Regulation of gene expression and adhesion in *Saccharomyces cerevisiae*

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Malte Kleinschmidt
aus
Hoya

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Korreferent: Prof. Dr. W. Liebl
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Summary

Adherence represents one important and initial virulence factor of fungal pathogenicity. In the model fungus *Saccharomyces cerevisiae* adherence to substrates or to other cells depends on nutrients and is part of complex developmental processes, such as haploid invasive growth or diploid pseudohyphal formation. Adherence *per se* can also be induced by amino acid starvation. This specific adaptation requires the adhesin Flo11p and the transcriptional activator of the general amino acid control system Gcn4p.

A genome-wide transcriptional analysis of Σ1278b yeast cells under adhesion-inducing conditions imposed by amino acid starvation was performed to identify specifically regulated genes. 22 novel genes were inducible by amino acid starvation. 72 genes of different functional groups showed a previously unrecognized dependence upon Gcn4p under adhesion-inducing conditions. In addition, several genes were identified as inducible by amino acid starvation in a Gcn4p-independent manner.

2D-DIGE experiments of Σ1278b yeast cells were carried out to identify regulated proteins under adhesion-inducing conditions. Seven protein spots displayed a highly increased intensity in response to amino acid starvation. These protein spots were identified by mass spectrometry as Cpc2p, Efb1p, His1p, Hsp60p, Sod1p, Tpi1p and Tpm1p. Comparisons with the respective transcriptional profiles revealed that the mRNA levels of the encoding genes were significantly increased only for the *HIS1* gene. Deletion of *CPC2*, which encodes a highly conserved Gβ-like WD-repeat protein, results in an adhesion deficient phenotype of amino acid-starved yeast cells. *CPC2* is also required for basal expression and activation of *FLO11* under amino acid starvation. The adherence-dependent developmental processes of haploid invasive growth and diploid pseudohyphal formation also depend on *CPC2*.

During utilization of the fermentable carbon source glucose, transcription of *CPC2* is induced. *CPC2* promoter analyses were performed to analyse regulation, and identified two upstream activation sequence elements required for basal expression and regulation of *CPC2*. The forkhead-like transcription factor Fhl1p and its co-factor Ifh1p were found as *trans*-acting elements. Deletion of *FHL1* reduces *CPC2* transcription significantly in presence of glucose, whereas increased amounts of Ifh1p induces *CPC2* transcription even under utilization of the non-fermentable carbon source ethanol.

Zusammenfassung

Adhäsion stellt einen wichtigen Faktor bei der Virulenz pathogener Pilze dar. In dem Modellorganismus *Saccharomyces cerevisiae* ist die Adhäsion an Substraten oder an anderen Zellen abhängig vom Nährstoffangebot und Bestandteil komplexer Entwicklungsprozesse, wie dem haploiden invasiven Wachstum oder der Bildung von Pseudohyphen diploider Zellen. Adhäsion *per se* kann auch durch einen Mangel an Aminosäuren induziert werden. Diese spezifische Adaption benötigt das Adhäsin Flo11p und den Transkriptionsaktivator der ‚Allgemeinen Kontrolle der Aminosäurebiosynthese' Gcn4p.

Um spezifisch regulierte Gene zu identifizieren, wurde Adhäsion durch Aminosäuremangel induziert und eine genomweite Transkriptionsanalyse von Σ1278b Hefezellen durchgeführt. 22 neue Gene waren durch Aminosäuremangel induzierbar. 72 Gene aus verschiedenen funktionellen Gruppen zeigten eine bisher nicht bekannte Abhängigkeit von Gcn4p unter zur Adhäsion führenden Bedingungen. Zusätzlich wurden zahlreiche Gene identifiziert, die durch Aminosäuremangel Gcn4p-unabhängig induziert werden.

Um regulierte Proteine unter zur Adhäsion führenden Bedingungen zu identifizieren, wurden 2-D-DIGE-Experimente mit Σ1278b Hefezellen durchgeführt. Sieben Proteinpunkte zeigten eine stark erhöhte Intensität unter Aminosäuremangel. Diese Proteinpunkte wurden durch Massenspektrometrie als Cpc2p, Efb1p, His1p, Hsp60p, Sod1p, Tpi1p und Tpm1p identifiziert. Ein Vergleich mit den entsprechenden Transkriptom-Daten ergab, dass unter Aminosäuremangel nur die mRNA Menge des *HIS1*-Gens signifikant erhöht ist. In Hefezellen führt eine Deletion des *CPC2*-Gens, welches für ein hoch konserviertes Gβ-artiges WD-Protein kodiert, zu einem Verlust der Adhäsion bei mangelhafter Versorgung mit Aminosäuren. Ein funktionales *CPC2*-Gen wird auch für die basale Expression von *FLO11* und dessen Aktivierung unter Aminosäuremangel benötigt. Die adhäsionsabhängigen Entwicklungsprozesse des haploiden invasiven und diploiden Pseudohyphen-Wachstums sind ebenfalls abhängig von *CPC2*.

Bei Verwertung der fermentierbaren Kohlenstoffquelle Glukose kommt es zur Induktion der *CPC2*-Transkription. *CPC2*-Promotoranalysen wurden durchgeführt, um diese Regulation zu untersuchen. Dabei wurden zwei stromaufwärts liegende Aktivierungselemente identifiziert, die für die basale Expression und Regulation von *CPC2* benötigt werden. Der ‚Forkhead'-artige Transkriptionsfaktor Fhl1p und sein Co-Faktor Ifh1p wurden als *trans*-agierende Elemente identifiziert. Ein Verlust von *FHL1* verringert die *CPC2* Transkription signifikant in Anwesenheit von Glukose, während erhöhte Mengen von Ifh1p auch bei Verwertung der nicht-fermentierbaren Kohlenstoffquelle Ethanol die *CPC2*-Transkription induzieren.

Chapter I

Introduction

1. Regulation of adhesion in *Saccharomyces cerevisiae*

Adherence is one of the most prominent determinants of fungal pathogenesis. It represents the crucial initial event of the fungus–host interaction and protects the fungus from removal by physical forces as wind or water. Furthermore, adherence is one of the prerequisites for the development of multicellular structures such as fungal filaments or biofilms. For the human pathogen *Candida albicans* it was shown that the transition of the unicellular yeast form to a hyphal form enhances virulence of the fungus (Lo *et al.*, 1997). In addition, biofilms of *C. albicans* are extremely resistant to antifungal drugs and act as a source of reinfections (Baillie and Douglas, 2000; Chandra *et al.*, 2001; Douglas, 2003; Lamfon *et al.*, 2004; Ramage *et al.*, 2005). Adherence to plastic surfaces such as prostheses or catheters can trigger hospital-acquired fungal infections, which are an increased clinical problem, especially for the group of immuno-compromised persons (Cormack *et al.*, 1999; Douglas, 2003; Sundstrom, 2002).

The non-pathogenic fungus *Saccharomyces cerevisiae* has also the ability for cell-cell and cell-surface adhesion in response to different environmental stimuli. Because of its easy handling and its high conservation of signal transduction pathways and of many proteins throughout the fungi, *S. cerevisiae* is appropriate to act as a model organism for adherence-dependent fungal infections.

1.1 Life cycle of *S. cerevisiae*

The budding yeast *S. cerevisiae* is able to live as a haploid (1n) or as a diploid (2n) organism. The switch between both genetic phases is mediated by conjugation of two haploid cells (1n to 2n) or sporulation of one diploid cell (2n to 1n) (Figure 1). Beside this alteration of genetic phases, cell morphology of haploid and diploid cells can also change in response to distinct environmental stimuli. Most of these developmental processes in the

life cycle of *S. cerevisiae* require the ability of the cell for cell-cell or cell-surface adhesion.

Haploid yeast cells exist in two different mating types, namely ‘a’ and ‘α’, which are able to conjugate with each other to form diploid (a/α) yeast cells. This process is initiated by small peptide pheromones that are constitutively secreted into the medium by haploid yeast cells. Haploid yeast cells with the opposite mating type sense these molecules and activate a signal cascade which induces alterations in different cellular processes including cell cycle arrest, polarity and morphology changes, and adherence. Adhesion represents one prerequisite for the initial contact of ‘a’ and ‘α’ cells finally resulting in cell and nuclear fusion (Cappellaro *et al.*, 1994; Roy *et al.*, 1991).

Diploid cells are able to grow as a yeast form with unicellular ellipsoid cell morphology, or in response to nitrogen starvation, to switch into a filamentous growth form consisting of chains of long, thin and elongated cells called pseudohyphae (Gimeno *et al.*, 1992; Mösch, 2000) (Figure 1). Beside these changes in morphology, pseudohyphal development also induces changes in the budding pattern from bipolar to unipolar distal, which results in linear filamentous chains of cells. In addition, pseudohyphal cells show an incomplete cell separation and an adhesive growth behaviour leading to a formation of long multicellular chains. A further consequence of enhanced adhesiveness is that pseudohyphal cells stick to the surface of substrates and grow there invasively.

A related phenotype is also observed in haploid yeast cells upon glucose limitation, namely haploid invasive growth (Cullen and Sprague, 2000; Roberts and Fink, 1994) (Figure 1). The dimorphic switch in haploids induces cell-cell adhesion and a direct substrate invasion, but only limited changes in the cell morphology. In contrast to pseudohyphal cells, haploid yeast cells growing invasively display a bipolar budding pattern.

A similar dimorphic mechanism has also been described for many pathogenic fungi. The dimorphic switch of pathogenic fungi results in an increased adherence when exposed to their host (San-Blas *et al.*, 2000; Sanchez-Martinez and Perez-Martin, 2001). Such regulated dimorphism and adherence has been established as an important virulence factor for colonization and invasion of the host by pathogenic fungi like *Candida albicans*, *Magnaporthe grisea* and *Ustilago maydis* (Lengeler *et al.*, 2000; Sanchez-Martinez and Perez-Martin, 2001).

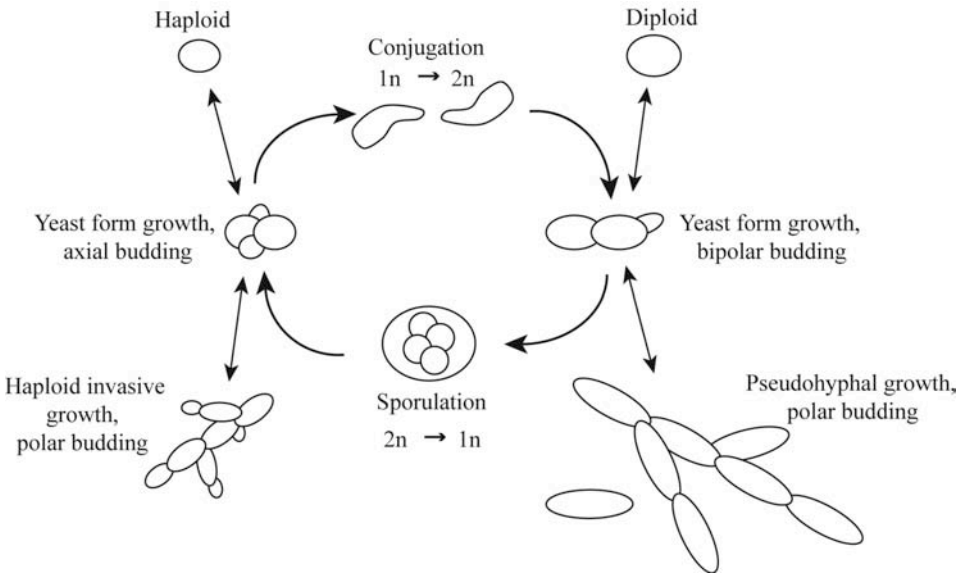

Figure 1: Life cycle of *S. cerevisiae* (adapted from Mösch, 2000).
Both haploids and diploids can grow vegetatively in the yeast form, switch to an invasive and adhesive grow mode or arrest growth in the stationary phase. In response to nitrogen starvation, diploids grow invasively as multicellular filaments called pseudohyphae. Invasively growing haploids develop small microfilaments upon glucose limitation. Haploids (1n) of the opposite mating types can conjugate to form diploids (2n), and these diploids can sporulate to form haploids.

1.2 Environmental stimuli and sensing systems

Adherence of *S. cerevisiae* is tightly controlled by environmental stimuli. Under laboratory conditions, haploid or diploid yeast cells are normally cultivated in medium containing the nitrogen source ammonium and the fermentable carbon source glucose. In diploid cells, starvation for nitrogen in presence of a fermentable carbon source induces pseudohyphal development. This pseudohyphal phenotype is not formed in medium containing standard amounts of ammonium, arginine, glutamine or glutamate, whereas standard concentrations of proline, histidine or uracil as sole nitrogen source are permissive for pseudohyphal development (Gimeno *et al.*, 1992). The sensor system that differentiates between different nitrogen components to control pseudohyphal growth is not completely understood. As sensor for the ammonium concentration, the high affinity ammonium permease Mep2p was identified (Lorenz and Heitman, 1998). Deletion of *MEP2* results in a pseudohyphal deficient phenotype suggesting that Mep2p is also involved in transmitting a signal to intracellular signalling pathways.

The fermentable carbon sources that promote pseudohyphal development are glucose, galactose, sucrose, maltose, and raffinose (Gimeno *et al.*, 1992; Kron *et al.*, 1994; Lorenz *et al.*, 2000). One sensor for different carbon sources is the cell surface G-protein Gpr1p (Kraakman *et al.*, 1999; Lemaire *et al.*, 2004; Lorenz *et al.*, 2000; Yun *et al.*, 1998). Gpr1p interacts with the G-alpha protein Gpa2p and with Plc1p, phosphatidyl-inositol-specific phospholipase C (Ansari *et al.*, 1999; Kraakman *et al.*, 1999; Lorenz *et al.*, 2000; Xue *et al.*, 1998). This complex is activated in response to glucose and stimulates pseudohyphal growth via the cAMP pathway. Another pathway for glucose sensing is the RAS/cAMP pathway (Broach, 1991a, 1991b; Jiang *et al.*, 1998). Activation of the small GTP-binding protein Ras2p results in a hyperfilamentous growth phenotype, but only upon nitrogen starvation. These data suggest that Ras2p is involved in regulation of pseudohyphal formation in response to glucose availability.

In haploids, cells grow invasively after a few days on rich medium, when nutrients have presumably been depleted. For this phenotype, the glucose availability seems to be the main stimulus. Lack of glucose induces haploid invasive growth, whereas the absence of fixed nitrogen do not cause invasion (Cullen and Sprague, 2000). Glucose limitation causes also the formation of biofilms in yeast (Reynolds and Fink, 2001).

Amino acid starvation represents an additional signal for adhesive growth. In amino acid-starved haploid and diploid yeast cells adhesion is induced even in the presence of glucose or ammonium (Braus *et al.*, 2003). Internal amino acid concentrations are sensed by the sensor kinase Gcn2p (Dever and Hinnebusch, 2005). Under amino acid starvation, uncharged t-RNA molecules accumulate in the cell. These t-RNA molecules are detected by Gcn2p, which activates a genetic network, called the general amino acid control (Hinnebusch and Natarajan, 2002). Deletion of *GCN2* results in an adhesion deficient phenotype upon amino acid starvation (Braus *et al.*, 2003).

1.3 Signal transduction pathways for adhesion

Adherence as part of haploid invasive growth and diploid pseudohyphal formation is controlled by complex regulatory pathways. The two main signal transduction pathways are the cAMP-dependent protein kinase A (PKA) pathway and the highly conserved so called filamentous mitogen-activated protein kinase (MAPK) cascade (Figure 2) (reviewed

by Elion *et al.*, 2005; Gancedo, 2001; Lengeler *et al.*, 2000; Mösch, 2000; Palecek *et al.*, 2002; Pan *et al.*, 2000; Rupp *et al.*, 1999).

In response to stimuli, the G-α protein Gpa2p or activated Ras2p can interact with the adenylat cyclase Cyr1p, which results in an increased concentration of intracellular cAMP. The high level of intercellular cAMP leads to an activation of the yeast protein kinase A (PKA) that is composed of an inhibitory subunit Byc1p and one of the catalytic subunits Tpk1p, Tp2p, or Tpk3p (Broach, 1991a). Only Tpk2p is required for filamentous growth (Robertson and Fink, 1998; Robertson *et al.*, 2000). Mutations in *TPK1* and *TPK3* lead to a hyper filamentous phenotype, suggesting that Tpk1p and Tpk3 are inhibitors of pseudohyphal formation (Pan and Heitman, 1999). Putative targets for Tpk2p are the transcription factors Flo8p and Sfl1p. Both transcription factors regulate expression of *FLO11*, encoding a glycosyl-phosphatidylinositol (GPI)-linked cell surface adhesin, which is essential for adherence during haploid invasive growth or diploid pseudohyphal formation (Lambrechts *et al.*, 1996; Lo and Dranginis, 1998). Thereby, Flo8p acts as a positive regulator of *FLO11*, whereas Snfl1p is negatively regulated by Tpk2p and represses *FLO11* transcription (Pan and Heitman, 1999; Robertson and Fink, 1998). Note, due to a nonsense mutation in the *FLO8* gene many *S. cerevisae* laboratory strains including the commonly used strain S288c have lost their ability to grow adhesively (Liu *et al.*, 1996), and therefore are not appropriate to study adhesion, differentiation or morphogenesis in yeast.

In addition to the cAMP pathway, activated Ras2p also stimulates the filamentous MAPK kinase cascade pathway. Ras2p is linked to the filamentous MAPK kinase cascade via a second small GTP binding protein, Cdc42p (Mösch *et al.*, 1996). Both GTPases are required for diploid pseudohyphal formation or haploid invasive growth (Mösch *et al.*, 1996; Mösch *et al.*, 1999; Mösch *et al.*, 2001). The MAPK cascade module contains the protein kinases Ste20p (MAPKKKK), Ste11p (MAPKKK), Ste7 (MAPKK) and Kss1p (MAPK). The function of this cascade is the phosphorylation of the transcription factor Ste12p in response to environmental stimuli. In absence of stimuli for adhesion, unphosphorylated Kss1p binds to Ste12p and inhibits thus Ste12p-dependent transcriptional activation (Bardwell *et al.*, 1998). Two additional proteins, Dig1p and Dig2p are also involved in Kss1p-mediated repression of Ste12p. After phosphorylation of Kss1p by Ste7p, Kss1p relieves its inhibition of Ste12p. For activation of its target genes, Ste12p acts together with Tec1p. Tec1p is a transcription factor of the TEA/ATTS DNA-

binding domain family and is required for diploid pseudohyphal formation as well as haploid invasive growth (Bürglin, 1991; Gavrias *et al.*, 1996; Mösch and Fink, 1997). Ste12p and Tec1p bind as heterodimers to specific filamentous response elements (FREs) in the promoter region of their target genes including *TEC1* itself (Madhani and Fink, 1997) and *FLO11* (Lo and Dranginis, 1998). In addition to this combinatorial control, Tec1p is also able to activate targets genes via Tec1p binding sites (TCS elements) in absence of Ste12p (Köhler *et al.*, 2002). Activation of the MAPK kinase cascade can also be modulated at the level of the MAPKKKK Ste20p and MAPKKK Ste11p. The two yeast 14-3-3 proteins, Bmh1p and Bmh2p, interact with Ste20p and are required for pseudohyphal growth (Roberts *et al.*, 1997).

