			
SRD2	Protein with similarity to Srd1p	3.1	+			
TPM2	Tropomyosin, isoform 2	(2.78)		+		
TUB2	Beta-tubulin	(2.61)		+		
WSC4	Protein required for secretory protein translocation, for maintenance of cell wall integrity, and for the stress response	2.7	+			
YLR194C	Probable cell wall protein	2.3	+			
YNL095C	Protein with similarity to Ecm3p	2.1	+			
YOL155C	Protein with similarity to *S. cerevisiae* glucan 1,4-alpha-glucosidase	2.1	+			

Unknown

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***	**Identified by miniarray hybridization**	**Identified by microarray hybridization (BASF-LYNX)[A]**	**At least 3 GCRE sites[B]**	**Known to be Gcn4p-dependent regulated[C]**
SDS24	Nuclear protein with similarity to *S. pombe* sds23/moc1 protein	3.0	+			
SLZ1	Sporulation-specific protein	3.9	+			
SRL1	Protein with similarity to Svs1p	2.0	+			
TOS4	Protein of unknown function	3.3	+			
TOS6	Probable cell wall protein, has weak similarity to Mid2p	3.2	+			
TOS11	Protein of unknown function	2.1	+			
YAE1	Essential protein of unknown function	3.5	+			
YAR068W	Protein with similarity to ICWP protein	2.7	+			
YAR070C	Protein of unknown function	(3.56)		+		
YBR047W	Protein of unknown function	(4.81)		+		

YBR052C	Protein of unknown function	(2.27)		+	
YBR053C	Protein of unknown function	(2.71)		+	
YBR056W	Protein of unknown function	(3.56)		+	
YBR068C	Protein of unknown function	(4.55)		+	
YBR111C	Protein of unknown function	(2.49)		+	
YBR126C	Protein of unknown function	(83.26)		+	
YBR137W	Protein of unknown function	(2.27)		+	
YBR145W	Protein of unknown function	(13.19)		+	
YBR147W	Protein of unknown function, has 7 potential transmembrane segments; transcription induced by the drug FK506 in a *GCN4*-dependent manner	3.6	+		+
YBR158W	Protein of unknown function	(2.41)		+	
YBR206W	Protein of unknown function; questionable ORF	2.2	+		
YBR209W	Protein of unknown function	2.3	+		
YBR214W	Protein of unknown function	(2.94)		+	
YBR230C	Protein of unknown function	(2.36)		+	
YBR248C	Protein of unknown function	(2.41)		+	
YBR249C	Protein of unknown function	(3.71)		+	
YBR256C	Protein of unknown function	(6.62)		+	
YBR272C	Protein of unknown function	(2.60)		+	
YCL042W	Protein of unknown function	2.3	+		
YCP4	Protein with similarity to *S. pombe* brefeldin A resistance protein obr1 and *E. coli* wrba protein which stimulates binding of Trp repressor to DNA	2.5	+		
YCR013C	Protein of unknown function	(2.94)		+	
YCR049C	Protein of unknown function	55.4	+		
YCR091W	Protein of unknown function	(3.40)		+	
YDL038C	Protein of unknown function	2.2	+		
YDL124W	Protein of unknown function	2.0 (4.49)	+	+	
YDL169C	Protein of unknown function	(2.88)		+	
YDL242W	Protein of unknown function	3.1	+		
YDR008C	Protein of unknown function	(9.39)		+	
YDR032C	Protein of unknown function	(2.23)		+	
YDR071C	Protein of unknown function	(2.23)		+	
YDR154C	Protein of unknown function	2.0	+		
YDR210W-A	Protein of unknown function	(2.15)		+	
YDR215C	Protein of unknown function	7.1	+		
YDR221W	Protein with similarity to the beta subunit of human glucosidase II	2.3	+		
YDR248C	Protein of unknown function	(2.72)		+	

YDR261C-D	Protein of unknown function	(2.14)		+	
YDR274C	Protein of unknown function	3.3	+		
YDR455C	Protein of unknown function	15.2	+		
YDR494W	Protein of unknown function	2.2	+		
YDR516C	Protein of unknown function	(8.71)		+	
YDR531W	Protein of unknown function	(2.28)		+	
YEL070W	Putative mannitol dehydrogenase with similarity to *E. coli* D-mannonate oxidoreductase; Yel070p and Ynr073p are identical proteins	3.1	+		
YEL073C	Protein of unknown function	4.2 (2.46)	+	+	
YER003C	Protein of unknown function	(2.59)		+	
YER063W	Protein of unknown function	(2.33)		+	
YER067W	Protein of unknown function	(7.20)		+	
YER128W	Protein of unknown function	2.0	+		
YER175C	Protein with predicted S-adenosylmethionine-dependent methyltransferase motif, has similarity to Yhr209p; transcription induced by the drug FK506 in a *GCN4*-dependent manner	3.9 (9.05)	+	+	+
YFL002W-B	Protein of unknown function	(2.17)		+	
YFR017C	Protein of unknown function	2.6	+		
YGL037C	Protein of unknown function	(7.36)		+	
YGL059W	Protein of unknown function	(8.32)		+	
YGL074C	Protein of unknown function	2.8	+		
YGL117W	Protein of unknown function; transcription induced by the drug FK506 in a *GCN4*-dependent manner	6.9 (17.05)	+	+	+
YGL121C	Protein of unknown function	3.0 (3.33)	+	+	
YGL177W	Protein of unknown function	(3.72)		+	
YGL262W	Protein of unknown function	3.3	+		
YGR069W	Protein of unknown function	(4.02)		+	
YGR111W	Protein of unknown function	(4.03)		+	
YHR029C	Protein with similarity to *Pseudomonas aureofaciens phz*c involved in phenazine antibiotic synthesis; transcription induced by the drug FK506 in a GCN4-dependent manner	2.1 (12.56)	+	+	+
YHR049W	Protein of unknown function	2.1	+		
YHR071W	Protein of unknown function	(12.28)		+	
YHR087W	Protein of unknown function	(7.09)		+	
YHR095W	Protein of unknown function	(2.82)		+	
YHR138C	Protein of unknown function	(2.57)		+	
YHR143W	Protein of unknown function	2.4	+		
YHR143W	Protein of unknown function	(3.75)		+	