A third signalling pathway for regulation of adhesive growth is the general amino acid control (Braus *et al.*, 2003). Under amino acid starvation haploid and diploid yeast cells interact by cell-cell and cell-surface adherence. In contrast to haploid invasive growth or diploid pseudohyphal formation, amino acid starvation-induced adhesion is independent of the filamentous MAPK cascade. It requires elements of the cAMP pathway and two elements of the general amino acid control system, namely Gcn2p and Gcn4p. Upon amino acid limitation, uncharged t-RNAs accumulate in the cell. The sensor kinase Gcn2p detects these molecules and phosphorylates the translation initiation factor eIF2, finally resulting in a translational de-repression of *GCN4* (Hinnebusch, 1997). *GCN4* encodes a transcription factor that activates transcription of over five hundred target genes including *FLO11* in response to amino acid starvation (Braus *et al.*, 2003; Natarajan *et al.*, 2001). Interestingly, high expression of Gcn4p under non-starvation conditions is not sufficient to induce adhesive growth and enhanced expression of *FLO11*, indicating that Gcn4p might control *FLO11* expression in concert with other transcription factors or by an indirect mechanism (Braus *et al.*, 2003). In addition to amino acid starvation-induced adhesive growth, Gcn4p is also required for haploid invasive growth and diploid pseudohyphal formation (Braus *et al.*, 2003). The impact of the general amino acid control on adherence-dependent differentiation processes has also been described for the human pathogen fungus *Candida albicans* (Garcia-Sanchez *et al.*, 2004; Tripathi *et al.*, 2002). A more detailed description of the yeast general amino acid control network and its transcriptional activator Gcn4p is given in part 2 of Chapter I.

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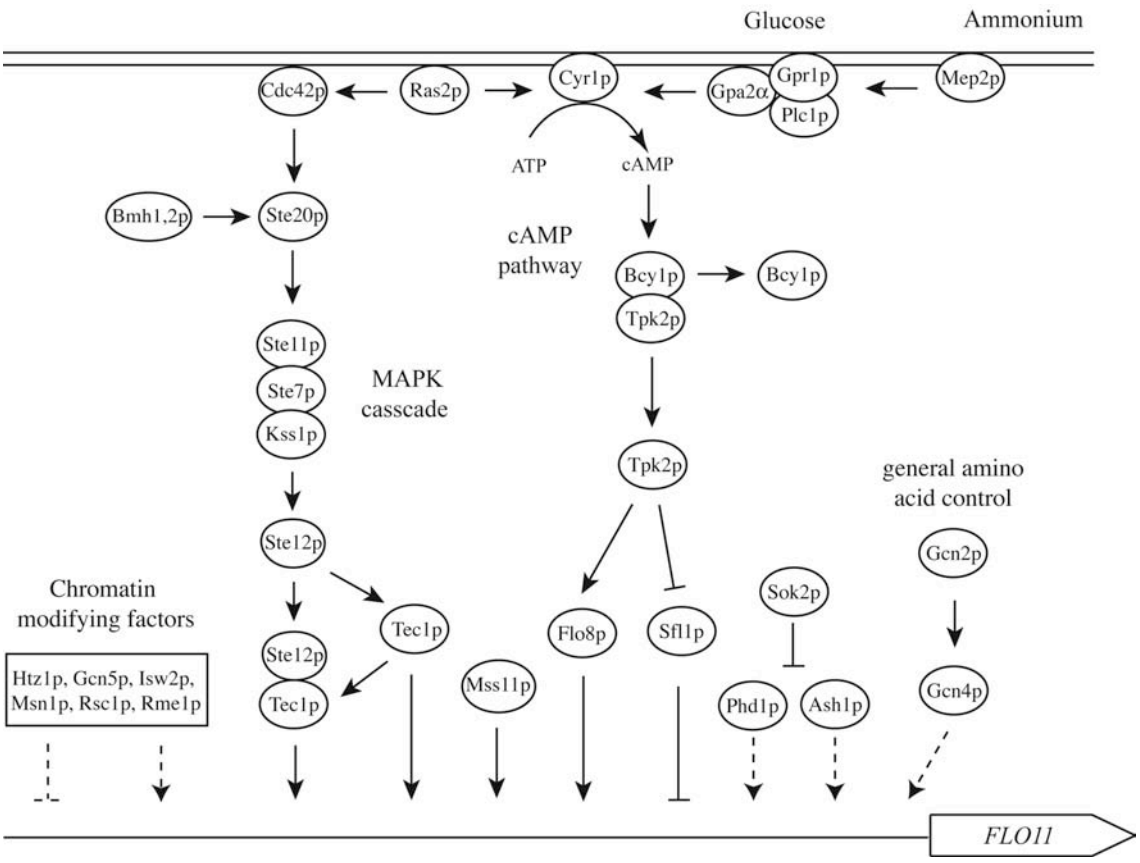

Figure 2: Model of signalling pathways regulating adherence in *S. cerevisiae* (see text for details).

Another important regulator for *FLO11* expression and adhesive growth is the transcriptional activator Mss11p (Gagiano *et al.*, 1999; van Dyk *et al.*, 2005). Genetic analyses to identify functional relationships between Mss11p and other *FLO11* regulators revealed that transcriptional induction of *FLO11* by overexpression of *TPK2*, *FLO8* or *TEC1* requires a functional *MSS11* gene. These data suggest that Mss11p plays a central role in regulation of *FLO11* transcription. Thereby, Mss11p activity is independent of the cAMP-dependent protein kinase A (PKA) pathway and the filamentous mitogen-activated protein kinase (MAPK) cascade.

A number of further transcription factors and chromatin modifying proteins involved in adhesion and differentiation have been identified. For instance, Sok2p negatively regulates *FLO11* expression and adhesion, whereas Phd1p and Ash1p are positive regulators (Gimeno and Fink, 1994; Mösch and Fink, 1997; Pan and Heitman, 2000; Ward *et al.*, 1995). Both *PHD1* and *ASH1* are induced in absence of Sok2p, suggesting that Sok2p acts upstream of Phd1p and Ash1p. Chromatin remodelling factors

such as Gcn5p, Isw2p, Msn1p, Rsc1p and Rme1p or the histone variant Htz1p affect also directly or indirectly *FLO11* transcription (Fischer *et al.*, 2005; Gagiano *et al.*, 1999; van Dyk *et al.*, 2003). Deletion of *GCN5, ISW2* or *RSC1* leads to a repression of *FLO11* transcription, whereas deletion of *HTZ1* results in a transcriptional activation of *FLO11*. The putative chromatin remodelling factors Msn1p and Rme1p activate *FLO11* transcription when expressed from multi-copy plasmids.

1.4 Cell surface adhesins in *S. cerevisiae*

The genome of *S. cerevisiae* contains a family of cell-wall glycoproteins related to adhesins of pathogenic fungi. The *FLO* (flocculation) genes of *S. cerevisiae* belong to this protein family and promote cell-cell and cell-surface adhesion (Guo *et al.*, 2000). The yeast genome sequence reveals the presence of five *FLO* genes, namely *FLO1, FLO5, FLO9, FLO10* and *FLO11*. Four of these *FLO* genes (*FLO1, FLO5, FLO9* and *FLO10*) are located adjacent to the telomeres and therefore these genes are transcriptionally silent (Figure 3) (Halme *et al.*, 2004; Liu *et al.*, 1996; Verstrepen *et al.*, 2004). Only *FLO11* is expressed in laboratory strains of *S. cerevisiae* with the result that Flo11p appears to be the most important flocculin for adhesion-specific phenotypes (Bayly *et al.*, 2005; Guo *et al.*, 2000; Lo and Dranginis, 1998). Interestingly, the *FLO11* promoter span at least 2.8 kb and is one of the largest promoters in the yeast genome, suggesting a complex regulation of *FLO11* expression. Analyses in a *flo11* mutant background show that the silent *FLO* genes are functional when they are expressed from a *GAL1* promoter. *FLO10* and *FLO11* promote filamentation and cell adherence to agar and plastic, whereas *FLO1* and, to lesser extent, *FLO10*, promote cell-cell adherence (Guo *et al.*, 2000).

Other related flocculins are Fig2p and Aga1p, which are induced during the conjugation process of two haploid cells (Erdman *et al.*, 1998; Roy *et al.*, 1991; Zhang *et al.*, 2002). Both proteins are not required for haploid invasive growth or diploid pseudohyphal formation, although overexpression of *FIG2* can partially substitute a *flo11* deletion (Guo *et al.*, 2000).

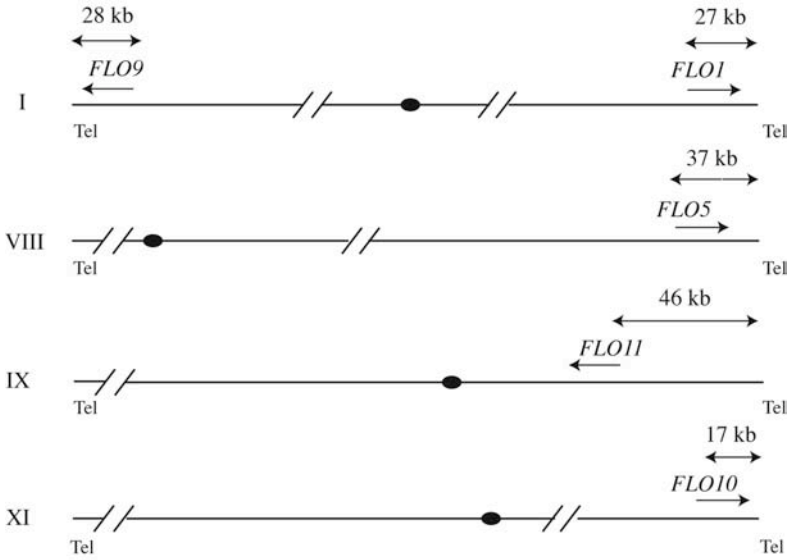

Figure 3: Chromosomal localization of *FLO* genes in *S. cerevisiae* (adapted from Verstrepen *et al*., 2004).
The numbers on the left indicate the yeast chromosome on which the *FLO* genes are located. The black dots represent the centromeres. The silent *FLO* genes are all located within the 40 kb of the telomeres (Tel). *FLO11* is neither centromeric nor telomeric.

2. Regulation of amino acid biosynthesis in *S. cerevisae*

Amino acids are essential building blocks for the ribosomal biosynthesis of proteins. To ensure a sufficient supply of amino acids, fungi are able to take up amino acids from the environment, to recycle amino acids by protein degradation or to synthesize all 20 amino acids *de novo*. Thereby, in numerous fungi the biosynthesis of amino acids is controlled by complex regulatory networks (reviewed by Braus *et al*., 2004).

The existence of regulatory networks for amino acid biosynthesis was first described for the ascomycetes *Neurospora crassa* and *Aspergillus nidulans* (Carsiotis and Jones, 1974; Carsiotis *et al.*, 1974; Piotrowska *et al.*, 1980). Further work on *Neurospora crassa* showed that starvation for a single amino acid leads to an activation of the majority of all 20 amino acid pathways (Barthelmess and Kolanus, 1990; Kolanus *et al.*, 1990). In *S. cerevisae* the regulatory network of amino acid biosynthesis is named general amino acid control and is well understood (Figure 4) (reviewed by Hinnebusch and Natarajan, 2002).

2.1 The general amino acid control of *S. cerevisiae*

In the budding yeast *S. cerevisiae* numerous genes were identified to be involved in the regulation of the general amino acid control network. These genes are subdivided into two groups. Mutations resulting in a loss of activation of the general amino acid control upon amino acid starvation are called *GCN* (general control non-derepressable). In contrast, mutations leading to a constitutively active general amino acid control are named *GCD* (general control derepressed) (Harashima and Hinnebusch, 1986).

The lack of just one amino acid or an amino acid imbalance causes the activation of the general amino acid control network (Hinnebusch, 1992). Beside its activation upon amino acid limitation, the general amino acid control is also induced in response to a limited supply of purines (Mösch *et al.*, 1991), tRNA synthetases (Meussdoerffer and Fink, 1983) or glucose (Yang *et al.*, 2000). In addition, cellular stress induced by uv-radiation (Engelberg *et al.*, 1994), high salinity (Goossens *et al.*, 2001), treatment with the drug rapamycin (Cherkasova and Hinnebusch, 2003; Kubota *et al.*, 2003; Valenzuela *et al.*, 2001) or the alkylating agent methyl methanesulfonate (MMS) (Natarajan *et al.*, 2001) stimulates activity of the general amino acid control (Figure 4). Under laboratory conditions, the basal expression of amino acid biosynthesis genes is sufficient to synthesize all 20 amino acids *de novo.* To induce amino acid starvation conditions, amino acid analoga can be added to the medium. The most prominent analoga are 3-amino-triazole (3AT) (Klopotowski and Wiater, 1965) or 5-methyl-tryptophan (5MT) (Schurch *et al.*, 1974).

Central element of this network is the transcriptional activator Gcn4p, a homologue of the human c-Jun protein, which induces transcription of several hundred genes in response to various environmental stimuli (Figure 4).

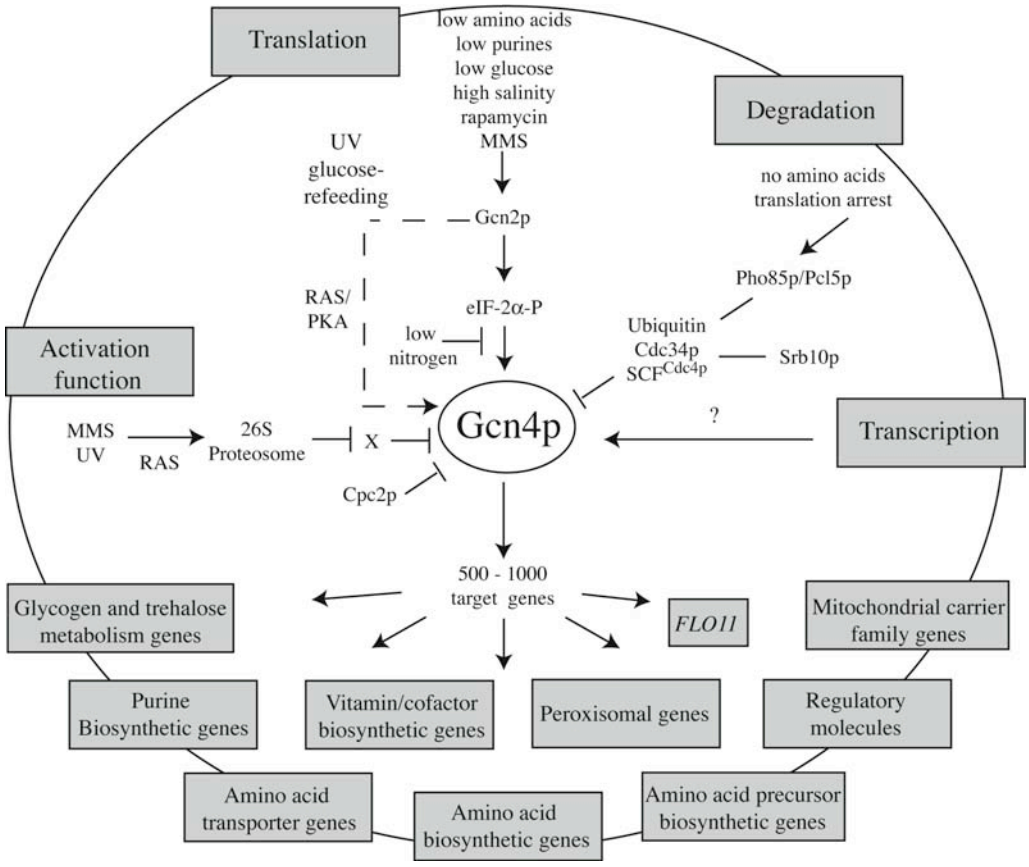

Figure 4: Model for the general amino acid control network in yeast.
The transcription factor Gcn4p is regulated at different levels in response to various environmental stimuli, which results in transcriptional activation of several hundred genes belonging to different pathways (see text for details).

2.2 Structure of the yeast Gcn4 protein

The central element of the general amino acid control is the transcription factor Gcn4p. Gcn4p consists of 281 amino acids and belongs to the family of the basic leucine zipper transcription factors (Hinnebusch, 1984; Thireos *et al.*, 1984). The sixty C-terminal amino acids of Gcn4p contain a leucine zipper domain for dimerization (LZ, amino acid 249 to 289) and a basic DNA binding domain (DB, amino acids 221 to 249) (Hope and Struhl, 1986) (Figure 5). Gcn4p binds as a homodimer to a specific 9 bp palindromic nucleotide sequence (5'-ATGA(C/G)TCAT-3') called Gcn4-protein responsive elements (GCRE) (Hope and Struhl, 1987; Oliphant *et al.*, 1989). For transcriptional induction, Gcn4p has also an activation domain spanning approximately half of the protein (Drysdale *et al.*,

1995) (Figure 5). This activation domain consists of a N-terminal activation domain (NTAD, amino acids 17 to 98) and a central acidic activation domain (CAAD, amino acid 107 to 144). Both activation domains have almost an identical activation potential. Between the two activation domains a so-called PEST-region is located which is responsible for the instability of the protein (Rechsteiner and Rogers, 1996). Under non-starvation conditions Gcn4p is an unstable protein with a half-life of 5 min (Kornitzer *et al.*, 1994). Deletion of the PEST-region results in a stabilization of the protein. In addition, a substitution of amino acid threonine 165 stabilizes Gcn4p under non-starvation conditions. To ensure a nuclear localization, Gcn4p has two nuclear localization sequences (NLS), namely NLS1 and NLS2 (Pries *et al.*, 2002) (Figure 5). The NLS1 consists of the amino acids 167 to 200 and acts as an ancillary motif. The NLS2 of Gcn4p is located within the leucine zipper domain (amino acids 231 to 249) and resembles a classical bipartite NLS-motif consisting of two basic clusters separated by a 10 amino acid spacer region. Nuclear import of Gcn4p requires also the presence of the karyopherins Srp1p and Kap95p (Pries *et al.*, 2004).

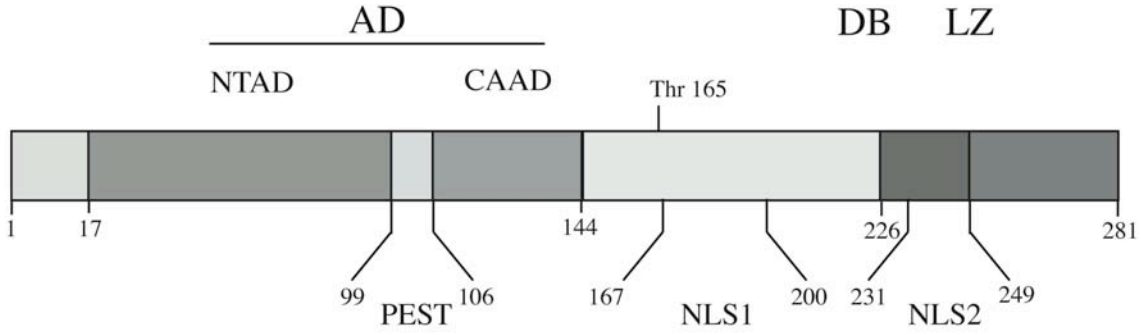

Figure 5: Schematic structure of Gcn4p.
The activation domain consists of a N-terminal activation domain (NTAD, aa 17-98) and a central acidic activation domain (CAAD, aa 107-144). Between these domains lies a PEST region (aa 99-106), which is responsible for the instability of the protein. The C-terminus of the protein comprises the DNA binding domain (DB, aa 226-249) and the dimerization domain (LZ, leucine zipper, aa 249-281). The two Gcn4p nuclear localization sequence motifs NLS1 and NLS2 consist of the amino acids 167 to 200 and 231 to 249, respectively.

2.3 Regulation of *GCN4* expression and its protein stability

The amount of Gcn4p in the cell is controlled by multiple mechanisms. Under amino acid starvation conditions, transcription of *GCN4* mRNA is two-fold induced resulting in an increased *GCN4* expression after 3-4 h of starvation (Albrecht *et al.*, 1998). But the principal means of inducing *GCN4* expression upon amino acid limitation operates at the level of *GCN4* mRNA translation.