YHR162W	Protein of unknown function	(2.31)		+
YHR214W	Protein of unknown function	2.7	+	
YIL059C	Protein of unknown function	(2.64)		+
YIL141W	Protein of unknown function; questionable ORF	2.4	+	
YJL066C	Protein of unknown function	2.4	+	
YJL135W	Protein of unknown function	3.2	+	
YJL202C	Protein of unknown function	3.4	+	
YJL207C	Protein of unknown function	2.8	+	
YJR008W	Protein of unknown function	(3.72)		+
YJR154W	Protein of unknown function	(7.69)		+
YKL023W	Protein of unknown function	(2.81)		+
YKL126W	Protein of unknown function	(2.81)		+
YKL151C	Protein of unknown function	(2.42)		+
YKL153W	Protein of unknown function, highly questionable ORF	2.2 (4.08)	+	+
YKL195W	Protein of unknown function	(2.26)		+
YKL202W	Protein of unknown function	(3.47)		+
YKR018C	Protein of unknown function	(2.62)		+
YKR040C	Protein of unknown function	(7.69)		+
YKR075C	Protein with similarity to Reg1p	4.4	+	
YLL030C	Protein with similarity to Ca2+-transporting ATPase	2.3	+	
YLR031W	Protein of unknown function	14.1	+	
YLR152C	Protein of unknown function	(5.78)		+
YLR159W	Protein of unknown function	5.6	+	
YLR254C	Protein of unknown function	2.5	+	
YLR257W	Protein of unknown function	(2.16)		+
YLR270W	Protein of unknown function	(5.07)		+
YLR290C	Protein of unknown function	(4.00)		+
YLR311C	Protein of unknown function	(2.74)		+
YLR349W	Protein of unknown function	2.1	+	
YLR381W	Protein of unknown function	3.1	+	
YLR400W	Protein of unknown function	2.6	+	
YLR410W-A	Protein of unknown function	(2.48)		+
YLR428C	Protein of unknown function	2.1	+	
YLR444C	Protein of unknown function	2.0	+	
YML002W	Protein of unknown function	2.2	+	
YMR031C	Protein of unknown function	(3.21)		+

YMR083W	Protein of unknown function	(3.03)		+
YMR095C	Protein of unknown function	(5.27)		+
YMR097C	Protein of unknown function	3.3 (4.60)	+	+
YMR173W-A	Protein of unknown function	(3.16)		+
YMR178W	Protein of unknown function	(2.74)		+
YMR184W	Protein of unknown function	(2.77)		+
YMR204C	Protein of unknown function	2.5	+	
YMR253C	Protein of unknown function, likely membrane protein	2.8	+	
YMR290W-A	Protein of unknown function	(2.85)		+
YMR291W	Protein of unknown function	(3.67)		+
YMR315W	Protein of unknown function	2.3	+	
YMR315W	Protein of unknown function	(2.53)		+
YMR321C	Protein nearly identical to a fragment of Sam4p, possibly a pseudogene	3.9 (2.59)	+	+
YNL017C	Protein of unknown function	3.1	+	
YNL028W	Protein of unknown function, questionable ORF	2.0	+	
YNL058C	Protein of unknown function	2.4	+	
YNL078W	Protein of unknown function	3.0	+	
YNL115C	Protein of unknown function	(2.29)		+
YNL129W	Protein of unknown function	(3.08)		+
YNL134C	Protein with similarity to *C. carbonum* toxd gene	2.1 (3.76)	+	+
YNL144C	Protein of unknown function	(3.59)		+
YNL179C	Protein of unknown function	(3.72)		+
YNL200C	Protein of unknown function	(3.67)		+
YNL208W	Protein of unknown function	4.9 (2.85)	+	+
YNL249C	Protein of unknown function	(2.93)		+
YNL274C	Protein of unknown function	(11.81)		+
YNL333W	Protein of unknown function	(6.64)		+
YNR009W	Protein of unknown function	2.1	+	
YNR022C	Protein of unknown function	2.2	+	
YNR066C	Protein of unknown function	(2.96)		+
YNR067C	Protein with similarity to *C. albicans* Eng1p, endo-1,3-beta-glucanase	13.0 (2.64)	+	+
YNR068C	Protein of unknown function	(3.33)		+
YOL031C	Protein of unknown function	(2.39)		+
YOL083W	Protein of unknown function	(2.81)		+
YOL085C	Protein of unknown function	5.2	+	
YOL117W	Protein of unknown function	(2.88)		+

YOL134C	Protein of unknown function	2.2	+	
YOL154W	Protein of unknown function	(2.78)		+
YOR120W	Protein of unknown function	(3.59)		+
YOR146W	Protein of unknown function	2.2	+	
YOR155C	Protein of unknown function	2.5 (4.66)	+	+
YOR189W	Protein of unknown function	2.1	+	
YOR199W	Protein of unknown function	2.2	+	
YOR215C	Protein of unknown function	(3.17)		+
YOR289W	Protein of unknown function	(2.86)		+
YOR314W	Protein of unknown function	9.5	+	
YOR343C-A	Protein of unknown function	(2.17)		+
YPL014W	Protein of unknown function	3.4	+	
YPL095C	Protein of unknown function	(3.73)		+
YPL141C	Serine/threonine protein kinase with similarity to Kin4p	4.0	+	
YPL250C	Protein of unknown function	(2.24)		+
YPL258C	Protein of unknown function	(2.46)		+
YPL264C	Protein of unknown function	2.8	+	
YPL273W	Protein of unknown function	(2.72)		+
YPR064W	Protein of unknown function	3.3	+	
YPR089W	Protein of unknown function	8.4	+	
YPR093C	Protein of unknown function	3.9	+	
YPR096C	Protein of unknown function	2.1	+	
YPR117W	Protein of unknown function	2.0	+	
YPR126C	Protein of unknown function, questionable ORF	2.4	+	
YPR130C	Protein of unknown function; questionable ORF	2.4	+	

Table 2. Gcn4p-dependent repressed genes identified by the evaluation of a comparative transcriptome analysis of a wild type and a *gcn4Δ* strain grown under amino acid starvation conditions.

The "ratio wt/*gcn4Δ*" is the ratio of the average intensity of the wild type hybridization signals divided through the average intensity of the corresponding *gcn4Δ* hybridization signals. "Description" of the genes' function was done according to the YPD database (Costanzo *et al*., 2001).