Under non-starvation conditions, the efficient translation of *GCN4* mRNA is prevented by four small upstream open reading frames (uORFs) within its 5'untranslated region (UTR) (reviewed by Hinnebusch, 1997; Hinnebusch and Natarajan, 2002). The first and the fourth uORF (from the 5'end) are sufficient for nearly wild-type translational control, whereas the second and the third uORF have only a weak influence on *GCN4* translation (Mueller and Hinnebusch, 1986). The 40S-ribosomal subunit and a ternary complex, consisting of the translation initiation factor eIF-2α, GTP and the initiation tRNA (Met-$tRNA_i^{MET}$), form a 43S preinitiation complex. This preinitiation complex binds near to the capped 5' end of *GCN4* mRNA, migrates downstream and joins with the 60S-ribosomal subunit at the AUG start codon of uORF1 to form an 80S initiation complex. During translation initiation, the GTP bound to eIF2 is hydrolysed and eIF2 is released as an inactive eIF2-GDP binary complex. At the stop codon of uORF1, the 80S ribosome dissociates, leaving about the half of the small subunits attached to the *GCN4* mRNA. To re-form a ternary complex for further translation initiation events, GDP bound to eIF-2 has to be replaced by GTP, which is mediated by the guanine nucleotide exchange factor eIF-2B. This event occurs before the 40S-ribosomal subunit reaches the uORF4. After translation of uORF4, the 80S ribosome dissociates again, which prevents *GCN4* translation (Figure 6).

Under amino acid starvation conditions, uncharged t-RNA molecules accumulate in the cell. The sensor kinase Gcn2p, consisting of a C-terminal histidyl-tRNA synthetase (HisRS) related domain and a N-terminal protein kinase domain, binds to these uncharged t-RNAs resulting in activation of its protein kinase domain (Dever *et al.*, 1992; Lanker *et al.*, 1992). Activation of Gcn2p by uncharged tRNAs also requires interaction between the N-terminus of Gcn2p and the Gcn1p-Gcn20p protein complex (Garcia-Barrio *et al.*, 2000; Kubota *et al.*, 2000; Kubota *et al.*, 2001; Sattlegger and Hinnebusch, 2005). After stimulation, Gcn2p phosphorylates the α subunit of the eukaryotic translation factor eIF-2-

GDP on serine 51 (Wek *et al.*, 1995; Zhu *et al.*, 1996). Phosphorylation of eIF-2α-GDP inhibits the guanine nucleotide exchange factor eIF-2B and prevents recycling of eIF-2α-GDP to eIF-2α-GTP. This results in reduced amounts of ternary complexes, so that many ribosomes scan the distance between uORF1 and uORF2 without rebinding the ternary complex. The bypass of uORF4 allows the ribosomes to reinitiate at the start codon of the *GCN4* ORF (Figure 6). Interestingly, under conditions of general nitrogen limitation, de-repression of *GCN4* translation is prevented even when cells are simultaneously starved for amino acids and eIF-2α is fully phosphorylated on Ser51 by Gcn2p (Grundmann *et al.*, 2001).

In addition to this control of *GCN4* expression, Gcn4p is also regulated at the level of protein stability (Irniger and Braus, 2003; Kornitzer *et al.*, 1994; Pries *et al.*, 2002). Under non-starvation conditions Gcn4p is a very instable protein with a half-life of 5 min or less. In amino acid-starved yeast cells, Gcn4p is stabilized in the nucleus with a half-life of up to 20 min. Rapid degradation of Gcn4p is mediated by two kinases, Srb10p and Pho85, which phoshorylate Gcn4p to mark the protein for ubiquitination by the SCF^{CDC4} ubiquitin ligase complex, finally resulting in its degradation at the 26S proteasome (Chi *et al.*, 2001; Meimoun *et al.*, 2000). Due to the fact that Srb10p is a component of the mediator complex associated to the RNA polymerase II (Myer and Young, 1998), it is supposed that Srb10p specifically phosphorylates promoter-bound Gcn4p. Thereby, Srb10p-dependent phosphorylation of Gcn4p seems to be independently of the amino acid supply. Pho85p is a cyclin-dependent protein kinase involved in multiple cellular functions including cell cycle progression and the metabolism of nutrients (Carroll and O'Shea, 2002; Toh and Nishizawa, 2001). It requires the cyclin Pcl5p for phosphorylation of Gcn4p (Shemer *et al.*, 2002). Pcl5p itself is a highly unstable protein. Upon starvation conditions, *PCL5* is transcriptionally induced by Gcn4p (Natarajan *et al.*, 2001), but not efficiently translated, because of a reduced overall translation efficiency and a limitation of amino acids. It is supposed that efficient accumulation of Pcl5p can only occur when amino acids are present in sufficient amounts and, as a consequence, Pho85p is inactive in amino acid-starved cells.

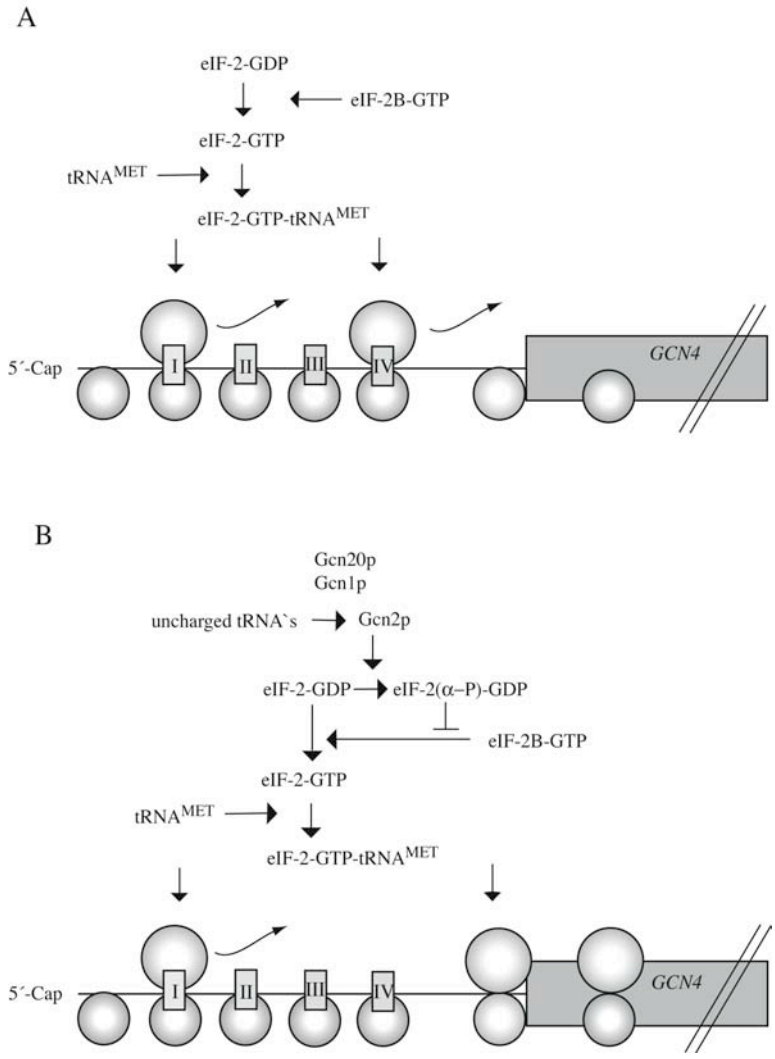

Figure 6: Translational control of *GCN4* expression in *S. cerevisiae*.
A Under non-starvation conditions four short open reading frames (uORF) at the 5' untranslated region of *GCN4* mRNA prevent efficient translation of *GCN4*. The 80S ribosome initiates translation at the start codon of uORF1 and dissociates from the mRNA at the uORF1 stop codon. Due to a sufficient amount of ternary complexes (eIF-2-GTP-$tRNA^{MET}$) in the cell, re-initiation of translation takes place at the uORF4. After translation of uORF4, the 80S-ribosome dissociates again, which prevents *GCN4* translation.
B Under amino acid starvation, uncharged t-RNAs are recognized by Gcn2p in cooperation with Gcn1p/Gcn20p. Gcn2p phosphorylates the α-subunit of the translation initiation factor eIF-2, which inhibits the guanine nucleotide exchange factor eIF-2B resulting in reduced amounts of ternary complexes in the cell. This reduced amount of ternary complexes leads to a delayed re-initiation of the reassembled ribosome, so that ribosomes bypass uORF4 and initiate translation at the *GCN4* start codon.

2.4 Gcn4p-dependent gene expression

Gcn4p activates transcription by binding as a homodimer to a specific 9 bp palindromic nucleotide sequence (5'-ATGA(C/G)TCAT-3') called Gcn4-protein responsive elements (GCRE) (Hope and Struhl, 1987; Oliphant *et al.*, 1989). Studies of Hollenbeck and Oakley (Hollenbeck and Oakley, 2000) show that a Gcn4p homodimer is also able to bind with high affinity to GCRE half sites *in vitro*. To activate transcription, Gcn4p function includes different effects on transcription such as re-organisation of chromatin, the modification of histones, and the recruitment of components of the transcriptional machinery to the target promoters (reviewed by Braus *et al*., 2004). For instance, one interaction of Gcn4p is TFIID, which binds to the TATA box. This interaction is mediated by a multiprotein bridging factor (Mbf1p), which is required for bridging Gcn4p to the TATA binding protein of TFIID (Takemaru *et al.*, 1998).

Transcriptional profiling experiments revealed for the adhesion deficient laboratory yeast strain S288c, that Gcn4p stimulates transcription of 539 genes by a factor of two and more in response to amino acid starvation induced by the histidin analogue 3-amino-triazole (Natarajan *et al.*, 2001). Of these genes, only 235 contain a GCRE site in their presumptive promoter region suggesting the presence of degenerate sequences or an indirect activation. 176 Gcn4p target genes can directly or indirectly assign to the amino acid and nitrogen metabolism. 78 of these genes encode amino acid or purine biosynthesis enzymes. With the exception of cysteine, every amino acid biosynthetic pathway is under the control of Gcn4p. But the biosynthesis of the cysteine precursors serine and homocysteine are also regulated in a Gcn4p-dependent manner, so that even the cysteine biosynthesis depends on Gcn4p. In addition, Gcn4p indirectly increases pathway activity by inducing genes encoding pathway specific transcription factors such as Ly14p, Leu3p, Met4p or Met28p. Further Gcn4p activated genes that are peripherally involved in amino acid biosynthesis encode for vitamin and co-factor biosynthetic enzymes, mitochondrial carrier proteins, and peroxisome biogenesis proteins (Figure 4). To enhance amino acid uptake from the environment, Gcn4p also induces transcription of several amino acid transporter genes upon amino acid limitation including *GAP1* and *APG1,* which encode general amino acid permeases.

In addition to this metabolic response, Northern hybridization experiments with the adherent yeast strain Σ1278b showed that Gcn4p also regulates adherence and

differentiation in *S. cerevisiae* (Braus *et al.*, 2003). Gcn4p induces expression of the adhesin-encoding gene *FLO11* in amino acid-starved yeast cells, which is required for adherence and developmental processes in yeast.

A proteomic analysis of the *S. cerevisiae* laboratory strain S288c identified 52 proteins, which are induced in response to 3AT treatment in a Gcn4p-dependent manner (Yin *et al.*, 2004). Of these proteins, 23 belong to 14 different amino acid biosynthetic pathways. Other Gcn4p-dependent induced proteins upon amino acid limitation are involved in the purine biosynthesis, the carbon metabolism or stress response. These changes in the *S. cerevisiae* proteome correlate with a rank correlation coefficient of 0.59 to the corresponding transcriptome data of Natarajan and co-workers (Natarajan *et al.*, 2001). Proteomic experiments with the human pathogen fungus *Candida albicans* showed a high degree of conservation with subtle differences in the Gcn4p-mediated response to amino acid starvation of *C. albicans* and *S. cerevisiae* (Yin *et al.*, 2004).

3. WD-repeat proteins in *S. cerevisiae*

The WD-repeat protein family is defined by a sequence repeat of 44 to 60 amino acids typically beginning with a glycine histidine pair and ending with a tryptophan aspartic acid pair (Neer *et al.*, 1994). WD-repeat proteins are found in all eukaryotes but not in prokaryotes. They have no enzymatic activity and are involved in regulation of many essential biological functions ranking from signal transduction, transcriptional regulation, cell cycle, to apoptosis. Furthermore, an association of WD-repeat proteins with several human diseases has been described (reviewed by Li and Roberts, 2001; Smith *et al.*, 1999).

In *S. cerevisiae* 63 proteins have been characterized as WD-repeat proteins. These proteins include proteins of a high functional diversity such as the general repressor of transcription Tup1p (Keleher *et al.*, 1992), the component of the TOR signalling pathway Lst8p (Chen and Kaiser, 2003), the cell cycle regulated activator of the anaphase promoting complex Cdc20p (Zachariae and Nasmyth, 1999), the actin interacting protein Aip1p (Voegtli *et al.*, 2003) or the putative translation factor Cpc2p (Chantrel *et al.*, 1998; Gerbasi *et al.*, 2004; Hoffmann *et al.*, 1999).

3.1 Structure of WD-repeat proteins

The WD-repeat comprises a 44-60 amino acid sequence motif that typically contains a glycine histidine pair (GH) at the N-terminus and a tryptophan aspartic acid pair (WD) at the C-terminus (Neer *et al.*, 1994; Smith *et al.*, 1999). Between the amino acids GH and WD is a conserved core sequence, which includes a variable region of 7-11 amino acids. Despite the high conservation, no amino acid in a WD-repeat motif is invariant. The WD-repeat motif can be present in 4-16 copies in a single protein.

The secondary and the tertiary structure of WD-repeat motifs of different proteins show a high identity among each other. The tertiary structure is named β-propeller, a highly symmetrical structure made up of repeats that each comprises a small four-stranded antiparallel β sheet (Lambright *et al.*, 1996; Smith *et al.*, 1999; Sondek *et al.*, 1996; Wall *et al.*, 1995). Each WD-repeat sequence motif corresponds to a structural motif of four β sheets. The first three β sheets represent one WD-repeat, whereas the fourth β sheet is part of the next. This arrangement generates a mechanism that allows a ring closure (Figure 7). The ring formation results in a stabilization of the protein, which is not affected by binding of interaction partners (Lambright *et al.*, 1996; Sondek *et al.*, 1996).

Figure 7: Hypothetical structure of a Gβ-like WD-repeat protein (modified from Smith *et al.*, 1999).
Gβ-like WD-repeat proteins are characterized by seven WD-repeat motifs (s1-s7). The seven WD-repeats are arranged in a ring to form a propeller structure with seven blades. Each blade of the propeller consists of a four-stranded antiparallel β sheet. The first three β sheets represent one WD-repeat, whereas the fourth β sheet is part of the next.

The general function of WD-repeat proteins is the interaction with proteins or with small ligands. It is supposed that these interactions are predominantly mediated by the top surface of the WD-repeat protein, including the central-tunnel opening (Smith *et al.*, 1999). Thereby, the specificity for different interaction partners depends on the number of WD-repeats and the variability in the amino acid sequence of the WD-repeats. In addition, the flanked regions of the β-propeller influence binding of specific interaction partners.

Gβ-like proteins represent one subgroup of the WD-repeat family. They consist of seven repeating WD motifs, each with an average length of 46 amino acids. One example for a Gβ-like protein in *S. cerevisiae* is Cpc2p.

3.2 The Gβ-like WD-repeat protein Cpc2p/Asc1p in *S. cerevisiae*

The yeast *CPC2* (cross pathway control) gene (also known as *ASC1*) encodes a Gβ-like WD-repeat protein of 319 amino acids with a high similarity to over 20 proteins present in organisms from yeast to human including the Cpc2 protein of *Schizosaccharomyces pombe*, the CPC2 protein of *Neurospora crassa* and the human RACK1 protein (Chantrel *et al.*, 1998; Hoffmann *et al.*, 1999). The open reading frame of *CPC2* is interrupted between the corresponding amino acid residues 179 and 180 by an intron of 273 nucleotides. This intron is located unusually close to the 3'-end and contains the coding sequence for the U24 small nuclear RNA (*SNR24*), which is required for site-specific 2'-o-methylation of 25S rRNA (Kiss-Laszlo *et al.*, 1996; Qu *et al.*, 1995). Expression analyses revealed for *CPC2* a high transcription rate (Velculescu *et al.*, 1997), finally resulting in estimated 330 000 Cpc2p molecules per cell (Ghaemmaghami *et al.*, 2003).

A deletion of *CPC2* suppresses the growth defect of a *gcn2* deletion strain upon amino acid starvation by increasing transcription of Gcn4p targets genes (Hoffmann *et al.*, 1999). A mutation in *cpc-2* of *N. crassa* also affects the cross pathway control in an unknown manner, resulting in a sensitivity of the mutant strain to amino acid starvation (Krüger *et al.*, 1990; Müller *et al.*, 1995). These data suggest for Cpc2p a regulatory role in the general amino acid control network of *S. cerevisiae*.

Polysome profiles and mass-spectrometry analyses identified for Cpc2p an association to the 40S-ribosomal subunit (Chantrel *et al.*, 1998; Gerbasi *et al.*, 2004; Link *et al.*, 1999), which is also described for the homologues Cpc2p in *S. pombe* (Shor *et al.*,

2003) and the human RACK1 (Ceci *et al.*, 2003). At the ribosomes, Cpc2p is required for interaction of RNA binding protein Scp160p with ribosomes depending also on the presence of mRNAs (Baum *et al.*, 2004; Frey *et al.*, 2001). Furthermore, it was shown that a *cpc2* deletion results in an increased translation of specific mRNAs suggesting that Cpc2p acts as a translational regulator in *S. cerevisiae* (Gerbasi *et al.*, 2004). In mammals RACK1 interacts with signal transduction pathways such as the protein kinase C pathway (Ron *et al.*, 1994) and Src (Chang *et al.*, 1998). It is supposed that RACK1p provides a physical and functional link between signalling pathways and translation (Ceci *et al.*, 2003; Nilsson *et al.*, 2004). In this way, RACK1 is involved in several different cellular processes such as cell spreading, the establishment of focal adhesions and cell–cell contacts (Nilsson *et al.*, 2004).

The impact of Cpc2p on different cellular processes in *S. cerevisiae* is supported by tandem affinity purification (TAP) experiments. Cpc2p was co-purified with eleven different multi protein complexes with functions in protein synthesis and turnover, transcription/DNA maintenance/chromatin structure, RNA-metabolism, membrane biogenesis and transport (Gavin *et al.*, 2002).

4. Aim of this work

Cells of *S. cerevisive* grow adhesively when limited in supply of amino acids. One aim of this work was to identify new regulated genes and proteins in amino acid-starved and adherent yeast cells. Therefore, transcriptional profiling experiments under adhesion-inducing conditions were performed with respect to the Gcn4p-mediated response to amino acid starvation. In addition, a proteomic approach was carried out to identify highly activated or de-repressed proteins upon amino acid limitation. Comparisons with the respective transcriptome data should reveal whether the increase in protein expression is based on a transcriptional or posttranscriptional regulation. Deletion strains of novel identified transcriptional or posttranscriptional regulated genes were generated, and the influence of these strains on amino acid starvation-induced adhesion was tested in adhesive growth assays.

Furthermore, the expression of the Gβ-like WD-repeat encoding gene *CPC2* was analysed under different growth conditions. Therefore, *CPC2* promoter deletion and

insertion constructs were generated to identify *cis*- and *trans*-acting factors involved in regulation of *CPC2*.