***GENE*/ ORF**	**Description**	**Ratio wt/*gcn4Δ***
AAP1	Alanine/arginine aminopeptidase, highly related to aminopeptidase Ape2p (yscII) and other zinc metalloproteases	0.4
ABD1	mRNA cap methyltransferase that methylates the terminal guanine residue of the mRNA cap	0.3
ACF2	Protein involved in cortical actin assembly, has similarity to *C. albicans* Eng2p, endo-1,3-beta-glucanase	0.3
ACO1	Aconitate hydratase (aconitase), converts citrate to cis-aconitate	0.4
ADK2	Adenylate kinase (GTP)	0.4
AEP2	Protein required for the expression of Atp9p	0.1
AFG1	Protein with weak similarity to members of the AAA family of ATPases	0.3
AFG3	Mitochondrial protein involved with Yta12p in proteolytic and chaperonin activities at the inner membrane, responsible for assembly of respiratory chain complexes, member of the AAA family of ATPases	0.3
ALK1	DNA damage-responsive protein	0.4
ALR1	Putative cytoplasmic magnesium and cobalt transporter, member of the metal ion transporter family of membrane transporters	0.2
AMD2	Protein with similarity to amidases	0.4
APC4	Component of the anaphase-promoting complex (APC), required for Clb2p degradation and for the metaphase-anaphase transition	0.3
APL3	Alpha-adaptin, large subunit of the clathrin-associated protein (AP) complex	0.3
ARE2	Acyl-CoA	0.3
ARF3	Protein with similarity to members of the arf family (ras superfamily) of GTP-binding proteins	0.3
ARL3	ADP-ribosylation factor-like protein, member of the arf-sar family in the ras superfamily	0.4
ARO7	Chorismate mutase; required for the synthesis of both phenylalanine and tyrosine	0.4
ARP5	Actin-related protein	0.2
ATP2	Beta subunit of F1-ATP synthase, three copies are present in each F1 complex	0.4
ATP5	Subunit 5 of F0-ATP synthase, oligomycin sensitivity-conferring subunit	0.3
AUR1C1	Phosphatidylinositol	0.4
AYT1	Protein with similarity to acetyltransferases	0.2
BAT2	Cytosolic branched-chain amino acid transaminase	0.3
BCY1	Regulatory subunit of cAMP-dependent protein kinases	0.3
BEM2	GTPase-activating (GAP) protein that regulates Rho1p and has a role in bud emergence and cell cycle-related cytoskeletal reorganization	0.3
BTT1	Alternate beta subunit of the nascent polypeptide-associated complex, localized bound to ribosomes	0.2
BUR2	Protein involved in negative regulation of basal trancription, causes chromosome loss when overproduced	0.1
CAJ1	Homolog of *E. coli* DnaJ, has a leucine zipper	0.2
CCA1	tRNA nucleotidyltransferase (tRNA CCA-pyrophosphorylase), carries out addition of CCA to 3' end without a template	0.4
CCR4	Component of the CCR4 transcriptional complex; has positive and negative effects on transcription	0.3
CCT8	Component of Chaperonin-containing T-complex	0.4
CDC37	Cell cycle protein required for spindle pole body duplication and passage through START, interacts with multiple protein kinase systems possibly as a targeting subunit	0.4
CDC54	Member of the MCM family of proteins, involved in DNA synthesis initiation	0.4
CDC95	Translation initiation factor 6	<0.1
CHA1	L-Serine/L-threonine deaminase	0.2
CHK1	Checkpoint kinase, required for metaphase DNA-damage checkpoint	0.3
CKA1	Casein kinase II (protein kinase CK2), catalytic (alpha) subunit	0.4
CNE1	Protein with similarity to mammalian calnexin and calreticulin, may be involved in a quality control process for secretory pathway proteins	0.4

COS1	Member of the COS family of subtelomerically-encoded proteins	0.4
COS3	Member of the COS family of subtelomerically-encoded proteins	0.2
COS4	Member of the COS family of subtelomerically-encoded proteins	0.3
COS6	Member of the COS family of subtelomerically-encoded proteins	0.3
COS7	Member of the COS family of subtelomerically-encoded proteins	0.3
COX15	Protein required for cytochrome oxidase assembly	0.3
COX4	Cytochrome-c oxidase subunit IV	0.2
COX6	Cytochrome-c oxidase subunit VI	0.2
CPR6	Cyclophilin (peptidylprolyl cis-trans isomerase or PPIase), interacts with Hsp82p, homolog of mammalian cyclophilin Cyp40	0.3
CRD1	Cardiolipin synthase, synthesizes cardiolipin from phosphatidylglycerol and CDP-diacylglycerol	0.4
CSE1	Karyopherin-beta involved in the nuclear export of karyopherin-alpha, possible kinetochore protein that interacts with centromeric element CDEII	0.2
CTH1	Protein of the inducible CCCH zinc finger family	0.4
CUP1A	Metallothionein (copper chelatin); identical to Cup1Bp	0.3
CUP1B	Metallothionein (copper chelatin); identical to Cup1Ap	0.4
CUP5	Vacuolar H(+)-ATPase (V-ATPase) dicyclohexylcarbodiimide (DCCD)-binding 16 kda proteolipid subunit (subunit c) of membrane (V0) sector	0.3
CYC7	Cytochrome-c isoform 2, predominant isoform during anaerobic growth	0.3
CYS3	Cystathionine gamma-lyase, generates cysteine from cystathionine	0.4
DAL2	Allantoicase, carries out the second step in the degradation of allantoin; allows purines to be used as nitrogen sources	0.4
DAL4	Allantoin permease, member of the uracil/allantoin permease family of membrane transporters	0.3
DBP8	Protein with similarity to DEAD box family of RNA helicases	0.3
DCG1	Protein possibly involved in cell wall structure or biosynthesis	0.1
DIN7	Mitochondrial inner membrane nuclease with a role in stabilizing the mitochondrial genome; expression is induced by DNA damage	0.4
DOG2	2-Deoxyglucose-6-phosphate phosphatase, converts 2-deoxy-D-glucose 6-phosphate to 2-deoxy-D-glucose and orthophosphate	0.3
DRS2	Membrane-spanning Ca-ATPase (P-type) required for ribosome assembly and involved in late Golgi function, member of the cation transporting (E1-E2) P-type ATPase superfamily	0.4
ECM27	Protein possibly involved in cell wall structure or biosynthesis, member of the Ca2+	<0.1
EDC1	Protein involved in mRNA decapping	0.3
EFR3	Protein with similarity to *Drosophila* cmp44E, conserved, ubiquitous membrane protein requiired for cell viability	0.3
EFT1	Translation elongation factor EF-2, contains diphthamide which is not essential for activity, identical to Eft2p	0.3
EKI1	Ethanolamine kinase I; has similarity to Cki1p choline kinase	0.3
EMP70	Endosomal membrane protein with similarity to human putative ion transporter EMP70	0.4
ENA1	P-type ATPase involved in Na+ and Li+ efflux, required for Na+ tolerance	0.2
ENP1	Essential nuclear protein with effects on N-glycosylation of proteins in the secretory pathway	0.4
EPT1	sn-1,2-Diacylglycerol ethanolaminephosphotransferase	0.4
ERG1	Squalene monooxygenase (squalene epoxidase), an enzyme of the ergosterol biosynthesis pathway	0.3
ERG12	Mevalonate kinase, generates mevalonate-5-phosphate from mevalonate	0.4
ERG13	3-Hydroxy-3-methylglutaryl coenzyme A synthase, functions in mevalonate synthesis	0.3
ERG24	C-14 Sterol reductase; carries out reduction of delta 14,15 double bond of the ergosterol precursor 4,4-dimethylcholesta-8,24,24 trienol	0.2
ERG4	Sterol C-24 (28) reductase	0.4
ERG5	Cytochrome P450	0.3
ERO1	Protein required for protein disulfide bond formation in the endoplasmic reticulum	0.4
ERV14	Protein of ER-derived vesicles, with similarity to *Drosophila melanogaster* cni protein	0.3
ETF-BETA	Electron-transferring flavoprotein, beta chain	0.4
FAA1	Long-chain fatty acid CoA ligase (fatty acid activator 1); can incorporate exogenous myristate into myristoyl-CoA and other fatty acids to the CoA derivatives	0.2
FAA3	Acyl-CoA synthase (long-chain fatty acid CoA ligase); activates endogenous but not imported fatty acids	0.2
FET5	Multicopper oxidase involved in ferrous iron transport	0.4
FLR1	Member of the multidrug-resistance 12-spanner (DHA12) family of the major facilitator superfamily (MFS-MDR)	0.1
FPS1	Glycerol channel protein, member of the major intrinsic protein (MIP) family of transmembrane channel proteins	0.2
FUI1	Uridine permease, member of the uracil/allantoin family of membrane transporters	0.4