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Chapter II

Transcriptional profile of *Saccharomyces cerevisiae* cells under adhesion-inducing conditions

Abstract

The ability to adhere to other cells is one of the most prominent determinants of fungal pathogenesis. Thus, adherence of fungi to human tissues or plastics triggers hospital-acquired fungal infections, which are an increased clinical problem, especially for immuno-compromised persons. In the model fungus *Saccharomyces cerevisiae* adhesion can be induced under amino acid starvation conditions and depends on the transcriptional activator of the general amino acid control system Gcn4p. However, not much is known about the transcriptional program that mediates adhesive growth under such conditions. In this study, we present a genome-wide transcriptional analysis of Σ1278b yeast cells that were subjected to adhesion-inducing conditions imposed by amino acid starvation. 22 novel genes were identified as inducible by amino acid starvation; 72 genes belonging to different functional groups, which were not previously known to be regulated by Gcn4p, require Gcn4p for full transcriptional induction under adhesion-inducing conditions. In addition, several genes were identified in Σ1278b cells that were inducible by amino acid starvation in a Gcn4p-independent manner. Our data suggest that adhesion of yeast cells induced by amino acid starvation is regulated by a complex, Σ1278b specific transcriptional response.

Introduction

Hospital-acquired fungal infections are an increased clinical problem, especially in immuno-compromised patients. These infections are linked to the ability of fungal cells to adhere to human tissues, plastic prostheses or catheters (Cormack, 1999; Sundstrom, 2002; Douglas, 2003). One major problem in therapy of such infections results from the adhesion-dependent formation of biofilms, which are extremely resistant to antifungal drugs and act as a source of new infections (Baillie and Douglas, 2000; Chandra, *et al*., 2001; Douglas, 2003).

In the fungal model organism *Saccharomyces cerevisiae*, the ability to adherence to other cells, or to the substratum, is a component of complex developmental processes like haploid invasive growth (Guo *et al*., 2000; Roberts and Fink, 1994), pseudohyphal development in diploids (Gimeno *et al*., 1992; Mösch and Fink, 1997) and biofilm formation (Reynolds and Fink, 2001). All these processes require the expression of the cell-surface flocculin Flo11p (Lo and Dranginis, 1998), which is regulated by the MAP kinase-dependent and cAMP-dependent signal transduction pathways (Mösch *et al*., 1999; Rupp *et al*., 1999). The signals for the activation of *FLO11* in haploid invasive growth and biofilm formation differ from those used during diploid pseudohyphal growth. In haploid *S. cerevisiae* cells lack of glucose causes invasive growth or biofilm formation (Cullen and Sprague, 2000, Reynolds and Fink, 2001), whereas in diploid *S. cerevisiae* cells starvation for nitrogen induces the pseudohyphal phenotype, when unicellular yeast cells switch to a polar pattern of growth and take on an elongated form (Liu *et al*., 1993; Lo and Dranginis, 1998; Robertson and Fink, 1998; Pan and Heitman, 1999).

Recent studies have identified novel stimuli of adhesion of *S. cerevisiae* yeast cells. Thus, starvation for amino acids induces adhesion and *FLO11* expression in haploid and diploid cells, even in the presence of glucose or ammonium (Braus *et al*, 2003). In contrast to haploid invasive growth or the diploid pseudohyphal formation, adhesion induced by amino acid starvation is independent of the MAP kinase signalling pathway. In addition to the transcription factor Flo8p and the protein kinase Tpk2p of the cAMP pathway, two elements of the general amino acid control system are essential for adhesion and *FLO11* expression under conditions of amino acid starvation (Braus *et al*., 2003). One is the transcription activator of the general amino acid control system, Gcn4p, which is also required for haploid invasive growth or diploid pseudohyhal development. The other

element is the sensor kinase Gcn2p, which regulates synthesis of Gcn4p (Hinnebusch, 1986). Under non-starvation conditions, efficient translation initiation at the *GCN4* start codon is prevented by the presence of four short upstream open reading frames (uORF) in the *GCN4* mRNA. Under conditions of amino acid starvation, Gcn2p detects uncharged t-RNA molecules and phosphorylates the α-subunit of the eukaryotic translation initiation factor eIF-2 (Dever *et al*., 1992; Wek *et al.*, 1995). This allows the ribosomes to scan past the uORFs and to initiate translation at the *GCN4* start codon (Hinnebusch, 1997). Gcn4p activates transcription as a homodimer by direct promoter binding at sequence-specific Gcn4p-responsive elements in promoters (Hope and Struhl, 1985; Oliphant *et al*., 1989). Target genes of Gcn4p are involved in biosynthetic pathways of several amino acids (Hinnebusch, 1992), the biosynthesis of amino acid tRNA synthetases (Meussdoerfer and Fink, 1983; Mirande and Waller, 1988) and in the purine biosynthesis (Mösch *et al*., 1991). However, microarray experiments have revealed that Gcn4p plays a more complex role in regulating metabolic pathways (Natarajan *et al.*, 2001). Thus, over 500 Gcn4p target genes were identified under amino acid starvation conditions in the adhesion deficient laboratory yeast strain S288c. The genetic background of the *S. cerevisiae* S288c laboratory strain differs from that of wild-type strains like Σ1278b and its derivatives. Cells with the S288c background have lost the ability of wild-type yeasts for haploid invasive growth or diploid pseudohyphal development (Liu *et al*. 1996). The S288c background is therefore not appropriate for the study of adhesion, differentiation or morphogenesis processes in yeast. The impact of the general amino acid control on differentiation processes like biofilm formation (Garcia-Sanchez *et al*., 2004) or filamentous growth (Tripathi *et al*., 2002) has also been described for the human pathogen *Candida albicans*. There, amino acid starvation induces filamentous growth, which requires the Gcn4p homologue CaGcn4p (Tripathi *et al*., 2002).

In this study, we have investigated the transcriptional profile of *S. cerevisiae* cells under adhesion-inducing conditions. We induced adherence by starving cells for histidine, resulting in a Gcn4p-mediated response. In this way numerous novel genes that are induced by, or otherwise dependent on, Gcn4p under adhesion-inducing conditions.

Materials and Methods

Yeast strains and growth conditions

All yeast strains used in this work are listed in Table 1. The deletion mutants *gpg1*Δ, *icy1*Δ, *mth1*Δ, *phd1*Δ and *cwp2*Δ were obtained by amplification and integration of deletion cassettes from the Euroscarf strain collection (Brachmann *et al*., 1998) in the Σ1278b background. All gene deletions were confirmed by Southern hybridization analysis (Ausubel *et al*., 1993). The S288c derivate strain RH3169 was generated by crossing the haploid strains RH1631 and RH1633 (our collection). For microarray and Northern hybridization experiments, the plasmid pRS316 was introduced into RH2656 and RH2658 using the lithium-acetate yeast transformation method (Ito *et al*., 1983). Strains were routinely cultivated in liquid synthetic minimal medium (YNB) with arginine at 30°C overnight, diluted and cultivated to mid log phase before isolation of total RNA. To impose starvation for histidine, 10 mM 3-amino-triazole (3AT) was added to cultures grown to mid-log phase and cells were incubated for 8 h. To test for adhesive growth, strains were grown in liquid or on solid (2% agar) YNB medium containing the appropriate supplements and 1 mM or 10 mM 3AT to induce amino acid starvation.

Table 1: *S. cerevisiae* strains used in this work.

Strain	Genotype	Reference
RH2656	Σ1278b *MAT**a**/α ura3-52/ura3-52 trp1::hisG/TRP1*	Braus *et al*., 2003
RH2658	Σ1278b *MAT**a**/α ura3-52/ura3-52 leu2::hisG/leu2::hisG trp1::hisG/TRP1 gcn4Δ::LEU2/gcn4Δ::LEU2*	Braus *et al*., 2003
RH2661	Σ1278b *MAT**a**/α ura3-52/ura3-52 flo11Δ::kanR/flo11Δ::kanR trp1::hisG/TRP*	Braus *et al*., 2003
RH3169	S288c *MAT**a**/α ura3-52/ura3-52*	This work
RH3170	Σ1278b *MAT**a**/α ura3-52/ura3-52 gpg1Δ::kanR gpg1Δ::kanR trp1::hisG/TRP1 LEU2/leu2::hisG*	This work
RH3171	Σ1278b *MAT**a**/α ura3-52/ura3-52 icy1Δ::kanR/icy1Δ::kanR trp1::hisG/TRP1 LEU2/leu2::hisG*	This work
RH3172	Σ1278b *MAT**a**/α ura3-52/ura3-52 mth1Δ::kanR/mth1Δ::kanR trp1::hisG/TRP1 LEU2/leu2::hisG*	This work
RH3173	Σ1278b *MAT**a**/α ura3-52/ura3-52 phd11Δ::kanR/phd1Δ::kanR trp1::hisG/TRP1 LEU2/leu2::hisG*	This work
RH3174	Σ1278b *MAT**a**/α ura3-52/ura3-52 cwp2Δ::kanR/cwp2Δ::kanR trp1::hisG/TRP1*	This work

Microarray hybridization experiments

Total RNA was isolated from four independent cultures for each described condition, following the protocol described by Cross and Tinkelenberg (Cross and Tinkelenberg, 1991). Aliquots (1μg, in water) of total RNA was added to 2 ng of oligo dT (10-20 mer mixture, Research Genetics, Groningen, Netherlands) in a final volume of 10 μl. After heat denaturation at 70°C for 10 min, the mixture was placed on ice. To initiate cDNA synthesis, 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) were added, and the reaction was incubated at 37°C for 90 min. The radiolabelled cDNAs were purified by using a Quick Spin Column Sephadex G50 Fine (Roche, Mannheim, Germany).

Two Miniarray GeneFilter sets (GF100, Research Genetics, Groningen, Netherlands) were used for four independent hybridization experiments for each described condition. Each set contains 6144 ORFs, individually amplified by PCR and spotted on nylon membrane filters. The filters 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 probes were 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. Filters were stripped by shaking for 20 min in 0.5% (w/v) SDS preheated to 100°C. More than 95% of the signal could be removed using this procedure.

The hybridization signals were detected with a Storm 860 Phosphorimager (Amersham Pharmacia Biotech, Freiburg, Germany). The data were transferred to the Pathway Software 2.01 (Research Genetics, Groningen, Netherlands) to analyse the transcriptional level of each gene, and exported to Microsoft Excel for final evaluation.

For each data the background corrected values were $\log_{10}$ transformed and the mean and standard derivation of the values were calculated. To test whether a gene is significantly differentially expressed in two sets we computed the p-value for each gene

(the probability that the data reflect the same expression level) using the t-test. Reproducibility was tested by comparison of two independent measurements of identical conditions (*GCN4*/*GCN4*) resulting in a correlation coefficient of 0.9758208.

Northern hybridization analysis

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

Adhesive growth assays

To assay for adhesive growth on plastic, yeast cells were grown in YNB medium containing supplements at 30°C overnight, 100µl aliquots of appropriate dilutions were transferred into wells of microtiter plates. Amino acid starvation conditions were induced by adding 10 mM 3AT. To visualize the adherence of cells to plastic, we modified the previously described protocol (O`Toole *et al*, 1999; Reynolds and Fink, 2001). First, 100 µl of 1% (w/v) crystal violet solution was added to the cells and the mixture was incubated for at least 15 min. Then the wells were washed carefully with water and photographed. Adhesive growth tests on solid YNB medium were performed essentially as described previously (Roberts and Fink, 1994). Strains were first grown for 20 h on solid YNB medium containing supplements. Then cells were patched onto fresh YNB containing supplements and 10 mM 3AT to induce amino acid starvation. After incubation for 1 day at 30°C, plates were photographed and then carefully washed under a stream of water. The plates were photographed once again to document adhering cells.

Results

Amino acid starvation induces a specific transcriptional profile in adhesive yeast cells

Diploid cells of the wild-type *S. cerevisiae* strain Σ1278b show cell-surface adhesion to agar or plastic when starved for amino acids by the histidine analogue 3-amino-triazole (3AT) (Braus *et al.*, 2003). This effect depends on the presence of the transcriptional activator Gcn4p. Transcriptional profiling experiments under amino acid starvation conditions suggest that Gcn4p is a master regulator for numerous pathways in *S. cerevisiae* S288c (Natarajan *et al*., 2001). However, diploid cells of this strain are unable to stick to surfaces in response to amino acid starvation, whereas Σ1278b cells adhere on plastic surfaces after amino acid starvation (Figure 8A). Flo11p and Gcn4p are required for adherence, and mutant strains carrying deletions in either of these genes do not display the induced adherence phenotype. Genes that are specifically affected by the presence of *GCN4* under non-starvation conditions are listed in Table 2 (see below for further discussion).

Genome-wide transcription profiling of *S. cerevisiae* Σ1278b was performed to analyse the transcriptional response of amino acid-starved and adherent yeast cells. Amino acid starvation and adhesion was induced by competitive inhibition of the histidine biosynthetic enzyme His3p by 3-amino-triazole (3AT), a widely used amino acid analogue, which facilitates a comparison with previous studies (Natarajan *et al*., 2001; Braus *et al*., 2003). We compared the transcriptional response of the diploid Σ1278b wild-type strain (RH2656) to amino acid starvation with that of the same strain under non-starvation conditions (wt +/- 10 mM 3AT; 8 h).

Figure 9A shows the results of this experiment. In order to focus on major differences, only the top scoring regulated genes were considered. These genes show a statistical significance (p-value of 0.01 or less, t-test) and at least a two-fold change in expression (black spots). Based on this comparison, 48 of 4605 genes evaluated were induced and seven genes were repressed during amino acid starvation-induced adhesive growth (wt +/- 10 mM 3AT; 8 h). Of these genes, 26 (including *AAD10, ADE1, BNA1, CTF13, GCV1, GLG1, HIS4, ICY1 MTG1, SNZ1, TMT1* and *YAH1*) had previously been reported to be induced in amino acid-starved haploid S288c cells (Marton *et al*., 1998;

Natarajan *et al.*, 2001). The remaining 22 genes have not previously been shown to be significantly activated in amino acid-starved yeast cells.

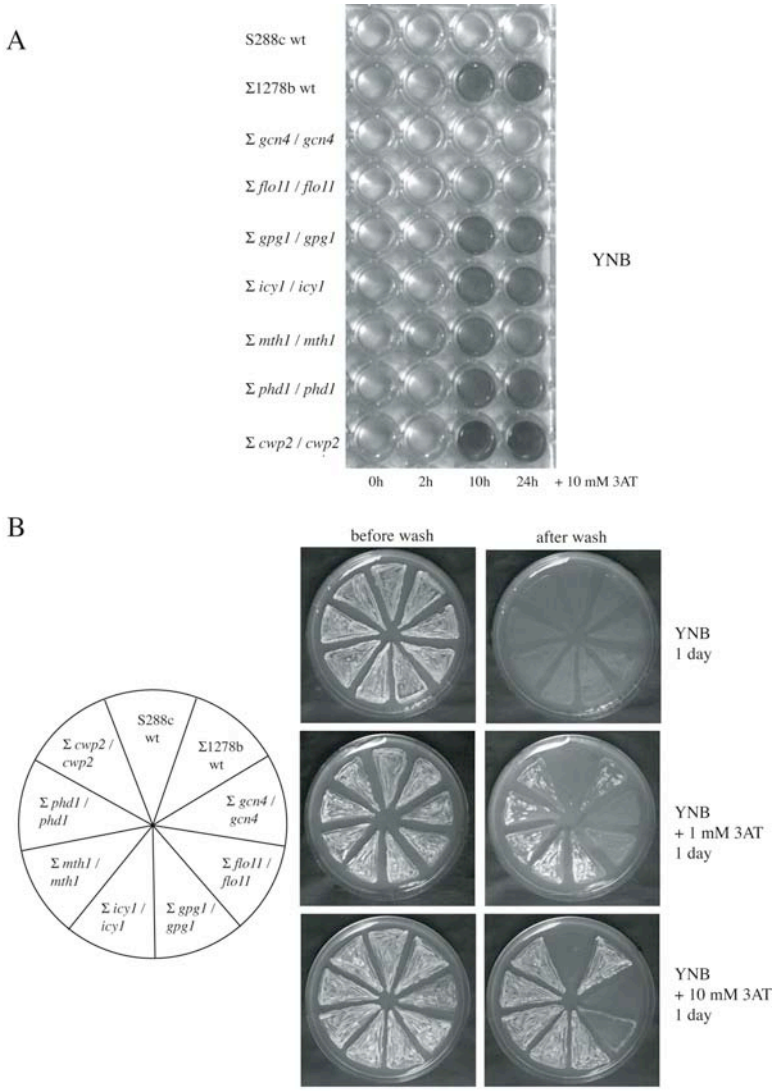

Figure 8: Adhesion phenotype induced in diploid *S. cerevisiae* Σ1278b by amino acid starvation.
A Wild-type *S. cerevisiae* cells of the S288c derivate RH3169 as control or the diploid Σ1278b derivatives RH2656 (wt), RH2665 (*gcn4/gcn4*), RH2661 (*flo11/flo11*), RH3170 (*gpg1/gpg1*), RH3171 (*icy1*/*icy1*), RH3172 (*mth1*/*mth1*), RH3173 (*phd1*/*phd1*) or RH3174 (*cwp2*/*cwp2*) were incubated in 100 µl portions of YNB (+ Ura, + Arg) medium in absence (0 h) or in presence of 10 mM of the histidine analogue 3-amino-triazole (3AT) for 2, 10 or 24 h, respectively, at 30°C in a microtiter plate. The optical density (OD_{600}) of the cultures was approximately 1.0, corresponding to about 1.5 x 10^6 cells. After staining with crystal violet, the culture was washed with water to remove cells that do not stick to plastic. The darkly stained wells contain cells that adhere to the surface of the plate. **B** The diploid S288c control strain RH3169 and the diploid Σ1278b yeast strains RH2656 (wt), RH2665

(*gcn4/gcn4*), RH2661 (*flo11/flo11*), RH3170 (*gpg1/gpg1*), RH3171 (*icy1*/*icy1*), RH3172 (*mth1*/*mth1*), RH3173 (*phd1*/*phd1*) or RH3174 (*cwp2*/*cwp2*) were patched on solid YNB (+ Ura, + Arg,) medium or solid YNB (+ Ura, + Arg,) containing 1 mM or 10 mM 3AT. After incubation for 1 day at 30°C plates were photographed before (total growth) and after washing (adhesive growth) with water. Non-adhesive growing cells were washed off the agar surface.

Table 2: Differentially expressed genes identified in the *GCN4*/*gcn4* comparison.

Gene and functional class	Expression ratio (*GCN4*/*gcn4*)	Function[A]
Amino acid metabolism		
ARG1	4.0	Argine biosynthesis
ARO10	3.6	Leucine catabolism
TRP1	2.9	Tryptophan biosynthesis
Vitamin and co-factor biosynthesis		
BIO3	4.0	Biotin biosynthesis
THI20	4.8	Thiamin biosynthesis
Ribosomal genes		
RPL11A	0.4	Protein biosynthesis
RPL43B	0.4	Protein biosynthesis
RPS12	0.5	Protein biosynthesis
RPS30B	0.4	Protein biosynthesis
Other[B]		
AAD10	2.6	Aldehyde metabolism
DDR48	4.0	DNA repair
GPD1	2.5	Accumulation of glycerol
GPG1	2.4	Signal transduction
GLK1	3.5	Carbohydrate metabolism
PNC1	2.6	Silencing at telomere
SDS24	3.5	Meiosis
YGL117w	2.5	Biological process unknown

[A] according to the annotation in the *Saccharomyces* Genome Database (http://www.yeastgenome.org).
[B] A complete list of genes is available in Supplementary Table 2.

In Table 3 some of these genes and the presumed functions of their products are listed. Two of these genes, *MTH1* and *TEC1*, are involved in Σ1278b specific developmental processes like haploid invasive growth or diploid pseudohyphal formation. *MTH1* encodes a repressor of *HXT* gene expression, and transposon insertion mutants of *MTH1* in a Σ1278b strain show no haploid invasive growth (Schulte and Ciriacy, 1995; Suzuki *et al*., 2003). The product of *TEC1* is a transcription factor of the TEA/ATTS DNA-binding domain family, which is required for haploid invasive growth as well as

diploid pseudohyphal development, but not for amino acid starvation-induced adhesive growth (Bürglin, 1991; Gavrias *et al.*, 1996; Madhani and Fink, 1997; Braus *et al*., 2003). Other significantly activated genes are involved in different cellular aspects like transport, signal transduction, splicing, sporulation, cell wall organization, or thiamin biosynthesis (see Table 3 and Supplementary Table 2). In recent studies it was shown by Northern analysis that *FLO11* is silenced in diploid Σ1278b cells under non-starvation but is transcriptionally induced upon imposition of amino acid starvation conditions (Braus *et al*., 2003). Silencing of *FLO11* under non-starvation conditions resulted in transcript values, which were similar to the filter background, and thus *FLO11* was eliminated from further consideration after correction of microarray data for background. The transcript levels of *FLO11* under amino acid starvation were significant higher than the filter background, confirming the activation of *FLO11* in adherent yeast cells.

Seven genes including *CTS2, DUN1, HXT1, SMP1, VPS65, YDR375c* and *YPS3* were identified as repressed in amino acid-starved and adherent yeast cells. *DUN1* was previously been described as being down-regulated in microarray experiments of amino acid-starved S288c cells (Natarajan *et al*., 2001). Repression of *HXT1* indirectly confirms our microarray data indicating the induction of *MTH1,* as the product of the latter gene is a repressor of *HXT* gene expression. Interestingly, *SMP1* was also repressed under adhesion-inducing conditions. *SMP1* codes for a transcription factor of the MADS box family which repression enhances ion tolerance and haploid invasive growth (Lamb and Mitchell, 2003).

The transcriptional profile of adherent Σ1278b yeast cells under amino acid starvation shows a series of regulated genes including 22 induced and six repressed genes, which had not yet been described. These results suggest the existence of a specific transcriptional profile of adhesively growing yeast cells.

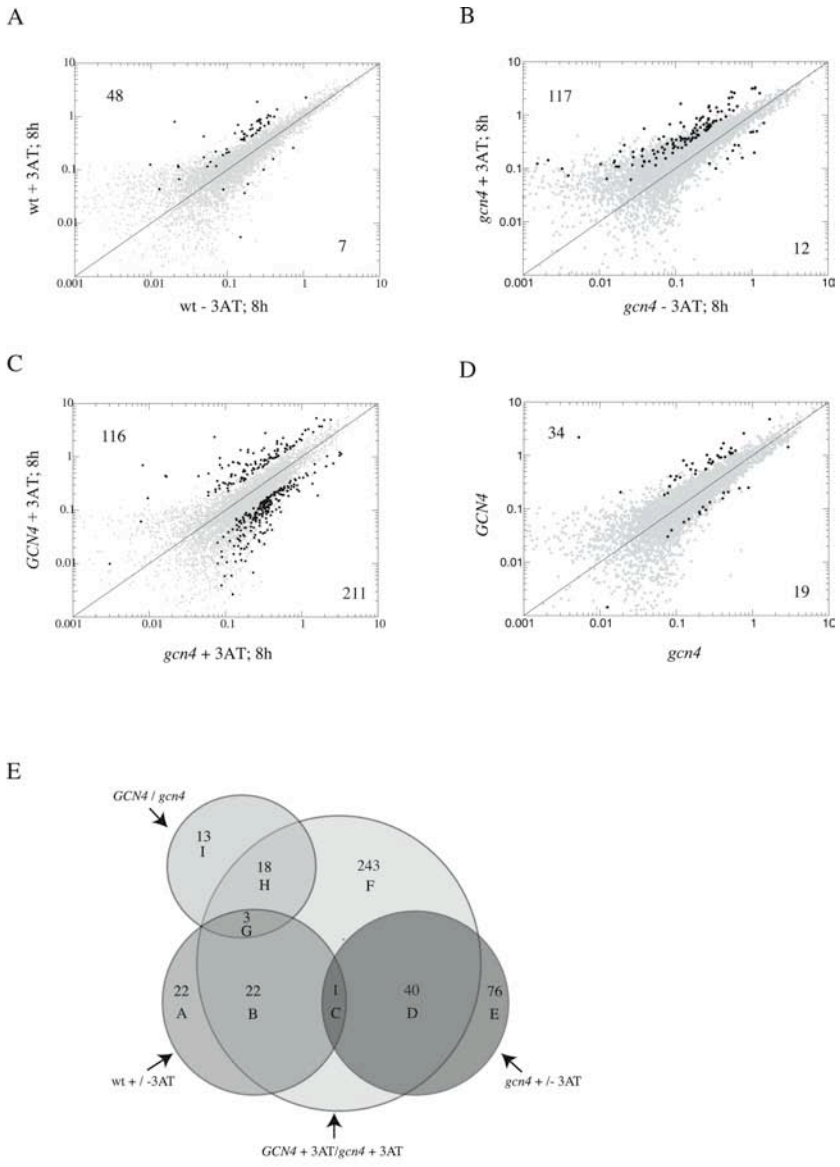

Figure 9: Transcriptional profiling experiments of adherent and non-adherent diploid yeast cells in the presence or absence of amino acid starvation.
The diploid Σ1278b yeast derivative strains RH2656 (wt) and RH2665 (*gcn4*/*gcn4*) were incubated in YNB medium (+ Arg) in presence or in absence of 10 mM of the histidine analogue 3-amino-triazole (3AT) for 8 h. Transcriptional profiles of cells cultivated under the two conditions were measured as described in Material and Methods. The averaged levels of mRNA determined for each gene in four independent measurements of each condition were plotted against the corresponding averaged levels obtained under a different condition. Genes were defined as being regulated if they showed at least a two-fold difference in each comparison at a p-value (t-test) of 0.01 or less (black spots), and all other genes disregarded. The numbers of black spots above (ratio ≥ 1) and below (ratio ≤ 1) the trend line are indicated. Unregulated genes that did not meet the selection criteria ('unregulated genes') are indicated by grey spots. **A** Profiles for RH2656 (wt) carrying a *GCN4* wild-type allele in presence or in absence of 3AT (wt +/- 10 mM 3AT; 8 h). **B** Profiles for the *gcn4* deletion strain RH2665 (*gcn4*/*gcn4*) in presence or in absence of 3AT (*gcn4* +/- 10 mM 3AT; 8 h). **C** Profiles for RH2656 (wt) and RH2665 (*gcn4*/*gcn4*) in

presence of 3AT (*GCN4* + 10 mM 3AT/*gcn4* + 10 mM 3AT; 8 h). **D** Profiles for RH2656 (wt) and RH2665 (*gcn4*/*gcn4*) deletion cells in absence of 3AT (*GCN4*/*gcn4*). **E** The degree of overlap between the regulated gene sets identified in the different transcriptional profiling experiments is displayed. The numbers of regulated genes in each class are indicated. Sectors A, B, C and G contain genes that are induced by 3AT in RH2656 (wt +/- 10 mM 3AT; 8 h). Genes in sections B, C and G are also more highly expressed in RH2656 (wt) than in RH2665 (*gcn4*/*gcn4*) cells in the presence of 3AT, respectively (*GCN4* + 10 mM 3AT vs. *gcn4* + 10 mM 3AT; 8 h). Genes in sections C, D and E are induced by 3AT in RH2665 (*gcn4*/*gcn4*) cells (*gcn4* +/- 10 mM 3AT; 8 h). Genes in section D are also more highly expressed in RH2665 (*gcn4*/*gcn4*) than in RH2656 (wt) cells in the presence of 3AT (*GCN4* + 10 mM 3AT vs. gcn4 + 10 mM 3AT; 8 h). The single gene in section C is induced by 3AT in RH2656 (wt) and RH2665 (*gcn4*/*gcn4*) cells, but requires Gcn4p for maximal induction (*GCN4* + 10 mM 3AT vs. *gcn4* + 10 mM 3AT; 8 h). Genes in sector G are induced by Gcn4p in absence of 3AT (*GCN4*/*gcn4*). Sections H and I contain genes that are induced by Gcn4p in absence and presence of 3AT. Section F contains genes identified in the comparison *GCN4* + 10 mM 3AT versus *gcn4* + 10 mM 3AT; 8 h.

Table 3: Novel genes induced by exposure of *GCN4* cells to 10 mM 3AT for 8 h.

Gene and functional class	Expression ratio (*GCN4*/*gcn4*)	Function[A]
Differentiation		
MTH1	3.3	Signal transduction
TEC1	2.1	Pseudohyhal growth
[*FLO11*[B]	>89	Adhesion; pseudohyphal growth]
Other[C]		
APM3	5.6	Golgi to vacuole transport
BBR2	2.4	5'-splice site cleavage
LCB4	4.4	Sphingolipid metabolism
RPI1	2.8	Thiamin biosynthesis
SNF3	2.3	Signal transduction
STP4	2.1	Biological process unknown
SUR7	2.2	Sporulation (sensu *Saccharomyces*)
WSC4	2.3	Cell wall organization and biogenesis

[A] According to the annotation in the *Saccharomyces* Genome Database (http://www.yeastgenome.org)
[B] Based on data from Northern analyses (Braus et al., 2003).
[C] A complete list of genes is available in Supplementary Table 2.

Transcription of *BAT2* and *MET10* is activated in amino acid-starved cells in absence of Gcn4p

Besides being necessary for cell-surface adhesion, Gcn4p is also required to ensure an adequate supply of amino acids in amino acid-starved Σ1278b cells. Cells lacking this

Gcn4p-mediated response to amino acid starvation require other strategies to survive temporary limitation of amino acids. To analyse the Gcn4p-independent response to amino acid starvation, the transcriptomes of the diploid *gcn4* deletion strain RH2658 were compared under amino acid starvation and non-starvation conditions (*gcn4* +/- 10 mM 3AT; 8 h). Figure 9B shows that 117 of 5084 considered genes were induced in amino acid-starved Σ1278b *gcn4*Δ cells, whereas 12 genes were repressed. Only the gene *MTG1*, which encodes a peripheral GTPase of the mitochondrial inner membrane, was also identified as activated in amino acid-starved Σ1278b wt cells carrying an intact *GCN4* allele (Figure 9E). These data indicate that the transcriptional response to amino acid starvation in a Σ1278b *gcn4*Δ background is completely different from the Gcn4p-mediated response in Σ1278b wild-type cells. A comparison with microarray experiments for haploid S288c *gcn4*Δ cells, which were treated with 100 mM 3AT for 1 h (*gcn4* +/- 100 mM 3AT; 1 h) showed that 30 induced genes, including *BTN2, DAL7, HSP42, HSP78, HSP104, PRM5, RPN4* and *SSA4*, were common to both sets (Natarajan *et al*., 2001). 87 novel genes were identified as being significantly induced by treatment of Σ1278b *gcn4*Δ cells with 10 mM 3AT (see the summary in Table 4). Two of these genes, *BAT2* and *MET10*, are involved in the amino acid biosynthesis, and have been shown to be activated in a Gcn4p-dependent manner in S288c yeast cells (Natarajan *et al*., 2001). Other genes that are significantly induced in amino acid-starved Σ1278b *gcn4* deletion cells are involved in protein folding (*CPR6, ERO1, MDJ1, SSA1, SSA2, SSE1, STI1*), protein degradation (*PEX4, RPT2, RPT4, RPN9, UBA1, UBC6*) and ergosterol biosynthesis (*ECM22, ERG3, ERG12, ERG26, ERG28*), respectively. Transcriptional profiling experiments, in which sulfometuron methyl (SM), an inhibitor of the branched chain amino acid biosynthesis, was used to impose amino acid starvation, confirm that some genes including *MET10, ERO1, MDJ1, SSE1, STI1* and *ERG3* (Table 4), are induced in amino acid-starved cells in a Gcn4p-independent manner (Jia *et al*., 2000).

Table 4: Novel genes induced by exposure of *gcn4* cells to 10 mM 3AT for 8 h.

Gene and functional class	Expression ratio (presence/absence of 3AT)	Function[A]
Amino acid biosynthesis		
BAT2	5.6	Branched chain family amino acid biosynthesis
MET10[B]	2.1	Sulfate assimilation
Vitamin and co-factor biosynthesis		
CPR6	4.2	Protein folding
ERO1[B]	3.8	Protein folding
MDJ1[B]	4.2	Protein folding
SSA1	5.0	Protein folding
SSA2	4.4	Protein folding
SSE1[B]	4.0	Protein folding
STI1[B]	4.6	Protein folding
Protein degradation		
PEX4	2.3	Polyubiquitination
RPT4	3.0	Ubiquitin-dependent protein catabolism
RPT2	2.4	Ubiquitin-dependent protein catabolism
RPN9	2.1	Ubiquitin-dependent protein catabolism
UBA1	2.3	Ubiquitin cycle
UBC6	2.3	Polyubiquitination
Ergosterol biosynthesis		
ECM22	2.8	Sterol biosynthesis
ERG3B	2.0	Ergosterol biosynthesis
ERG12	2.5	Ergosterol biosynthesis
ERG26	2.9	Ergosterol biosynthesis
ERG28	2.1	Ergosterol biosynthesis
Other[C]		
CUP1-1[B]	3.0	Response to copper
CUP1-2[B]	2.9	Response to copper
YGL117w	2.5	

[A] According to the annotation in the *Saccharomyces* Genome Database (http://www.yeastgenome.org).
[B] Gcn4p-independent induced genes after treatment with sulfometuron methyl (SM) (Jia *et al*., 2000).
[C] A complete list of genes is available in Supplementary Table 3.

Twelve down-regulated genes were identified in *gcn4*Δ cells after 3AT treatment. These include genes to be involved in amino acid biosynthesis (*ARG3, LYS20*), chromatin assembly (*HTA2*), phosphatidylcholine biosynthesis (*OPI3*) and nucleic acid metabolism (*SRL1*), respectively (see Supplementary Table 3).

A number of novel genes were found to be activated in a Gcn4p-independent manner in amino acid-starved Σ1278b cells. The most prominent examples for these genes

are *BAT2* and *MET10*. Thus, these two genes can be activated in a Gcn4p-dependent and a Gcn4p-independent manner in response to amino acid limitation.

Full transcriptional induction of 116 genes depends on Gcn4p in amino acid-starved and adherent yeast cells

To analyse dependency of the response to amino acid starvation upon Gcn4p, the transcriptional profile of wild-type Σ1278b cells under adhesion-inducing conditions was directly compared with that of a *gcn4*Δ under conditions of amino acid starvation (*GCN4* + 10 mM 3AT versus *gcn4* + 10 mM 3AT; 8 h). Figure 9C shows that 116 of 4954 tested genes were more highly expressed in wild-type cells than in *gcn4*Δ cells under amino acid starvation. Of these genes, 26 had previously been identified as activated in amino acid-starved Σ1278b wild-type cells (wt +/- 10 mM 3AT; 8 h) (Figure 9E).

A comparison with microarray experiments in the S288c background (*GCN4* + 100 mM 3AT versus *gcn4* + 100 mM 3AT; 1 h) revealed overlap that 44 of the 116 identified genes are common to both strains, including *AAD10, ADE1, ARG1, ARG3, ARO10, BIO2, BIO3, BIO4, BNA1, DDR48, GAT1, GLG1, GPG1, HIS4, HOM2, HOM3, ICY1, LYS20, LYS21, MET17, PCL5, SDS24, SNZ1, THR1, TMT1* and *YAH1* (Natarajan *et al*., 2001).

In all, 72 novel genes were identified as being expressed at higher levels upon amino acid limitation in wild-type Σ1278b as compared to Σ1278b *gcn4*Δ cells. Five of these genes have been reported to be involved in specific processes connected to filamentous growth. These are the cyclin encoding gene *CLN1* and *BMH1* (a member of the conserved eukaryotic 14-3-3 gene family), both of which are required for diploid pseudohyphal development (Roberts *et al*., 1997; Madhani *et al*., 1999; Oehlen and Cross, 1998), *FLO11* itself, *MTH1* and *PHD1*. *PHD1* encodes a transcription factor with a highly conserved helix-loop-helix motif. Although a *phd1* deletion strain shows no obvious defect in pseudohyphal growth, overexpression of *PHD1* enhances the *FLO11* expression and the pseudohyphal development even on nitrogen containing rich medium (Gimeno and Fink, 1994; Pan and Heitman, 2000).

In addition, four genes involved in cell wall biosynthesis or maintenance were more highly expressed in amino acid-starved wild-type Σ1278b cells than in similarly treated *gcn4*Δ cells. *CIS3* and *CWP2* code for cell wall mannoproteins, which are structural

constituents of the cell wall (Ram *et al.*, 1998; Klis *et al.*, 2002). The *CTS1* gene encodes an endochitinase that is required for the separation of mother and daughter cells (Kuranda and Robbins, 1991). The gene product of *PSA1* is a GDP-mannose pyrophosphorylase (Hashimoto *et al.*, 1997), which provides GDP-mannose, an important substrate for biosynthesis of cell wall mannoproteins and GPI-anchors (Abeijon and Hirschberg, 1992). Other genes that show a significant dependence upon Gcn4p in amino acid-starved Σ1278b cells are involved in thiamin (*RPI1, THI7, THI20*) and glycine metabolism (*GCV1, GCV3*), gluconeogenesis (*FBA1*) or purine biosynthesis (*MTD1*). A screen for Gcn4p binding sites in the entire yeast genome performed by Schuldiner *et al.* (Schuldiner *et al.*, 1998) confirms the presence of three putative Gcn4p binding sites in the promoter region of *THI7, GCV3, FBA1* and *MTD1*, and thus corroborates our microarray data.

Figure 9C shows that 211 genes are expressed at significantly higher levels in amino acid-starved Σ1278b *gcn4*Δ cells than in similarly treated Σ1278b *GCN4* cells. 40 of these genes were also induced in amino acid-starved Σ1278b *gcn4*Δ cells (*gcn4* +/- 10 mM 3AT; 8 h) (Figure 9E). Furthermore 55 genes, including *ARO7, ATF2, BTN2, CPR6, CUP1-1, CUP1-2, NRG1, SSE1* and *SSA2*, show a similar expression pattern in microarray experiments with haploid S288c cells (*GCN4* + 100 mM 3AT versus *gcn4* + 100 mM 3AT; 1 h) (Natarajan *et al.*, 2001). Interestingly, *NRG1* codes for a repressor of *FLO11* expression, haploid invasive growth or diploid pseudohyphal formation in adherent Σ1278b cells (Kuchin *et al.*, 2002; Kuchin *et al.*, 2003). Two other genes for putative negative effectors of haploid invasive growth were newly identified as being induced in amino acid-starved Σ1278b *gcn4*Δ cells. Haploid strains carrying a *bem2* or *zuo1* mutation display a hyperinvasive phenotype in Σ1278b cells (Palecek *et al.*, 2000; Suzuki *et al.*, 2003), which implies a regulatory function for these genes in haploid invasive growth of Σ1278b cells.

To validate the microarry data, Northern hybridization experiments were performed for several of the newly identified regulated genes. In accordance with the array data, *CIS3, CTS1, CWP2* and *PSA1* were expressed at higher levels in amino acid starved Σ1278b wild-type cells than in amino acid starved Σ1278b *gcn4*Δ cells (Figure 10). This regulation predominantly results from reduced transcription in amino acid-starved Σ1278b *gcn4*Δ cells. For *CWP2* significant activation was also detected in amino acid-starved Σ1278b wild-type cells. This indicates that, during amino acid starvation-induced adhesive

growth, *CWP2* is directly induced by Gcn4p. We also tested the weakly expressed *MTH1* and *PHD1* genes, but due to the low sensitivity of Northern hybridization technique no transcripts could be detected (data not shown). As an example for genes that were more expressed in amino acid-starved Σ1278b *gcn4*Δ cells than in similarly treated *GCN4* cells, *ZUO1* was subjected to Northern analysis. The data shown in Figure 10 confirm the increased levels of *ZUO1* transcripts in Σ1278b *gcn4*Δ cells after amino acid starvation.