FUR4	Uracil permease, member of the uracil/allantoin permease family of membrane transporters	0.1
GBP2	Potential telomere-associated protein that binds single-stranded G-strand telomere sequence, has three RNA recognition (RRM) domains	0.3
GCD1	Translation initiation factor eIF2B (guanine nucleotide exchange factor), 58 kda (gamma) subunit	0.4
GCD11	Translation initiation factor eIF2 gamma subunit	0.2
GCD6	Translation initiation factor eIF2B (guanine nucleotide exchange factor), 81 kda (epsilon) subunit	0.3
GCN1	Component of a protein complex required for activation of Gcn2p protein kinase in response to starvation for amino acids or purines	0.2
GCN20	Component of a protein complex required for activation of Gcn2p protein kinase in response to amino acid starvation, member of the non-transporter group within the ATP-binding cassette (ABC) superfamily	0.2
GIN4	Serine/threonine-protein kinase required for septin organization at the bud neck, has similarity to Ycl024p	0.2
GIP1	Glc7p-interacting protein, possible regulatory subunit for the PP1 family protein phosphatases	0.3
GLY1	Threonine aldolase, required for glycine biosynthesis	0.2
GOG5	Golgi GDP-mannose transporter, member of the nucleotide-sugar transporter (NST) family of membrane transporters	0.3
GPI1	Protein involved with Gpi2p and Gpi3p in the first step in glycosylphosphatidylinositol (GPI) anchor synthesis	0.2
GPI8	Probable component, with Gaa1p, of a glycosylphosphatidylinositol (GPI) transamidase involved in the attachment of GPI anchors to proteins	0.4
GPM3	Phosphoglycerate mutase; has similarity to Gpm1p and Gpm2p	0.4
GRX4	Glutaredoxin, has similarity to Grx3p and Grx5p	0.3
GSG1	High molecular weight subunit of the Transport Protein Particle (TRAPP) complex that is involved in the vesicular transport from the endoplasmic reticulum to the Golgi	0.3
HCA4	Probable RNA helicase CA4, involved in ribosomal RNA processing	0.4
HCH1	Protein of unknown function	0.3
HEM2	Porphobilinogen synthase (delta-aminolevulinic acid dehydratase), second step in heme biosynthesis pathway	0.2
HEM3	Porphobilinogen deaminase (pre-uroporphyrinogen synthase), catalyzes the third step in porphyrin biosynthesis pathway	0.1
HOG1	MAP kinase (MAPK) central to the high-osmolarity signal transduction pathway	0.4
HSN1	High-copy allele-specific suppressor SEC4	0.2
HSP104	Heat shock protein required for induced thermotolerance and for resolubilizing aggregates of denatured proteins, important for the [psi-] to [PSI+] prion conversion	0.3
HSP78	Heat shock protein of the ClpB family of ATP-dependent proteases, mitochondrial	0.2
HSP82	Heat-inducible chaperonin homologous to *E. coli* HtpG and mammalian HSP90	0.4
HXT13	Member of the hexose transporter family of the major facilitator superfamily (MFS)	0.4
HXT9	Hexose transporter, member of the hexose transporter family of the major facilitator superfamily (MFS)	<0.1
IMP3	Component of U3 snoRNP, required for pre-18S rRNA processing	0.2
INO2	Basic helix-loop-helix (bHLH) transcription factor required for derepression of phospholipid synthetic genes	0.2
IOC3	Protein of unknown function	0.4
IST3	Protein with a role in sodium tolerance, has one RNA recognition (RRM) domain	0.3
JEN1	Pyruvate and lactate-proton symporter, member of the major facilitator superfamily (MFS)	0.3
KAP122	Nuclear transport factor, member of karyopherin-beta family	0.2
KEL1	Protein involved in cell fusion and morphology, contains six Kelch repeats	0.4
KEX1	Carboxypeptidase specific for C-terminal Arg or Lys, involved in processing precursors of alpha-factor and K1 and K2 killer toxins	0.2
KKQ8	Serine/threonine protein kinase of unknown function	0.2
KNS1	Serine/threonine protein kinase with unknown role	0.3
LAG1	Protein required with Lac1p for ER-to-Golgi transport of GPI-anchored proteins, has indirect effect on cell longevity	0.4
LCB2	Subunit of serine C-palmitoyltransferase, first step in sphingolipid biosynthesis, and suppressor of calcium-sensitivity of csg2	0.3
LCP5	Protein required for ribosomal RNA processing	0.4
LST4	Protein required for regulated transport of nitrogen-regulated permeases from the Golgi to the plasma membrane	0.4
LTV1	Protein required for viability at low temperature	0.4
LYP1	Lysine-specific permease; high affinity	0.2
MAK11	Protein essential for replication of M double-stranded RNA (dsRNA) virus; member of the WD (WD-40) repeat family	0.4
MAK5	Probable pre-mRNA splicing RNA-helicase of the DEAD box family, involved in maintenance of M double-stranded RNA (dsRNA) killer plasmid	0.4
MAL32	Maltase	0.3