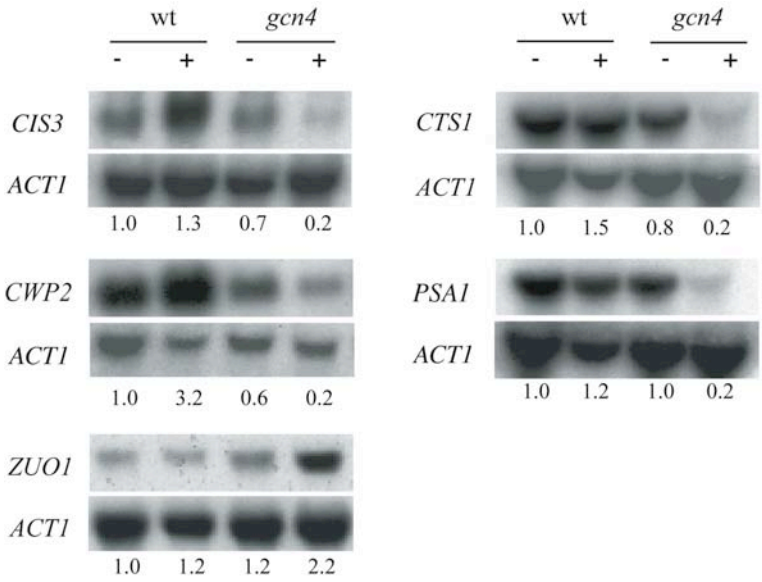

Figure 10: Gcn4p-dependent gene expression in *S. cerevisiae* Σ1278b under conditions of amino acid starvation.
The diploid Σ1278b yeast strains RH2656 (wt) and RH2665 (*gcn4*/*gcn4*) were incubated on YNB medium (+ Arg) in absence (-) or in presence (+) of 10 mM 3AT for 8 h at 30°C. Alliquots (20 μg) of total RNAs from each sample were used for Northern hybridization analysis. The numbers indicate levels of *CIS3, CTS1, CWP2, PSA1* and *ZUO1* transcripts relative to the internal standard *ACT1*.

A list of the genes that are subjected to Gcn4p-dependent regulation in amino acid-starved Σ1278b cells is presented in Table 5 (see also Supplementary Table 4).

Direct comparison between the Gcn4p-independent and dependent responses to amino acid starvation reveals that, in the absence of Gcn4p, nearly twice (211) as many genes are significantly induced than in the presence of Gcn4p (116). Of these regulated genes, 72 show a previously unrecognized dependence upon Gcn4p under adhesion-inducing conditions.

Table 5: Novel genes that are differentially expressed in *GCN4* versus *gcn4* cells exposed to 10 mM 3AT for 8 h.

Gene and functional class	Expression ratio (*GCN4* + 3AT/*gcn4* + 3AT)	Function[A]
Differentiation		
BMH1	2.5	Pseudohyphal growth
CLN1	2.3	G1/S transition of mitotic cell cycle
FLO11	6.4	Pseudohyphal growth
MTH1	6.3	Signal transduction
PHD1	2.1	Pseudohyphal growth
Cell wall maintenance or biosynthesis		
CIS3	2.5	Cell wall organization and biogenesis
CTS1	3.0	Cytokinesis, completion of separation
CWP2	2.7	Cell wall organization and biogenesis
PSA1	2.4	Cell wall mannoprotein biosynthesis
Thiamine metabolism		
RPI1	3.3	Thiamin biosynthesis
THI7	2.5	Thiamin transport
THI20	5.0	Thiamin biosynthesis
Glycine metabolism		
GCV1	3.1	One-carbon compound metabolism
GCV3	2.2	One-carbon compound metabolism
Histones		
HTA2	6.2	Chromatin assembly/disassembly
HTB2	2.1	Chromatin assembly/disassembly
HHF1	2.3	Chromatin assembly/disassembly
HHF2	2.2	Chromatin assembly/disassembly
Other[B]		
BAT2	0.3	Branched chain family amino acid biosynthesis
BEM2	0.3	Actin cytoskeleton organization and biogenesis
FBA1	2.1	Gluconeogenesis
MTD1	2.0	One-carbon compound metabolism
ZUO1	0.4	Protein folding

[A] According to the annotation in the *Saccharomyces* Genome Database (http://www.yeastgenome.org)
[B] A complete list of genes is available in Supplementary Table 4.

Gcn4p is required for efficient transcription of over 30 genes in untreated and non-adhering cells

The *gcn4* null mutant is viable but requires arginine for normal growth on minimal medium. This phenotype shows that a basal Gcn4p level in the cell is necessary for a basal-

level transcription of some genes. To analyse the role of Gcn4p under non-starvation conditions, we compared the transcriptional profiles of wild-type diploid Σ1278b cells and diploid Σ1278b *gcn4*Δ cells (*GCN4*/*gcn4*). Figure 9D shows that 34 of 4713 genes considered required a basal Gcn4p level for basal expression. 19 genes were more highly expressed in Σ1278b *gcn4*Δ cells, suggesting that the presence of Gcn4p directly or indirectly repress transcription of these genes. Table 2 lists the genes that are regulated in Gcn4p-dependent manner under non-starvation conditions (see also Supplementary Table 1). Over 52% (18 genes) of all genes stimulated by a basal Gcn4p level are also dependent on Gcn4p under amino acid starvation conditions (*GCN4* + 10 mM 3AT versus *gcn4* + 10 mM 3AT; 8 h). These include *AAD10, ARG1, ARO10, BIO3, DDR48, GPG1, PNC1, SDS24, THI20, TRP1, YGL117w* and *YLR004c* (Figure 9E). Three of these genes *AAD10, YGL117w* and *YLR004c* were also shown to be induced by amino acid starvation (wt +/- 10 mM 3AT; 8 h) (Figure 9E). Interestingly, four of the 19 genes that were more highly expressed in Σ1278b *gcn4*Δ cells encode ribosomal proteins (*RPL11A, RPL43B, RPS12, RPS30B).*

The identification of several regulated genes that are Gcn4p-dependent under non-starvation conditions shows that small amounts of Gcn4p are required and sufficient for basal transcriptional activation of numerous pathways.

Adherence of yeast is independent of *MTH1, PHD1* and *CWP2* function

The diploid Σ1278b yeast deletion strains RH3170 *(gpg1*/*gpg1),* RH3171 (*icy1*/*icy1),* RH3172 *(mth1/mth1),* RH3173 (*phd1/phd1)* and RH3174 (*cwp2/cwp2*) were tested for adhesive growth to analyse the relationship between Gcn4p-dependent gene activation and adhesion in amino acid-starved yeast cells. All the genes deleted in these strains are involved in haploid invasive growth, pseudohyphal development or cell wall structure.

The activation of *MTH1, PHD1* and *CWP2* in amino acid-starved and adherent Σ1278b cells was shown to be dependent on Gcn4p. Transposon mutants of *MTH1* are defective for haploid invasive growth (Suzuki *et al.*, 2003). Overexpression of *PHD1* triggers pseudohyphal development and *FLO11* expression, irrespective of the nitrogen source (Gimeno and Fink, 1994; Pan and Heitman, 2000). *CWP2* codes for a GPI-anchored cell wall protein, which is covalently linked to the β-1.6-glucan portion of the cell wall

(Ram *et al*., 1998). Therefore, we wondered whether these three genes might be essential for adherence under conditions of amino acid starvation. We also tested two Gcn4p-dependent genes (*GPG1* and *ICY1*) that were induced in both adherent and non-adherent (S288c) cells. *GPG1* and *ICY1* were strongly induced in amino acid-starved non-adherent S288c and adherent Σ1278b cells in a Gcn4p-dependent manner (Natarajan *et al*., 2001). Loss of *GPG1* in Σ1278b cells leads to a modest reduction in haploid invasive growth, pseudohyphal development or *FLO11* expression (Harashima and Heitman, 2002), whereas mutants of *ICY1* are deficient in haploid invasive growth (Suzuki *et al*., 2003). All Σ1278b derivates constructed were tested for adhesive growth on plastic and on solid agar medium in presence or absence of 3AT (Figure 8). As expected, all tested strains were non-adhesive under non-starvation conditions. After starving the cells for amino acids for 10h, all strains, with the exception of the S288c strain and the *gcn4/gcn4* and *flo11/flo11* Σ1278b controls, were found to adhere on the surface of the microtiter plate with the exception of the S288c strain and the *gcn4/gcn4* and *flo11/flo11* control Σ1278b strains (Figure 8A). On solid medium all tested strains became adhesive in presence of 1 mM 3AT and showed a strong adherence in presence of 10 mM 3AT (Figure 8B). These results indicate that none of the five genes tested (*MTH1*, *PHD1*, *CWP2*, *GPG1* and *ICY1*) is required for amino acid starvation-induced adhesion.

These data corroborate the array data and suggest that the transcriptional response to amino acid starvation in adherent yeast strains is complex. With the exception of *FLO11* and *GCN4*, none of the genes that are specifically induced under these conditions has yet been identified as indispensable for the adherence response.

Discussion

Transcriptional profiling analysis has identified major transcriptional changes in adherent yeast Σ1278b cells starved for amino acids relative to non-adherent yeast cells grown under conditions of amino acid sufficiency. We identified 22 novel genes as being induced by amino acid limitation imposed by the histidine analogue 3AT in Σ1278b cells. In the non-adherent S288c cells, a significantly larger number of induced genes have been described by (Natarajan *et al.*, 2001). In S288c, 294 genes are activated by a factor of two and more after incubation with 10 mM 3AT for 1 h, and about 1000 genes are induced within 1 h if the concentration of 3AT is increased to 100 mM 3AT, whereas only 48 genes were induced in amino acid-starved Σ1278b cells. The higher number of induced genes in the S288c background is no doubt partly attributable to the differences in experimental conditions (haploid vs. diploid cells, 1 h vs. 8 h incubation with 100 mM or 10 mM 3AT, the use of glass slides vs. membrane filters for microarray construction). In addition, our threshold for data evaluation was more strict (p-value $\leq$ 0.05 vs. p-value $\leq$ 0.01). The use of the threshold p-value $\leq$ 0.05 increases the number of induced genes by a factor of two (to 95) in amino acid-starved Σ1278b cells. However, this increase corresponds to a five-fold increase in the probability of false positives. The 22 novel genes described here suggest that there are also general differences between adherent and non-adherent yeast cells. One reason for these differences might be that the basal level of Gcn4p under non-starvation conditions is significantly higher in cells of the Σ1278b background (Grundmann *et al.*, 2001). This higher basal activity of Gcn4p is associated with lower relative induction values under amino acid starvation. As previously reported for S288c and its derivates, the general control usually activates target genes by a factor of 2.0 to 3.0 (based on Northern hybridization data; Hinnebusch, 1992), and several known Gcn4p targets were under threshold of two-fold activation in our experiments due to the higher basal levels of Gcn4p in Σ1278b cells. Recent microarray experiments have directly compared the transcriptome of a Σ1278b yeast strain to that of the adhesion deficient W303 yeast strain in the absence of amino acid limitation (Breitkreutz *et al.* 2003). In this study 496 genes were expressed at significantly higher levels in the W303 background, whereas only 112 genes show enhanced expression in the Σ1278b background. The majority of these differentially expressed genes are involved in metabolism, transport or

unknown processes, which support our observation of general differences between different *S. cerevisiae* genetic backgrounds.

Besides the 22 novel genes, which were induced in adherent yeast cells upon amino acid starvation, 72 novel genes were found to require Gcn4p for full transcriptional induction in amino acid-starved Σ1278b cells. All these genes are putative targets of Gcn4p, and this finding underlines the impact of the general control system on adhesion of yeast cells. Mutations for three of these genes were tested in adhesive growth assays. Single *cwp2*, *mth1* and *phd1* mutants retain the ability to grow adhesively. At present, *GCN4* and *FLO11* are the only genes, which have been shown to be essential for amino acid starvation-induced adherence (Braus *et al.*, 2003). We cannot exclude the possibility that various combinations of mutations in the genes identified as being induced in this study might result in an impairment of adherence. *EFG1*, the *C. albicans* homologue of *PHD1*, is required for the amino acid starvation-induced filamentous growth of the human pathogen *C. albicans* (Tripathi *et al.*, 2002). This suggests either that Phd1p and Efg1p play different roles in *S. cerevisiae* and *C. albicans* under conditions of amino acid starvation, or that *S. cerevisiae* possesses additional genes whose functions overlap with Phd1p. A transcriptome analysis of the invasive growth of haploid *S. cerevisiae* cells has shown a similar relationship between gene activation and the corresponding phenotype to that revealed by this study. There, only expression of *FLO10* is consistently correlated with haploid invasive growth and filamentous morphology cells (Breitkreutz *et al.*, 2003), but *flo10Δ* strains are still competent for invasion (Guo *et al.*, 2000).

Several down-regulated genes were also identified in amino acid-starved and adherent yeast cells. Two of these genes, *SMP1* and *NRG1*, have been described as co-ordinately repressed by the transcription factor Rim101p during induction of haploid invasive growth (Lamb and Mitchell, 2003). The identification of *SMP1* and *NRG1* as genes that are repressed under adhesion-inducing conditions suggests a similar mechanism for stimulation of amino acid starvation-induced adhesive growth. However, the absence of putative Gcn4p binding sites in the promoters of most of the repressed genes indicates that the contribution of Gcn4p to down-regulation is probably indirect.

Amino acid starvation of yeast cells that lack Gcn4p imposes harsh conditions because it combines nutrient stress with the absence of Gcn4p. Accordingly, significantly more genes are induced in comparison to amino acid starvation in cells that retain an intact general control system due to a functional Gcn4p. The fact that only one gene (*MTG1*) was

regulated in both *GCN4* and *gcn4*Δ cells starved for histidine suggests, that the dual stress (no functional Gcn4p and simultaneous amino acid starvation) induces a completely different response to that evoked by amino acid starvation of *GCN4* cells. The back-up system consists of genes (*HSP42, HSP78, HSP104, SSA4),* which are stereotypically regulated under diverse stress conditions and mediate an 'environmental stress response' (Gasch *et al*., 2000). The products of other genes that respond more specifically to amino acid starvation enhance the refolding and reactivation of previously denatured and aggregated proteins or the recycling and biosynthesis of amino acids.

Interestingly, also genes involved in ergosterol biosynthesis were induced in amino acid-starved *gcn4*Δ cells. As the major sterol of the yeast plasma membrane, ergosterol is required for targeting amino acid permeases to the plasma membrane (Umebayashia and Nakando, 2003) and is also linked to the oxidative stress response in yeast (Higgens *et al*., 2003; Thorpe *et al.,* 2004). Alterations in membrane composition presumably affect the uptake or efflux of drugs like 3AT into or out of the cell. An additional reason for activation of ergosterol biosynthetic genes could be protection against oxidative stress, which results from the inhibition of catalase activity by 3AT (Halliwell and Gutteridge, 1989). In microarray experiments of *C. albicans,* ergosterol biosynthetic genes were also found to be induced during biofilm formation (Garcia-Sanchez *et al*., 2004). It is assumed, that an enrichment or redistribution of sterols in biofilm membranes could explain their resistance to azole-derived antifungal drugs.

In summary, we identified numerous novel genes as being induced under adhesion-inducing conditions. These newly described genes will be the focus of future experiments, which should throw light on the molecular mechanisms required for the establishment and regulation of the adherence phenotype as one of the initial steps in biofilm formation.

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Chapter III

The yeast RACK1 homologue *CPC2/ASC1* is required for *FLO11*-dependent adhesive growth of *Saccharomyces cerevisiae*

Abstract

Cells of the eukaryotic model organism *Saccharomyces cerevisiae* interact by cell-cell or cell-surface adhesion during limited supply of amino acids. This adherence growth phenotype requires the expression of the adhesin encoding gene *FLO11* and of Gcn4p, the global transcription factor of the general control of amino acid biosynthesis. In this study, we present 2D-DIGE experiments of amino acid-starved Σ1278b yeast cells to identify additional highly de-repressed or activated proteins. Seven protein spots display a highly increased intensity in response to amino acid starvation in comparison to sated yeast cells. These protein spots were identified by mass spectrometry and have previously been reported to be involved in translation (Cpc2p/Asc1p, Efb1p), stress response (Hsp60p, Sod1p), metabolism (His1p; Tpi1p) or cytoskeleton (Tpm1p). Comparisons with recent transcriptome data revealed that the mRNA levels of the encoding genes were significantly increased only for the *HIS1* gene. Deletion of the yeast RACK1 homologue *CPC2/ASC1* results in an adhesion deficient growth phenotype for amino acid-starved yeast cells. In addition, *CPC2* is also required for basal transcription and activation of *FLO11* upon amino acid limitation. Further we demonstrate that other Flo11p-dependent phenotypes such as haploid invasive growth and diploid pseudohyphal development are also *CPC2*-dependent. These data suggest that the translation factor Cpc2p controls the synthesis of either a protein, which is required for *FLO11* transcription, or of Flo11p itself.

Introduction

The colonization of hosts by pathogenic fungi often requires the ability of cells to stick to each other or to surfaces. This ability for adherence protects the fungus from removal by physical forces as wind or water. In addition, adherence represents the crucial initial event of the fungus–host interaction in case of humans or plants pathogens, and is therefore an important target for defence or therapy of fungal infections.

In the model fungus *Saccharomyces cerevisiae*, cell-cell or cell-surface adherence are parts of complex developmental processes as haploid invasive growth (Guo *et al.*, 2000; Roberts and Fink, 1994) or diploid pseudohyphae formation (Gimeno *et al.*, 1992; Mösch and Fink, 1997). These adherence growth phenotypes are induced by distinct environmental signals. The lack of glucose represents a signal, which results in invasive growth of haploid yeast cells (Cullen and Sprague, 2000). In diploid yeast cells, starvation for nitrogen induces the pseudohyphal growth phenotype, a switch from unicellular yeast cells to elongated cells with polar cell growth (Cullen and Sprague, 2000; Lo and Dranginis, 1998; Pan and Heitman, 1999; Robertson and Fink, 1998). Both developmental processes depend on expression of the cell-surface adhesin Flo11p (Lo and Dranginis, 1998), which is under the control of the MAP kinase and cAMP signal transduction pathways (Elion *et al.*, 2005; Lengeler *et al.*, 2000; Mösch *et al.*, 1999; Rupp *et al.*, 1999).

Amino acid starvation represents an additional signal for adhesive growth. In amino acid-starved haploid and diploid yeast cells, adhesion and *FLO11* expression is induced even in the presence of glucose or ammonium (Braus *et al.*, 2003). The cAMP signal transduction pathway, but not the MAP kinase pathway is required for amino acid starvation-induced adherence. In addition, two elements of the general amino acid control are required for regulation of *FLO11* expression and amino acid starvation-induced adhesive growth, namely the transcriptional activator Gcn4p (Hinnebusch and Natarajan, 2002) and the amino acid sensor kinase Gcn2p (Dever and Hinnebusch, 2005). Transcriptional profiling experiments in the adhesion deficient laboratory yeast strain S288c revealed that Gcn4p induces transcription under amino acid starvation of several hundred genes belonging to different metabolic pathways (Natarajan *et al.*, 2001). Recent transcriptome analyses in the adherent yeast strain Σ1278b show that Gcn4p regulates additional genes that have earlier been described to be involved in haploid invasive growth or pseudohyphal formation upon amino acid limitation (Kleinschmidt *et al.*, 2005).

Transcriptional profiling experiments do not reflect regulatory events on the protein level, which might also play important roles in the response of yeast cells to amino acid starvation conditions. Until now, only a proteome analysis of non-adherent S288c yeast cells was performed to identify such regulatory events under conditions of amino acid limitation (Yin *et al.*, 2004).