MAL33	Maltose fermentation regulatory protein, has a Zn[2]-Cys[6] fungal-type binuclear cluster domain	0.4
MDJ1	Protein involved in mitochondrial biogenesis and protein folding, member of DnaJ family of protein chaperones	0.4
MDJ2	Protein involved in import and folding of mitochondrial proteins; has similarity to *E. coli* DnaJ and other DnaJ-like proteins, function partially overlaps that of Mdj1p	0.4
MET4	Transcriptional activator of the sulfur assimilation pathway; member of basic leucine zipper (bZIP) family	0.4
MET7	Folylpolyglutamate synthetase, involved in methionine biosynthesis and maintenance of mitochondrial genome	0.3
MHT1	Putative cobalamin-dependent homocysteine S-methyltransferase	0.4
MIP6	Protein with similarity to Pes4p and Pab1p in the N-terminal region, has 4 RNA recognition (RRM) domains	0.4
MIS1	C1-tetrahydrofolate synthase (trifunctional enzyme), mitochondrial	0.3
MLF3	Serine-rich protein conferring resistance to immunosuppresive drug leflunomide	0.3
MOD5	tRNA isopentenyltransferase; cytoplasmic and mitochondrial forms have alternative translational start sites	0.1
MON1	Protein of unknown function	0.4
MRS4	Splicing protein and member of the mitochondrial carrier (MCF) family, suppresses mitochondrial splicing defects	0.4
MSH3	Component of DNA mismatch binding factor along with Msh2p; involved in mismatch repair involving microsatellite (short repeat) sequences	0.3
MSI1	Chromatin assembly complex, subunit 3, involved in nucleosome assembly linked with DNA replication, has WD (WD-40) repeats	0.3
MSS116	Mitochondrial RNA helicase of the DEAD box family, required for splicing of group II introns of COX1 and COB	0.3
MSY1	Tyrosyl-tRNA synthetase, mitochondrial	0.4
MUP3	Low-affinity methionine amino acid permease	0.4
MUS81	Protein involved in repair of DNA damage; found in a complex with Rad54p	<0.1
MXR1	Peptide methionine sulfoxide reductase involved in cellular antioxidation	0.4
NAN1	Nucleolar protein associated with Net1p	0.3
NCA3	Protein involved in regulation of synthesis of Atp6p and Atp8p	0.2
NFU1	Protein with similarity to Anabaena nitrogen fixing protein nifU	0.3
NOP12	Protein of unknown function	0.4
NRD1	Protein that controls transcriptional elongation, interacts with the C-terminal domain of RNA polymerase II large subunit and binds DNA through a RNA recognition (RRM) domain	0.4
NTH2	Putative secondary neutral trehalase (alpha, alpha-trehalase)	0.3
NUP1	Nuclear pore protein (nucleoporin) with XFXFG motifs; related to Nsp1p	0.4
NUP159	Nuclear pore protein (nucleoporin) with XFXFG repeats	0.3
NUP84	Nuclear pore protein	0.2
OPY1	Protein that imparts Far- phenotype	0.4
ORC6	Origin recognition complex (ORC), sixth subunit, binds domain A consensus sequence	0.4
ORM1	Protein of unknown function	0.4
PAF1	Protein associated with RNA polymerase II, involved in positive and negative regulation	0.4
PAT1	Protein required for faithful chromosome transmission and normal translation initiation	0.3
PAU2	Member of the seripauperin (PAU) family	0.4
PCH2	Protein required for cell cycle arrest at the pachytene stage of meiosis in a zip1 mutant, has similarity to Rpt5p and NSF vesicular fusion protein and other members of the AAA family of ATPases	0.4
PEA2	Protein involved in oriented growth toward mating partner	0.4
PET100	Protein required for assembly of cytochrome c oxidase	0.4
PET9	ADP/ATP carrier protein of the mitochondrial carrier family (MCF) of membrane transporters	0.3
PFK27	6-Phosphofructose-2-kinase, isozyme 2	0.3
PGM1	Phosphoglucomutase, minor isoform, interconverts Glc-1-P and Glc-6-P	0.4
PHO13	4-Nitrophenylphosphatase	0.4
PHO87	Member of the phosphate permease family of membrane transporters	0.2
POR2	Outer mitochondrial membrane porin; voltage-dependent anion-selective channel	0.4
POT1	3-Ketoacyl-CoA thiolase, peroxisomal (acetyl-CoA acyltransferase), catalyzes last step of peroxisomal fatty acid beta-oxidation	<0.1
POX1	Acyl-CoA oxidase, first and rate-limiting enzyme of peroxisomal beta-oxidation	0.4
PPA1	Proteolipid of the vacuolar H(+)-ATPase (V-ATPase)	0.3
PPH3	Protein serine/threonine phosphatase, member of the PPP family of protein phosphatases and related to PP2A phosphatases	0.1
PPX1	Exopolyphosphatase, degrades polyphosphate with conversion of ADP to ATP	0.3

PPZ2	Protein serine/threonine phosphatase involved in osmoregulation, member of the PPP family of protein phosphatases and related to PP1 phosphatases	0.4
PRI1	DNA primase small subunit (DNA polymerase alpha 48 kda subunit) involved in the synthesis of RNA primers for Okazaki fragments	0.4
PRM5	Protein of unknown function regulated by pheromone	0.4
PRM8	Protein with strong similarity to subtelomerically-encoded proteins such as Cos5p, Ybr302p, Cos3p, Cos1p, Cos4p, Cos8p, Cos6p, Cos9p, and Ycr007p	0.4
PRP16	Pre-mRNA splicing factor and an ATP-dependent RNA helicase of DEAH box family	0.4
PRP43	Pre-mRNA splicing factor, member of the DEAH-box RNA helicase family	0.4
PRP6	U4/U6.U5 tri-snRNP-associated splicing factor, contains tetratricopeptide (TPR) repeats	0.3
PRS1	Phosphoribosylpyrophosphate synthetase, synthesizes phosphoribosylpyrophosphate (PRPP) from ribose-5' phosphate and ATP, involved in pseudohyphal growth	0.4
PRS3	Phosphoribosylpyrophosphate synthetase (ribose-phosphate pyrophosphokinase II); component of yeast 20S proteasome, with a role in cell cycle regulation	0.4
PSD2	Phosphatidylserine decarboxylase; Golgi and vacuolar isozyme, converts phosphatidyl-L-serine to phosphatidylethanolamine	0.4
PTC3	Protein serine/threonine phosphatase of the PP2C family	0.3
PUS4	Pseudouridine synthase that creates pseudouridine-55 in cytoplasmic and mitochondrial tRNAs	0.1
PUT2	Delta-1-pyrroline-5-carboxylate dehydrogenase (P5C dehydrogenase), carries out the second step in proline degradation; allows proline to be used as a nitrogen source	0.2
QRI1	UDP-N-acetylglucosamine pyrophosphorylase	0.3
RAD55	Component of recombinosome complex involved in meiotic recombination and recombinational repair; with Rad57p promotes DNA strand exchange by Rad51p recombinase	0.4
RAD59	Homolog of Rad52p involved in homologous recombination and DNA repair	0.4
RAI1	Protein that binds and enhances function of nuclear exonuclease Rat1p	0.2
RAS1	GTP-binding protein involved in regulation of cAMP pathway, homolog of mammalian proto-oncogene ras	0.3
RBK1	Ribokinase, member of a family of sugar kinases that includes Pfk2p	0.3
RCK1	Serine/threonine protein kinase with similarity to Cmk1p, Cmk2p, and Cmk3p	0.2
RFC3	Replication factor C, 40 kda subunit, member of the DEAD box family of putative helicase proteins	0.4
RFT1	Protein involved required for N-linked glycosylation	0.3
RGD1	GTPase-activating protein (GAP) for Rho3p and Rho4p	0.4
RGP1	Protein required for proper mitotic growth	0.4
RHO3	GTP-binding protein involved in control of actin cytoskeleton and exocytosis, member of the rho family in the ras superfamily	0.4
RIF1	Protein involved in telomere length regulation and transcriptional silencing, interacts with Rap1p	<0.1
RIO1	Protein with similarity to *Aspergillus nidulans* SUDD suppressor of bimD6 chromosome-spindle attachment defect	0.4
RKI1	Ribose 5-phosphate ketol-isomerase	0.3
RNA1	GTPase-activating (GAP) protein for Gsp1p (Ran), involved in nuclear export	0.4
RNH1	Ribonuclease H, endonuclease that degrades RNA in RNA-DNA hybrids	0.4
RNH35	Ribonuclease H, endonuclease that degrades RNA in RNA-DNA hybrids	0.2
RNP1	Ribonucleoprotein 1, contains two RNA recognition (RRM) domains which include RNP-1 octamer and RNP-2 hexamer motifs	<0.1
ROK1	ATP-dependent RNA helicase required for rRNA processing, member of DEAD box family	0.4
RPA49	RNA polymerase I third largest subunit	0.4
RPC53	RNA polymerase III, fourth-largest essential subunit (C53)	0.4
RPL16B	Ribosomal protein L16 (rp23), nearly identical to Rpl16Ap	0.4
RPL31B	Ribosomal protein L31 (yeast L34; YL36; YL28; rat L31), nearly identical to Rpl31Ap	0.3
RPS19B	Ribosomal protein S19 (rp55; YS16B; rat S19), nearly identical to Rps19Ap	0.4
RPS7B	Ribosomal protein S7 (rp30; human S7; *Xenopus* S8), nearly identical to Rps7Ap	0.3
RRN9	Component of the Upstream Activation Factor (UAF) complex, involved in activation of RNA polymerase I	0.1
RRP3	Helicase required for maturation of pre-rRNA	0.2
RRP4	3'-5' exoribonuclease required for 3' processing of ribosomal 5.8S rRNA, component of both the nucleolar and cytoplasmic forms of the 3'-5' exosome complex	0.4
RSC8	Component of abundant chromatin remodeling complex (RSC)	0.3
RTG2	Protein involved in inter-organelle communication between mitochondria, peroxisomes, and nucleus	0.1
RTS2	Protein with similarity to mouse KIN17 protein, has a single C2H2-type zinc finger	0.1
RVB1	Putative 3' to 5' DNA/RNA helicase involved in ribosomal RNA processing	0.3
SAG1	Alpha-agglutinin involved in cell-cell interactions during mating	0.3

SCH9	Serine/threonine protein kinase involved in nutrient-sensing signaling pathway, probably parallel to cAMP pathway	0.4
SCT1	Suppressor of a choline transport mutant	0.3
SDA1	Essential protein required for normal organization of the actin cytoskeleton	0.3
SDS3	Suppressor of silencing defect	0.4
SEC13	Component of the COPII coat of vesicles involved in endoplasmic reticulum to Golgi transport, contains six WD (WD-40) repeats	0.4
SEC21	Coatomer (COPI) complex gamma chain (gamma-COP) of secretory pathway vesicles; required for retrograde Golgi to endoplasmic reticulum transport	<0.1
SEC34	Protein involved in retention of proteins in the Golgi apparatus	0.3
SEC4	GTP-binding protein involved in vesicular transport between Golgi and plasma membrane, member of the rab family in the ras superfamily	0.2
SET1	Protein involved in diverse cellular processes including transcription and chromatin structure, member of the trithorax family of SET domain-containing proteins	0.1
SFI1	Protein required for cell cycle progression, suppressor of partial adenylate cyclase deficiency	0.4
SHC1	Protein involved in cell wall chitin synthesis or deposition	0.2
SHU1	Protein of unknown function	0.2
SKI7	Antiviral protein that represses replication of double-stranded RNA viruses	0.3
SKI8	Protein involved in meiotic recombination and in protection from double-stranded RNA (dsRNA) viruses; member of the WD (WD-40) repeat family	0.1
SMF3	Possible metal transporter, has similarity to Smf1p and Smf2p	0.3
SMP2	Protein involved in plasmid maintenance and respiration	0.2
SNG1	Probable transport protein, confers resistance to MNNG and nitrosoguanidine	0.2
SNU71	U1 snRNA-associated protein	0.3
SPO11	Catalytic subunit of the meiotic double strand break transesterase	<0.1
SPP381	Protein required for efficient pre-mRNA splicing, component of the U4/U6.U5 tri-snRNP complex	0.4
SPS100	Sporulation specific protein involved in spore wall formation, first appears at 12h of sporulation	0.4
SPS2	Protein involved in middle/late stage of sporulation	0.3
SPT10	Protein that amplifies the magnitude of transcriptional regulation at various loci	0.4
SRB2	Component of the RNA polymerase II holoenzyme and Kornberg's mediator (SRB) subcomplex	0.2
SRD1	Nucleolar protein involved in pre-rRNA processing, but does not bind to small nucleolar RNA (snoRNA)	0.3
SRP101	Signal recognition particle receptor, alpha subunit, interacts with beta subunit, Srp102p	0.4
SSA1	Cytoplasmic chaperone and heat shock protein of the HSP70 family	0.3
SSA2	Cytoplasmic chaperone; member of the HSP70 family	0.3
SSA3	Chaperone of the HSP70 family, heat-induced cytoplasmic form not expressed under optimal conditions	0.2
SSA4	Protein chaperone of the HSP70 family, cytoplasmic heat-induced form that is not expressed under optimal conditions	0.2
SSE1	Heat shock protein of the HSP70 family, multicopy suppressor of mutants with hyperactivated ras/cAMP pathway	0.3
SSE2	Heat shock protein of the HSP70 family, present at low abundance at 23 deg but greatly induced after shift to 37 deg	0.1
SSF1	Protein with a potential role in mating	0.4
SSL2	DNA helicase component of RNA polymerase II transcription initiation factor TFIIH	0.1
STE11	MAP kinase kinase kinase (MAPKKK or MEKK), component of pheromone response, filamentous growth, high-osmolarity sensing, and STE vegetative growth pathways	<0.1
STE2	Pheromone alpha-factor G protein-coupled receptor (GPCR), member of the GPCR or seven transmembrane segments (7-TMS) superfamily	0.3
STI1	Stress-induced protein required for optimal growth at high and low temperature, has tetratricopeptide (TPR) repeats	0.3
SUA5	Protein involved in translation initiation, mutation suppresses the effect of an aberrant upstream ATG in CYC1	0.3
SUN4	Protein involved in cell separation and in the aging process	0.3
SYS1	Multicopy suppressor of ypt6 null mutation	0.3
TAF145	Component of the TAF(II) complex (TBP-associated protein complex); required for activated transcription by RNA polymerase II	0.3
TAF60	Component of TAF(II) complex (TBP-associated protein complex) and SAGA complex (Spt-Ada-Gcn5-acetyltransferase); required for activated transcription by RNA polymerase II	0.4
TAF90	Component of the TAF(II) complex (TBP-associated protein complex) and SAGA complex (Spt-Ada-Gcn5-acetyltransferase); required for activated transcription by RNA polymerase II, member of WD (WD-40) repeat family	0.3
TEP1	Protein tyrosine phosphatase (PTPase), has similarity to human tumor suppressor gene TEP1/MMAC/PTEN1	0.1
TFB2	Component of RNA polymerase II transcription initiation TFIIH (factor b), 55 kda subunit	0.2