Under amino acid starvation, uncharged t-RNA molecules accumulate in the cell. The sensor kinase Gcn2p, which includes a C-terminal histidyl-tRNA synthetase (HisRS) related domain and an N-terminal protein kinase domain, senses these uncharged t-RNAs and phosphorylates the α subunit of the eukaryotic translation factor eIF2 on serine 51 (Dever *et al.*, 1992; Wek *et al.*, 1995; Zhu *et al.*, 1996). The mechanism of amino acid limitation sensing by Gcn2p is highly conserved and has also recently been identified in the mammalian piriform cortex in response to a deficiency of indispensable amino acids for protein synthesis (Hao *et al.*, 2005). In yeast, phosphorylation of eIF2 results in reduced levels of ternary complexes eIF2-GTP-Met-$tRNA_i^{MET}$ in the cell and finally in a reduced overall translation efficiency. Only a limited number of mRNAs carrying specific 5' untranslated regions are increasingly translated. One of these mRNAs is the *GCN4* mRNA carrying four short upstream open reading frames (uORFs) in its 5' leader sequence, which inhibits translation under non-starvation conditions (Hinnebusch, 1997). As consequence of the reduced level of cellular ternary complexes upon amino acid starvation, ribosomes scan past the uORFs and translate the mRNA of the *GCN4* gene. In addition to this translational control of *GCN4* expression, Gcn4 protein is stabilized in the nucleus of amino acid-starved yeast cells from a half-life of about 5 minutes or less to up to 20 minutes (Kornitzer *et al.*, 1994; Pries *et al.*, 2002).

In this work we sought for additional highly de-repressed or activated proteins possibly affecting amino acid starvation-induced adhesive growth of *S. cerevisiae*. Therefore, 2D-differential-gel-electrophoresis (2D-DIGE) analyses of amino acid-starved Σ1278b yeast cells were performed. One of the protein spots found to be up-regulated upon amino acid starvation was identified as Cpc2p. The *CPC2* gene (also known as *ASC1*) encodes a highly conserved Gβ-like ribosome-associated WD protein (Hoffman *et al.*, 1999; Chantrel et al., 1998; Gerbasi et al., 2004) and is a homologue of the *cpc-2* gene of *Neurospora crassa* (Krüger *et al.*, 1990; Müller *et al.*, 1995), and of the human RACK1 encoding a receptor of activated protein kinase C (Ron *et al.*, 1994). In yeast, a *cpc2* deletion suppresses the growth phenotype of a *gcn2* mutant under amino acid starvation

conditions (Hoffmann *et al.*, 1999), whereas deletion of *cpc-2* in *N. crassa* results in a sensitivity to amino acid starvation (Krüger *et al.*, 1990; Müller *et al.*, 1995). Here we show that a functional *CPC2* gene is required for *FLO11* expression and adhesive growth.

Materials and Methods

Yeast strains and growth conditions

Yeast strains used in this work are derivates of *S. cerevisiae* Σ1278b and listed in Table 6. Deletion mutants for *CPC2* (*cpc2*) were obtained by using the *cpc2* deletion plasmid pRSBH1-14 (Hoffmann *et al.*, 1999). Transformations were carried out using the lithium-acetate yeast transformation method (Ito *et al.*, 1983). Standard methods for crosses were performed as described previously (Guthrie and Fink, 1991). For non–starvation conditions, strains were cultivated in liquid synthetic minimal medium (YNB) containing respective supplements at 30°C overnight, diluted and cultivated to mid-log phase before isolation of protein extracts or total RNAs. For amino acid starvation conditions, 10 mM of the histidine analogue 3-amino-triazole (3AT) was added to yeast cultures grown to mid-log phase and cells were incubated for 1, 6 or 10 h at 30°C, respectively. For adhesive growth tests, strains were grown on solid (2% agar) YNB medium containing respective supplements and 10 mM 3AT to induce amino acid starvation. For haploid invasive growth tests, cells were grown on solid (2% agar) yeast extract-peptone dextrose (YPD) medium or on solid (2% agar) synthetic minimal medium (YNB) containing 2% galactose. Qualitative pseudohyphal growth was assayed on SLAD plates (Gimeno *et al.*, 1992).

Table 6: *S. cerevisiae* strains used in this work.

Strain	Genotype	Reference
RH2817	*MATα ura3-52 his3::hisG trp1::hisG HIS*$^+$	Strittmatter *et al*., 2005
RH2656	*MATa/α ura3-52/ura3-5 trp1::hisG/TRP1*	Braus *et al*., 2003
RH2661	*MATa/α ura3-52/ura3-52 flo11Δ::kanR/flo11Δ::kanR trp1::hisG/TRP1*	Braus *et al*., 2003
RH2662	*MATa ura3-52 flo11::kanR*	Braus et al., 2003
RH3220	*MATα ura3-52 sfl1::kanR his3::hisG trp1::hisG HIS*$^+$,	Fischer *et al*., 2005
RH3222	*MATa/α ura3-52/ura3-52 sfl1::kanR/sfl1::kanR trp1::hisG/TRP1*	Fischer *et al*., 2005
RH3263	MAT*α ura3-52 trp1::hisG leu2::hisG cpc2::LEU2*	This work
RH3264	*MATa/α GCRE6-lacZ::URA3/ura3-52 trp1::hisG/ trp1::hisG leu2::hisG/leu2::hisG cpc2::LEU2/ cpc2::LEU2*	This work

2D-DIGE analysis

Two serial 10 ml YNB pre-cultures, the first incubated overnight and the second for 2 hours, were used to inoculate 100 ml YNB medium and were grown to an OD_{600} between 0.6 and 0.8 for harvesting cells. Amino acid starvation was induced at an OD_{600} of 0.3 by adding 3-aminotriazole (3-AT) to a final concentration of 10 mM. Cell disruption was done using the Y-PERS reagent of Pierce Biotechnology Inc. (Rockford, IL). Protein concentrations were determined with the Pierce BCA-kit according to the supplier's manual. Recommended amounts of cell extracts were purified by methanol-chloroform extraction and differentially labelled with the fluorescent dyes Cy2, Cy3, or Cy5 according to the supplier's protocol (GE Healthcare). The protein standard originates from equal aliqoutes of each extract used for the analysis, and was labelled with Cy2. This Cy2-labelled standard and extracts of different culture conditions labelled with either Cy3 or Cy5 were combined for IEF and PAGE. Differentially labelled proteins of 2D-gels were imaged by serially scanning the gel with the respective laser/filter combination on a Typhoon scanner (GE Healthcare). Images were normalized against the standard and analysed by using the DeCyder software of GE Healthcare (Yan *et al.*, 2002).

Growth tests and photomicroscopy

Amino acid starvation-induced adhesive growth tests on solid YNB medium were performed as described previously (Braus *et al.*, 2003). Strains were pre-grown on solid YNB medium containing respective supplements for 20 h. Cells were patched on fresh YNB containing supplements and 10 mM 3AT to induce amino acid starvation. After incubation for one day at 30°C, plates were photographed and then carefully washed under a stream of water. The plates were photographed again to document adhesive growing cells. Haploid invasive growth was assayed as described previously (Roberts and Fink, 1994). After five days of growth on solid YPD medium or solid YNB medium carrying 2% galactose, cells were washed by water and photographed to document haploid invasive cells. For qualitative diploid pseudohyphal development assays cells were grown on solid SLAD medium for five days. Pseudohyphal colonies were viewed with an Axiovert microscope (Carl Zeiss, Jena, Germany) and photographed using a Xillix microimager digital camera with the Improvision Openlab software (Improvision, Coventry, United Kingdom).

Northern hybridization analysis

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

β-galactosidase assay

Assays were performed with extracts grown in liquid medium. Specific β-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 and Botstein, 1983). Assays were performed for at least three independent cultures.

Results

Seven protein spots are highly enhanced after treatment with the histidine analogue 3-amino triazole

Cells of the wild-type *Saccharomyces cerevisiae* strain Σ1278b grow adhesively on surfaces when limited in supply of amino acids (Braus *et al.*, 2003). This adherence phenotype requires a functional *GCN4* gene encoding the global transcriptional activator of numerous pathways in amino acid-starved yeast cells (Kleinschmidt *et al.*, 2005; Natarajan *et al.*, 2001). The amount of Gcn4p in the cell is regulated on a posttranscriptional level in response to amino acid starvation. Translation of *GCN4* mRNA is specifically de-repressed as a result of a phosphorylated translation initiation factor eIF2 upon amino acid limitation (Hinnebusch and Natarajan, 2002).

2D-differential-gel-electrophoresis (2D-DIGE) experiments of amino acid-starved and adherent yeast cells were performed to identify further highly de-repressed or activated proteins, which are potentially involved in adhesive growth. Comparisons with respective transcriptome data should show whether the increase in protein expression is based on a transcriptional or posttranscriptional regulation.

We induced amino acid starvation by adding the histidine analogue 3-amino-triazole (3AT) and incubated the diploid Σ1278b wild-type strain RH2656 in absence (0 h) or in presence of 10 mM 3AT for 1 h, 6 h or 10 h at 30°C. Protein extracts were isolated from three independent cultures of each condition and labelled with the fluorescent dye Cy3 (0 h; 10 h) or with Cy5 (1 h; 6 h). For normalization of the different 2D-DIGEs, a mixed internal standard pool consisting of equal aliquots of all twelve individual extracts was labelled with the fluorescent dye Cy2. By this way two groups of 2D-DIGE were obtained, namely 0 h vs. 10 h (gels 1-3) and 1 h vs. 6 h (gels 1-3), with all gels also comprising the same amount of Cy2-standard extract. After evaluation, protein spots, which displayed an at least eight-fold induction and a statistical significance of less than 0.001 (t-test), were considered as highly de-repressed or activated. Further we excluded protein spots appearing in less than 27 of 33 gel images. The protein spots matching these criteria are illustrated in Figure 11, and specifications listed in Table 7.

Table 7: Highly up-regulated protein spots after 3AT treatment in *S. cerevisiae.*

Protein	Amino acid starvation			Number of spot appearances (Total number of gels)
	1 h Factor (t-test)	6 h Factor (t-test)	10 h Factor (t-test)	
His1p	2.3 (0.0046)	1.7 (0.0049)	13.5 (0.00019)	33 (33)
Tpm1p	3.4 (0.054)	3.6 (0.015)	15.7 (2.9e-006)	33 (33)
Sod1p	1.5 (0.3)	2.0 (0.014)	8.0 (0.00013)	27 (33)
Hsp60p	3.4 (0.15)	2.3 (0.16)	14.6 (6.1e-005)	30 (33)
Efb1p	3.0 (0.097)	3.1 (0.026)	20.0 (0.0011)	33 (33)
Tpi1p	3.5 (0.44)	3.6 (0.17)	37.2 (5.4e-005)	30 (33)
Cpc2p	4.7 (0.011)	4.9 (0.006)	9.0 (8.3e-005)	33 (33)

Figure 11 shows that a Cpc2p protein spot was nine-fold up-regulated after 10 h of amino acid starvation. Microarray experiments revealed for *CPC2* no transcriptional induction under amino acid starvation. In contrast, *CPC2* transcription was reduced to the level of 0.75 in adherent Σ1278b yeast cells when starved for amino acids (Kleinschmidt *et al.*, 2005). Cpc2p is a ribosomal Gβ-like WD protein, which influences translation of specific mRNAs (Gerbasi *et al.*, 2004). A deletion of *CPC2* suppresses the growth phenotype of a *gcn2* mutant under amino acid starvation conditions (Hoffmann *et al.*, 1999), suggesting an association for Cpc2p to the general control of amino acid biosynthesis.

In addition to Cpc2p, also the translation factor Efb1p was identified as significantly up-regulated by a factor of twenty upon 10 h amino acid starvation (Figure 11). No transcriptional regulation was detected by transcriptional profiling of adherent yeast cells under amino acid starvation (Kleinschmidt *et al.*, 2005). The essential protein Efb1 is part of the translation elongation factor eEF1. Efb1p is the GDP-GTP exchange factor that regenerates eEF1-α-GTP for following elongation cycles.

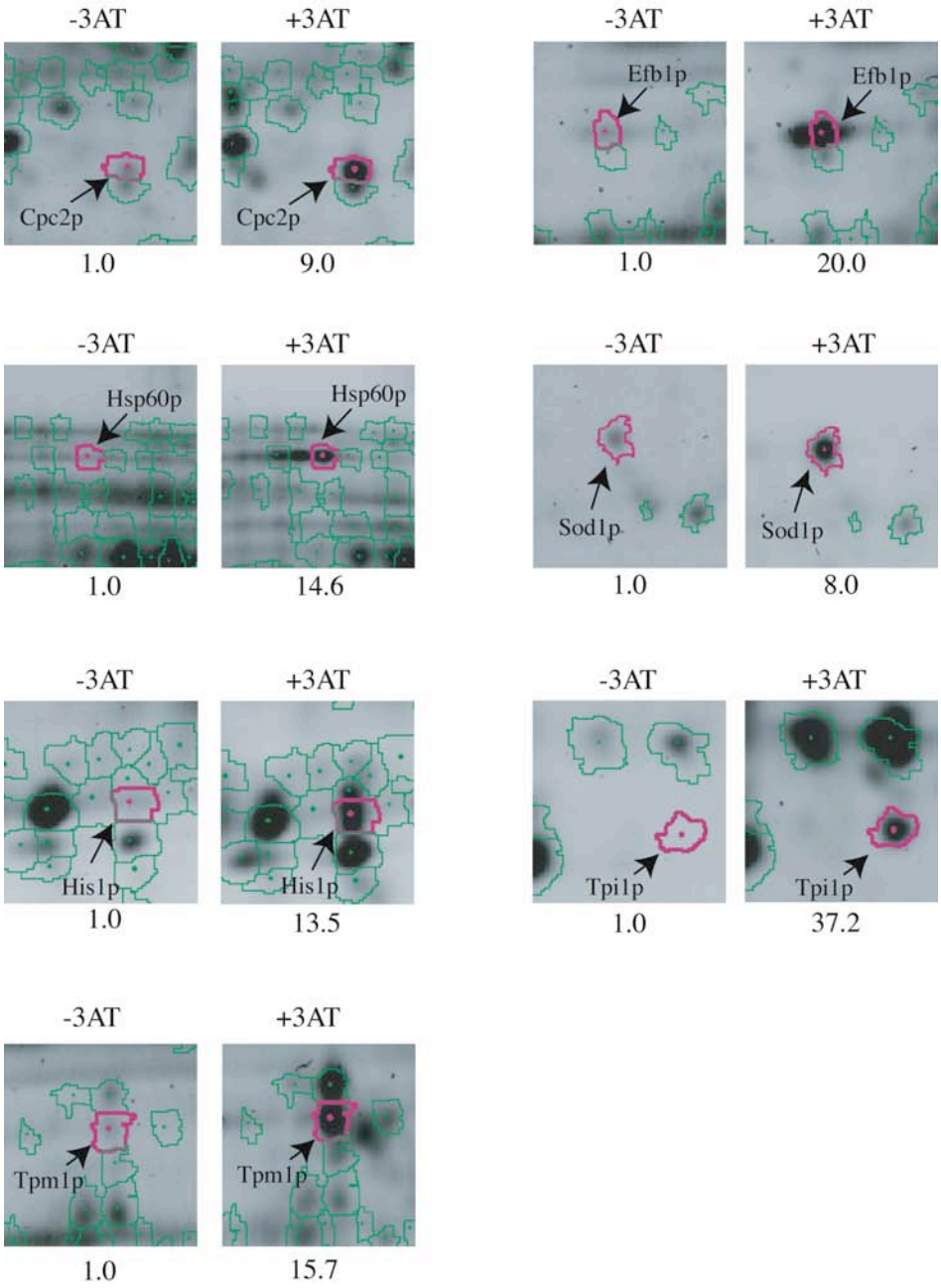

Figure 11: Protein spots with highly increased intensity after 3 amino-triazole (3AT) treatment.
The *S. cerevisiae* Σ1278b strain RH2656 was cultivated in YNB (Ura) medium in absence or in presence of the histidine analogue 3 amino-triazole (3AT) for 10 h at 30° C. Proteins of these cultures were isolated and labelled with Cy3 (-3AT) or with Cy5 (+ 3AT). For normalization a standard pool containing equal amounts of all samples were labelled with Cy2. All labelled samples were used for 2D-DIGE experiments. By running three replicates of each 2D-DIGE experiment at least twelve separate physical gels were generated, each of them representing a gel triplet. Arrows indicate name of the protein spot on a section of a representative 2D-gel. Protein identification was done by nano-LCMS/MS2 of tryptically in-gel digested proteins.

The analysis also determined an up-regulation for two proteins involved in the oxidative stress response of yeast cells. Hsp60p was up-regulated after treatment with 3AT for 10 h by factor 14, whereas the protein spot identified as Sod1p displayed an eight-fold intensity increase (Figure 11). The comparison with the respective transcriptional profiles revealed a two-fold reduced transcription for *HSP60,* and a weak transcriptional induction by a factor of 1.4 for *SOD1* in amino acid-starved and adherent yeast cells (Kleinschmidt *et al*., 2005). In non-adherent S288c cells no significant transcriptional induction was observed upon amino acid limitation (Natarajan *et al*., 2001). The heat shock factor Hsp60p is an essential tetrameric mitochondrial chaperon, which is highly conserved throughout eukaryotes. The protein is required for folding of precursor polypeptides and complex assembly (Cheng *et al.,* 1989; Johnson *et al.,* 1989). It has been described to be involved in the oxidative stress defence mediated by protection of several Fe/S proteins (Cabiscol *et al.,* 2002; Reverter-Branchat *et al.,* 2004). Sod1p is a superoxide dismutase, which protects yeast cells against oxidative stress. Up-regulation of Hsp60p and Sod1p might be the result of a side effect of 3AT, which inhibits in addition to the histidine biosynthesis also the catalase activity, therefore inducing both amino acid starvation and oxidative stress (Ueda *et al.,* 2003).

Also two spots of proteins involved in metabolism, namely His1p and Tpi1p, were identified as highly induced after 3AT treatment (Figure 11). The His1p protein spot was up-regulated by factor 13.5 after 10h of amino acid starvation. Transcription of *HIS1* had previously been described as induced upon amino acid starvation by a factor of about five in non-adherent S288c cells (Natarajan *et al.,* 2001) and by factor 1.6 in adherent Σ1278b cells (Kleinschmidt *et al*., 2005). These data suggest that the increased His1p level in amino acid-starved yeast cells is at least partially a consequence of a transcriptional induction of *HIS1*. His1p catalyzes the first reaction step of the *de novo* biosynthesis of histidine and purines, which is the condensation of ATP and 5-phosphoribosyl-1-pyrophosphate to form N'-5'-phosphoribosyl-ATP (Hinnebusch and Fink, 1983).

The protein spot identified as Tpi1p was up-regulated by a factor of 37 after 10h of amino acid starvation (Figure 11), whereas no transcriptional regulation has been reported for the *TPI* gene in amino acid-starved yeast cells (Kleinschmidt *et al*., 2005; Natarajan *et al*., 2001). Tpi1p is a triosephosphate isomerase that catalyzes the aldose-ketose isomerization of two triose phosphates of the glycolytic pathway (Krietsch, 1975).

Another highly increased protein spot was identified as Tpm1p. After 10h of amino acid starvation Tpm11p showed an increased intensity of a factor 15 (Figure 11). In contrast to this increased protein level, the amount of *TPM1* mRNA has been characterized to be three-fold reduced under conditions of amino acid starvation (Kleinschmidt *et al.*, 2005). Tropomyosin isoform 1, Tpm1p, is the major isoform of tropomyosin, an actin binding protein that stabilizes actin filaments (Liu and Bretscher, 1989). Interestingly, a putative connection between an intact cytoskeleton and the functional and spatial organisation of glycolytic enzymes as Tpi1p was drawn by co-localization studies in mammals and genetic interactions in yeast (Götz *et al.*, 1999)

By means of 2D-DIGE experiments highly increased intensities for seven protein spots after treatment with 3AT were determined. These protein spots were identified as proteins involved in translation, stress response, metabolism or cytoskeleton. The comparison with the respective transcriptome analyses revealed only for *HIS1* a significant transcriptional induction, suggesting a posttranscriptional regulation upon amino acid limitation for the other six identified proteins. One of these protein spots was identified as Cpc2p, a protein that has previously been described to be associated to the general control of amino acid biosynthesis in yeast.