THP1	Protein of unknown function	0.4
THS1	Threonyl-tRNA synthetase, cytoplasmic, member of the class II family of aminoacyl-tRNA synthetases	0.4
TID3	Protein of the spindle pole body with similarity to myosin heavy chain, possible coiled-coil	0.3
TIM44	Mitochondrial inner membrane protein required in transport across the inner membrane	0.4
TIR2	Cold-shock induced protein, member of the seripauperin (PAU) family	0.4
TPD3	Protein serine/threonine phosphatase 2A (PP2A) regulatory subunit A, ceramide-activated protein phosphatase A subunit	0.4
TRM2	Uridine methyltransferase that catalyzes the formation of ribothymidine at position 54 in cytoplasmic and mitochondrial tRNA	0.4
UBP9	Ubiquitin C-terminal hydrolase, has similarity to Ubp13p	0.1
UFD4	Probable ubiquitin-protein ligase E3 enzyme functioning in ubiquitin fusion degradation pathway	0.4
URA1	Dihydroorotate dehydrogenase, catalyzes fourth step in pyrimidine biosynthesis pathway	0.1
URK1	Uridine kinase, converts ATP and uridine to ADP and UMP	0.3
VAN1	Vanadate resistance protein; component of mannan polymerase M-Pol I, which includes Mnn9p and Van1p	0.3
VID28	Protein involved in vacuolar import and degradation	0.2
VPH1	Vacuolar H(+)-ATPase (V-ATPase) 94 kda subunit of membrane (V0) sector, essential for vacuolar acidification and vacuolar H(+)-ATPase (V-ATPase) activity	0.3
VPS38	Protein involved in vacuolar sorting	0.3
VPS5	Member of the sorting nexin family involved in Golgi retention and vacuolar sorting	0.4
VPS8	Protein involved in vacuolar sorting	0.4
VTC4	Putative polyphosphate synthetase	0.3
WBP1	Oligosaccharyltransferase beta subunit, member of a complex of 8 ER proteins that transfers core oligosaccharide from dolichol carrier to Asn-X-Ser/Thr motif	0.4
YAL004W	Protein of unknown function; questionable ORF	0.4
YAL019W	Protein of the Snf2p family with a DNA-dependent ATPase domain, involved in resistance to UV radiation	0.4
YAR1	Protein with 2 ankyrin repeats	0.1
YBL009W	Protein of unknown function	0.2
YBL046W	Protein of unknown function	0.4
YBL060W	Protein of unknown function	0.4
YBL111C	Protein of unknown function; subtelomerically encoded	0.3
YBR005W	Protein of unknown function, mRNA abundance is reduced by the drug FK506 in a calcineurin- and immunophilin-dependent manner	0.3
YBR013C	Protein of unknown function	0.3
YBR096W	Protein of unknown function, has a role in pseudohyphal growth and in resistance to elevated concentrations of NaCl and H2O2	0.4
YBR101C	Protein involved in resistance to H2O2	0.3
YBR187W	Protein with similarity to ND5 and PSB2	0.4
YBR210W	Protein of unknown function, has similarity to Erv14p, a protein of ER-derived vesicles	0.2
YBR266C	Protein of unknown function	0.4
YCL002C	Protein of unknown function	0.3
YCL041C	Protein with similarity to human papilloma virus (HPV) E6 protein	0.4
YCR023C	Member of the multidrug-resistance 12-spanner (DHA12) family of the major facilitator superfamily (MFS-MDR)	0.3
YCR044C	Suppressor of cdc1-1 temperature-sensitive growth defect, involved in Mn2+ homeostasis	0.3
YCR045C	Protein with similarity to protease B (Prb1p) and subtilisin family proteases	<0.1
YCR079W	Protein phosphatase of the PP2C family	0.2
YCR105W	Protein with similarity to bovine alcohol dehydrogenase	0.4
YDL010W	Protein with similarity to Ybr014p and glutaredoxins	0.4
YDL015C	Protein with similarity to rat synaptic glycoprotein SC2	0.2
YDL036C	Protein with similarity to Rib2p	0.3
YDL037C	Protein with similarity to glucan 1,4-alpha-glucosidase	0.3
YDL060W	Protein of unknown function	0.4
YDL085W	Mitochondrial NADH dehydrogenase that catalyzes the oxidation of cytosolic NADH	0.1
YDR003W	Protein with similarity to Ybr005p	0.3
YDR036C	Member of the enoyl-coA hydratase/isomerase protein family	0.4
YDR049W	Protein of unknown function, has a single C2H2-type zinc finger	0.3

YDR060W	Protein required for 60S ribosomal subunit biogenesis	0.4
YDR061W	Protein with similarity to *E. coli* photorepair protein	0.4
YDR083W	Protein involved in cleavage at site A2 in pre-rRNA in the pathway of ribosomal RNA processing	0.3
YDR084C	Protein of unknown function	0.4
YDR101C	Protein of unknown function	0.4
YDR105C	Protein of unknown function	0.4
YDR111C	Putative alanine aminotransferase	0.4
YDR132C	Protein of unknown function, putative pseudogene	0.4
YDR165W	Protein of unknown function	0.4
YDR179W	Protein of unknown function	0.3
YDR223W	Protein of unknown function	0.2
YDR267C	Protein with similarity to Sec13p and other proteins with WD (WD-40) repeats	0.3
YDR307W	Protein with similarity to Pmt1p	0.4
YDR333C	Protein of unknown function	0.2
YDR370C	Protein of unknown function	0.3
YDR372C	Protein of unknown function	0.3
YDR398W	Protein of unknown function	0.4
YDR412W	Cytoplasmic protein of unknown function	0.3
YDR426C	Protein of unknown function	<0.1
YDR428C	Protein of unknown function	0.4
YDR534C	Protein of unknown function	0.3
YDR541C	Protein of unknown function, member of a family (GRE2, YGL039W, YGL157W, YDR541C) having similarity to plant dihydroflavonol-4-reductases	0.4
YEL015W	Protein of unknown function	0.4
YEL047C	Cytoplasmic soluble fumarate reductase	0.3
YEL072W	Protein of unknown function	0.1
YEL075C	Protein with similarity to other subtelomerically-encoded proteins including Yhl049p, Yil177p, and Yjl225p	0.1
YER024W	Protein with similarity to Yat1p	0.4
YER034W	Protein of unknown function	0.1
YER049W	Protein of unknown function	0.4
YER113C	Protein with similarity to Emp70p	0.2
YER135C	Protein of unknown function	0.3
YER186C	Protein of unknown function	0.2
YFL027C	Protein of unknown function	0.3
YFR038W	Member of the Snf2/Rad54 subfamily of NTP-dependent DNA helicases	0.4
YFR045W	Member of the mitochondrial carrier family (MCF) family of membrane transporters	0.4
YFR046C	Protein of unknown function	0.4
YGL015C	Protein of unknown function	0.1
YGL046W	Protein of unknown function	0.4
YGL060W	Protein of unknown function	0.2
YGL114W	Protein member of the oligopeptide transporter (OPT) family of membrane transporters	0.3
YGR031W	Protein of unknown function	0.4
YGR043C	Protein of unknown function, may be involved in signal transduction; has strong similarity to Tal1p	0.4
YGR089W	Protein with similarity to human desmoplakin I PIR	0.3
YGR103W	Nuclear protein of unknown function with similarity to zebrafish pescadillo	0.4
YGR110W	Protein of unknown function, induced during aerobic growth	<0.1
YGR126W	Protein of unknown function	0.4
YGR127W	Protein with similarity to mouse T10 protein PIR	0.3
YGR169C	Protein of unknown function	0.3
YGR176W	Protein of unknown function	0.4
YGR198W	Protein of unknown function	0.2
YGR223C	Protein of unknown function	<0.1
YGR242W	Protein of unknown function	<0.1

YGR266W	Protein of unknown function	0.4
YGR283C	Protein of unknown function	0.4
YHL029C	Protein of unknown function	0.3
YHL049C	Protein with similarity to other subtelomerically-encoded proteins including Yer189p, Yml133p, and Yjl225p, coded from a subtelomeric Y' region	0.1
YHR035W	Protein with weak similarity to Sec23p, expressed under nitrogen-starvation conditions	0.1
YHR045W	Protein of unknown function, has 5 potential transmembrane domains	0.3
YHR056C	Protein of unknown function, encoded from the CUP1A and CUP1B repeat region	0.3
YHR130C	Protein of unknown function	0.3
YHR149C	Protein of unknown function	0.4
YHR151C	Protein of unknown function	0.4
YHR196W	Protein of unknown function	0.4
YHR199C	Protein with similarity to Yhr198p	0.4
YHR202W	Protein of unknown function	0.2
YHR204W	Protein with similarity to alpha-mannosidase and other glycosyl hydrolases	0.4
YHR212C	Protein identical to Yar060p/Raa19p	0.4
YHR218W	Protein with near identity to subtelomerically-encoded proteins including Yhr219p and Yfl065p, possible pseudogene	0.4
YIL011W	Member of the seripauperin (PAU) family of possible cell wall mannoproteins	0.3
YIL028W	Protein of unknown function, possible pseudogene	0.4
YIL039W	Protein of unknown function	0.3
YIL040W	Protein of unknown function	0.2
YIL090W	Protein of unknown function	0.1
YIL091C	Protein of unknown function	0.3
YIL092W	Protein of unknown function	0.4
YIL102C	Protein of unknown function	0.4
YIL137C	Protein with similarity to aminopeptidases	0.4
YIL145C	Protein with similarity to *E. coli* pantoate beta-alanine ligase (pantothenate synthetase)	0.4
YIL163C	Protein of unknown function	0.3
YIP1	Protein involved in vesicular transport; interacts with transport GTPases Ypt1p and Ypt31p at the Golgi membrane	0.3
YIR042C	Protein of unknown function	0.3
YJL038C	Protein of unknown function	0.4
YJL171C	Protein of unknown function	0.3
YJL195C	Protein of unknown function, questionable ORF	0.2
YJL204C	F-box protein involved in endocytic membrane traffic and recycling out of an early endosome	0.3
YJR013W	Protein with weak similarity to human angiotensin II type 1b receptor	0.4
YJR084W	Protein of unknown function	0.4
YJR098C	Protein of unknown function	0.4
YJR146W	Protein of unknown function	0.3
YKL027W	Protein with similarity to *E. coli* molybdopterin-converting factor	0.4
YKL034W	Protein of unknown function	0.2
YKL051W	Protein of unknown function	0.3
YKL077W	Protein of unknown function	0.4
YKL174C	Protein with similarity to Hnm1p and other permeases	0.4
YKR015C	Protein of unknown function	0.2
YKR087C	Protein with a putative zinc-binding motif of neutral zinc metallopeptidases	0.3
YLL005C	Protein of unknown function	0.4
YLR003C	Protein of unknown function	0.4
YLR007W	Protein of unknown function	0.4
YLR008C	Protein of unknown function	0.1
YLR016C	Protein of unknown function	0.4
YLR033W	Protein with similarity to human TFIIIC90	0.3
YLR050C	Protein with similarity to C-terminal region of human MAC30	0.4
YLR064W	Protein of unknown function	0.4

YLR184W	Protein of unknown function	0.3
YLR187W	Protein of unknown function	0.3
YLR217W	Protein of unknown function	0.1
YLR218C	Protein of unknown function	0.3
YLR219W	Protein of unknown function	0.4
YLR225C	Protein of unknown function	0.4
YLR339C	Protein of unknown function	0.4
YML080W	Protein with weak similarity to *Azospirillum brasilense* nifR3 protein	0.3
YML087C	Protein of unknown function	0.3
YMR048W	Protein of unknown function	0.3
YMR115W	Protein of unknown function	0.4
YMR210W	Protein of unknown function	0.3
YMR251W	Putative paralog of Ecm4p	0.3
YMR252C	Protein of unknown function	0.1
YMR266W	Possible membrane transporter involved in tunicamycin sensitivity	0.2
YNL065W	Member of the multidrug-resistance 12-spanner (DHA12) family of the major facilitator superfamily (MFS-MDR)	0.2
YNL080C	Protein of unknown function	0.4
YNL101W	Putative membrane transporter of the amino acid/auxin permease (AAAP) family	0.3
YNL119W	Protein possibly involved in cytoplasmic ribosome function	0.4
YNL120C	Protein of unknown function, questionable ORF	<0.1
YNL158W	Protein of unknown function	0.4
YNL182C	Protein of unknown function	0.2
YNL190W	Protein of unknown function	0.4
YNL266W	Protein with weak similarity to NADH dehydrogenases	0.3
YNL288W	Protein of unknown function	0.2
YNR004W	Protein of unknown function	0.4
YNR047W	Serine/threonine protein kinase of unknown function	0.4
YNR048W	Protein of unknown function	0.3
YNR053C	Nuclear protein of unknown function, has similarity to human breast tumor-associated autoantigen	0.4
YOL014W	Protein of unknown function	0.4
YOL024W	Protein of unknown function	0.1
YOL032W	Protein of unknown function	0.4
YOL035C	Protein of unknown function	0.1
YOL057W	Protein with similarity to rat dipeptidyl peptidase III which is a zinc-binding metalloproteinase	0.3
YOL063C	Protein of unknown function, has WD (WD-40) repeats	0.4
YOL128C	Serine/threonine protein kinase with strong similarity to Mck1p	0.4
YOR137C	Protein of unknown function	0.4
YOR161C	Protein of unknown function	0.1
YOR164C	Protein of unknown function	0.3
YOR258W	Protein of unknown function	0.4
YOR273C	Member of the multidrug-resistance 12-spanner (DHA12) family of the major facilitator superfamily (MFS-MDR)	0.3
YOR292C	Protein of unknown function	0.2
YOR390W	Protein of unknown function	0.4
YPK2	Serine/threonine protein kinase with similarity to Ypk1p	0.4
YPL041C	Protein of unknown function	0.2
YPL060W	Protein with similarity to Mrs2p	0.3
YPL107W	Protein of unknown function	0.3
YPL108W	Protein of unknown function	0.4
YPL113C	Protein with similarity to *E. coli* 2-hydroxyacid dehydrogenase (SP	0.4
YPL150W	Serine/threonine protein kinase with unknown role	0.1
YPL272C	Protein of unknown function	0.1
YPT11	Protein of unknown function, member of the rab family in the ras superfamily of small GTP-binding proteins	0.1

YSR3	Sphingoid base-phosphate phosphatase, putative regulator of sphingolipid metabolism and stress response	0.4
ZPR1	Zinc finger protein of unknown function	0.2
ZRT2	Low-affinity zinc transport protein, member of ZIP family of metal ion transporters	0.4
ZUO1	Zuotin (Z-DNA-binding protein), has region of similarity to *E. coli* DnaJ chaperonin	0.4

References

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