Cpc2p is required for amino acid starvation-induced adhesive growth of yeast cells

Amino acids are essential building blocks of translation. Because protein spots of the translation factors Cpc2p and Efb1p were detected as highly increased and posttranscriptionally induced upon amino acid limitation, we wondered whether a translation factor is required for amino acid starvation-induced adhesive growth.

As a highly conserved Gβ-like WD protein, Cpc2p was shown to influence translation of specific mRNAs (Gerbasi *et al.*, 2004). Interestingly, a deletion of *cpc2* suppresses the growth defect of a *gcn2* mutant under amino acid starvation conditions (Hoffmann *et al.*, 1999). Therefore, we tested the haploid *cpc2* deletion strain (RH3263) and the diploid *cpc2* deletion strain (RH3264) for adhesive growth. In addition to wild-type (*CPC2*) cells, also *flo11* deletion and *sfl1* deletion cells were used as controls for these experiments. The GPI-anchored cell wall adhesin Flo11p is required for cell surface adhesion in amino acid-starved yeast cells (Braus *et al.*, 2003), and therefore *flo11* deletion

cells act as a negative control. *SFL1* encodes a DNA-binding repressor of *FLO11*, and its deletion results in a hyper adhesive phenotype (Robertson and Fink, 1998).

Figure 12 shows that with the exception of the hyper adhesive *sfl1* deletion strains all tested strains were non-adhesive under non-starvation conditions. After starving for amino acids for one day, wild-type strains carrying intact *CPC2* alleles became adhesive. Haploid and diploid *cpc2* deletion cells show no adhesive growth under amino acid limitation comparable with the *flo11* negative control strains.

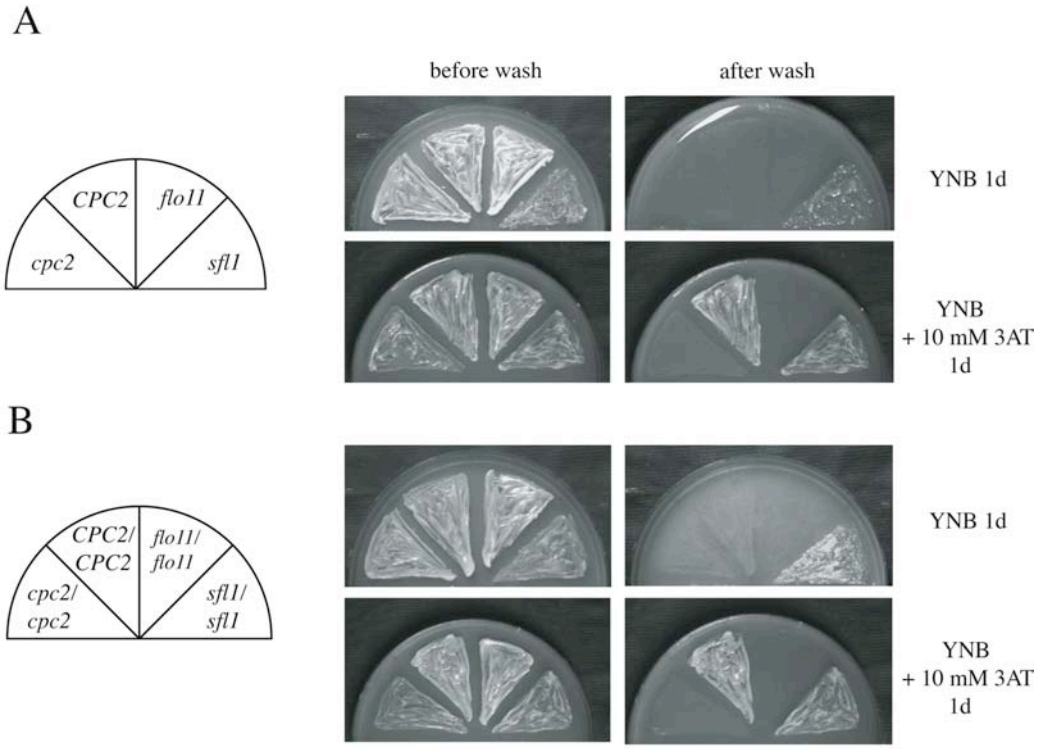

Figure 12: Requirement of *CPC2* for amino acid starvation-induced adhesive growth.
A The haploid Σ1278b yeast strains RH2817 (*CPC2*), RH3220 (*sfl1*), RH2662 (*flo11*) or RH3263 (*cpc2*) and **B** the diploid Σ1278b yeast strains RH2656 (*CPC2/CPC2*), RH3222 (*sfl1*/*sfl1*), RH2661 (*flo11/flo11*) or RH3264 (*cpc2/cpc2*) were patched on solid YNB medium (+ Ura, Trp) or solid YNB (+ Ura, Trp) containing 10 mM 3AT, respectively. After incubation for 1 day at 30°C plates were photographed prior (total growth) and after washing (adhesive growth) by water. Non-adhesive growing cells were washed off the agar surface.

In summary, a Cpc2p protein spot is posttranscriptionally induced upon amino acid starvation. Loss of a functional *CPC2* gene results in an adhesion deficient phenotype in amino acid-starved haploid and diploid yeast cells.

Basal and amino acid starvation-induced transcription of *FLO11* in *S. cerevisiae* depend on *CPC2*

We showed that the ribosome-associated protein Cpc2p is required for amino acid starvation-induced adhesion, and that its protein spot intensity is highly increased upon amino acid starvation. To analyse whether *CPC2* influences the expression of the adhesin encoding gene *FLO11*, Northern hybridization experiments were performed to determine *FLO11* transcript levels under non-starvation and amino acid starvation conditions.

Figure 13A shows a basal amount of *FLO11* mRNAs in haploid wt cells (*CPC2*) in absence of 3AT. After 3AT treatment to induce amino acid starvation, the level of *FLO11* mRNAs increases about a factor of three. In contrast, in haploid *cpc2* deletion cells no *FLO11* transcript was detectable neither in absence nor in presence of 3AT. β-galactosidase activity assays using the *flo11-lacZ* reporter plasmid B3782 (2μm; Rupp *et al.*, 1999) led to similar results to those obtained from Northern hybridization experiments (Figure 13B). For haploid wild-type (*CPC2*) cells a low basal specific β-galactosidase activity was assayed under non-starvation conditions, whereas specific β-galactosidase activity of the *flo11-lacZ* reporter was strongly increased after 3AT treatment. In *cpc2* deletion cells specific β-galactosidase activity is reduced to almost background activity.

The yeast *CPC2* gene encoding a ribosome-associated protein is required for both basal and induced *FLO11* transcription in amino acid-starved yeast cells. The control of *FLO11* transcription by a ribosomal protein might be explained by the regulation of the synthesis of a *FLO11* transcription factor that controls *FLO11* expression.

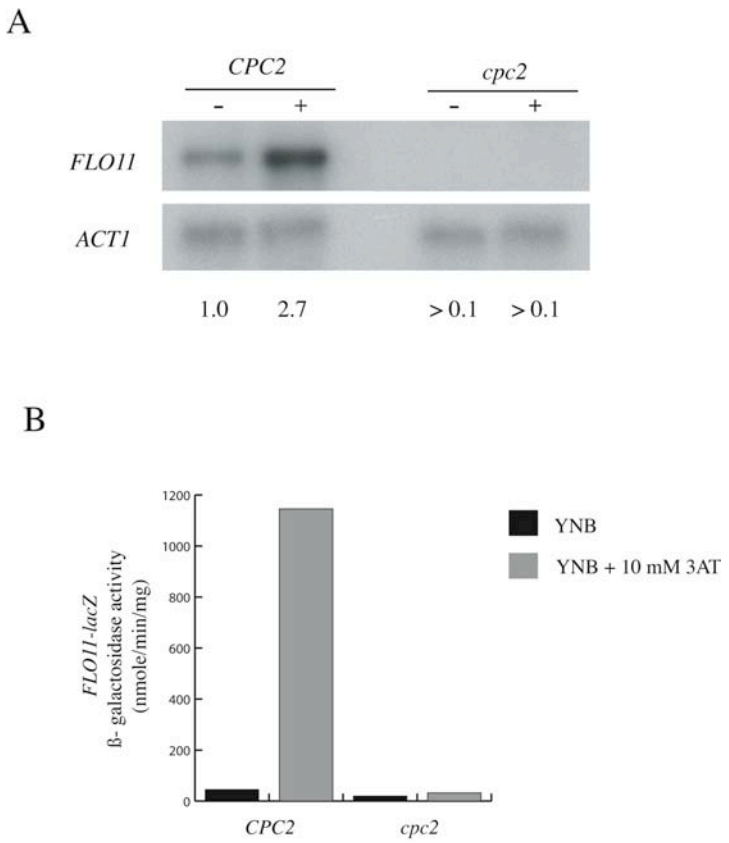

Figure 13: *FLO11* expression depends on *CPC2* in amino acid-starved yeast cells.
A 20 μg of total RNA of the haploid yeast strains RH2817 (*CPC2*) and RH3263 (*cpc2*) cultivated in liquid YNB medium (+ Ura, Trp) in absence (-) or presence (+) of 10 mM 3AT were used for Northern hybridization analysis. Numbers indicate relative transcript levels of *FLO11* to the internal standard *ACT1*.
B The yeast strains RH2817 and RH3263 *(cpc2*) each carrying a *FL011-lacZ* reporter were grown to logarithmic phase in YNB (Trp) in absence (black bars) or in presence (grey bars) of 10 mM 3AT before specific β-galactosidase activities were assayed. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. Bars depict means of at least three independent measurements with a standard deviation not exceeding 20%.

Haploid invasive growth and diploid pseudohyphal development in *S. cerevisiae* require a functional *CPC2* gene

Beside amino acid starvation-induced adhesive growth expression of *FLO11* is also required for other adhesion-dependent growth phenotypes such as haploid invasive growth upon glucose limitation and diploid pseudohyphal development under nitrogen starvation conditions (Lo and Dranginis, 1998). Thereby, *FLO11* transcription is controlled by the

MAP kinase and cAMP signal transduction pathway (Mösch *et al.*, 1999; Rupp *et al.*, 1999). To analyse a putative impact of *CPC2* on these phenotypes, *cpc2* deletion cells were assayed for haploid invasive growth and diploid pseudohyphal development.

For the haploid invasive growth assay, the *cpc2* deletion and control strains were cultivated on YPD plates for five days to induce local glucose limitation and thus invasive growth. The wash test showed that *cpc2* deletion cells failed to grow invasively and were washed away from the agar surface (Figure 14A). We further tested whether overexpression of *TEC1* as downstream transcription factor of the MAP kinase signal transduction pathway can restore invasive growth in *cpc2* deletion cells. Overexpression of *TEC1* had previously been described to strongly induce *FLO11* transcription and thus invasive growth in haploid yeast cells (Köhler *et al.*, 2002). We transformed haploid wild-type and *cpc2* deletion yeast cells with plasmid pME2071 (*GAL1*(p)::*TEC1* fusion in pRS316; Köhler *et al.*, 2002) and cultivated these strains on YNB plates containing 2% galactose for five days. Figure 14B shows that overexpression of *TEC1* induces invasive growth in wild-type haploid yeast cells, but not in haploid *cpc2* deletion cells. These data show that *CPC2* is required for haploid invasive growth induced by glucose limitation or *TEC1* overexpression.

Diploid pseudohyphal development was induced by growth on nitrogen starvation medium (SLAD) for five days. Figure 14C shows that the cell morphology of diploid wild-type cells (*CPC2/CPC2*) changes to elongated cells with polar budding pattern forming filament-like structures after five days on nitrogen starvation medium. In contrast, diploid cells lacking *CPC2* (*cpc2/cpc2*) show a pseudohyphal growth defect (Figure 14C).

Taken together, *FLO11* transcription and different *FLO11*-dependent processes of both haploid and diploid yeast cells require a functional *CPC2* gene.

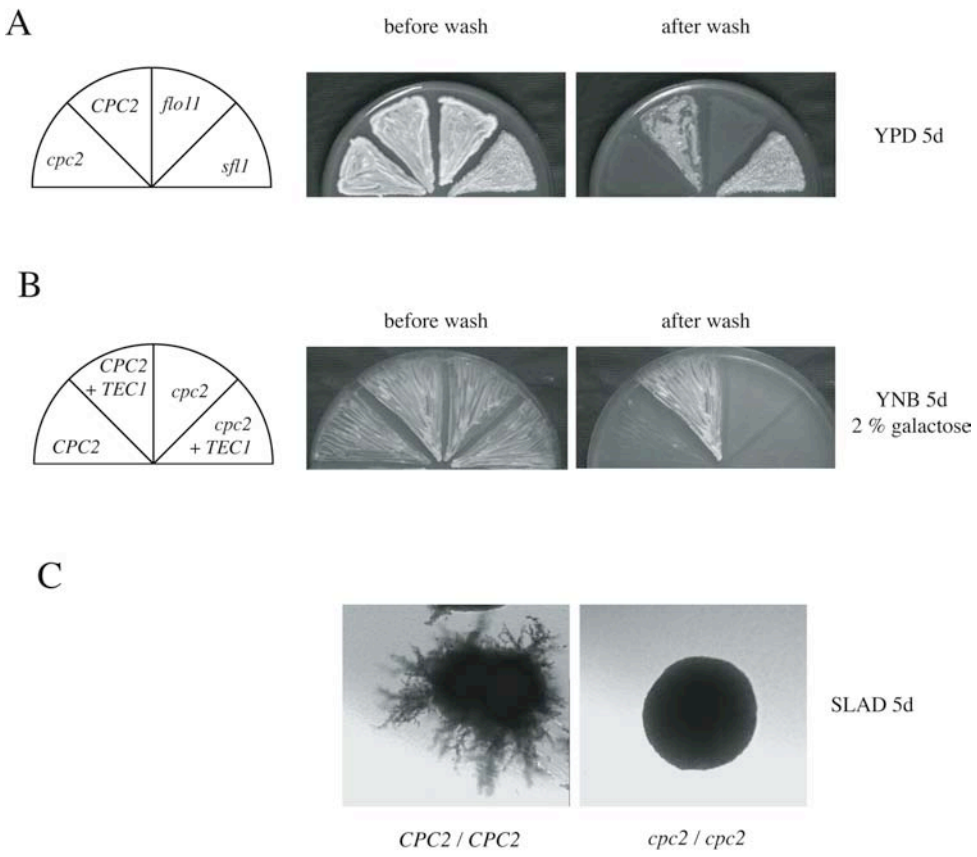

Figure 14: Requirement of *CPC2* for haploid invasive growth and diploid pseudohyphal development.
A The haploid Σ1278b yeast strains RH2817 (*CPC2*), RH3220 (*sfl1*), RH2662 (*flo11*) or RH3263 (*cpc2*) were patched on solid YPD medium for 5 days at 30°C. Cells were photographed before (total growth) and after washing (invasive growth) with water. Non-invasive growing yeast cells were washed off the agar surface. **B** The haploid yeast strains RH2817 (*CPC2*) and RH3263 (*cpc2*) were transformed with plasmids pME2071 (*TEC1*) or pRS316. Strains were cultivated on solid YNB (Trp) medium with 2% galactose (*TEC1*) for 5 days at 30°C. Plates were photographed before (total growth) and after washing (invasive growth) by water. Non-invasive growing cells were washed off the agar surface. **C** The yeast strains RH2656 (*CPC2/CPC2*) and RH3264 (*cpc2*/*cpc2*) carrying plasmids pRS316 or pRS314 were cultivated on nitrogen starvation plates (SLAD) for 5 days at 30°C to stimulate diploid pseudohyphal development.

Discussion

The availability of amino acids as protein building blocks strongly influences gene expression. Transcriptome data revealed that transcription of numerous genes is increased under amino acid starvation conditions (Kleinschmidt *et al.*, 2005; Natarajan *et al.*, 2001). Central element for this transcriptional response is Gcn4p, the transcription factor of the general control of amino acid biosynthesis. Gcn4p activates genes belonging to different functional groups including genes encoding amino acid biosynthetic proteins, vitamin and co-factor biosynthetic enzymes, peroxisomal and mitochondrial proteins, amino acid transporters, transcription factors, and protein kinases and phosphatases (Natarajan *et al.*, 2001). Recent proteome analyses with *S. cerevisiae* determined a correlation coefficient of 0.59 between transcriptome and proteome data of non-adhesive S288c cells upon amino acid starvation (Yin *et al.*, 2004). However, the transcriptional profiles of two *S. cerevisiae* strains with different genetic backgrounds differ significantly as recently shown for the adhesively growing Σ1278b strain (Kleinschmidt *et al.*, 2005). Although transcriptional profiling enables a global view on the mRNA level of each gene under the analyzed growth conditions, it does not reflect the regulatory response as a whole because this also encompasses posttranscriptional regulation mechanisms.

In this work, seeking for highly activated or de-repressed proteins at adhesion-inducing amino acid starvation conditions, only one of seven top-scoring proteins, namely His1p, have been expected beforehand to be significantly up-regulated from the respective transcriptome data (Kleinschmidt *et al.*, 2005; Natarajan *et al.*, 2001). In contrast, the transcriptome data did not suggest that highly up-regulated protein spots identified as components of the translation machinery (Cpc2p, Efb1p), of stress response (Hsp60p, Sod1p), of glycolysis (Tpi1p) and of the cytoskeleton (Tpm1p), were induced (Kleinschmidt *et al.*, 2005; Natarajan *et al.*, 2001). To decipher the dynamic interactions between components acting on different levels of gene regulation, and to consider the specific nature of any global experimental readout will be a challenging prerequisite for the computational modelling of cellular processes as it is aimed by recent system biology projects (Wolkenhauer *et al.*, 2005).

Posttranscriptional up-regulation of Sod1p and the chaperon Hsp60 might be required for coping with cellular oxidative stress caused as a side effect of 3AT and its inhibitory effect on catalase activity. Detoxification and chaperon activity are cell-fate

determining aspects, which have to be ensured also during conditions of an overall reduced mRNA translation, and therefore, are possibly provided by alternative regulation mechanisms on the level of translation. Also activation of the glycolytic enzyme Tpi1p was previously shown to be the result of cellular stress (Rodriguez-Vargas *et al.*, 2002).

Tropomyosin isoform 1, Tpm1p, an actin binding protein that stabilizes actin filaments has been reported to be essential for polar growth forms such as pseudohyphal formation upon nutrient limitation (Kang and Jiang, 2005; Mösch and Fink, 1997). Tpm1p activation or de-repression in amino acid-starved yeast cells might be required for polarity and cell wall changes, which probably also depend on a reorganized actin cytoskeleton structure.

Amino acid starvation is an efficient signal for yeast cells to grow adhesively. This phenotype requires Cpc2p. In addition, expression of the adhesin encoding gene *FLO11*, responsible for adhesion of Σ1278b cells, strictly depends on a functional *CPC2* gene. Due to the fact that Cpc2p is a ribosome-associated Gβ-like WD protein (Chantrel *et al.*, 1998; Hoffmann, *et al*., 1999), which has recently been reported to influence translation of specific mRNAs (Gerbasi *et al*., 2004), it is surprising that in absence of Cpc2p no *FLO11* mRNA is detectable. In contrast to bacteria, where the progression of transcription is spatially and mechanistically linked to the process of mRNA translation, such coupled mechanisms, like e.g. attenuation, are not practical for eukaryotes where both processes are spatially and mechanistically separated. However, more indirectly, the translation efficiency of mRNAs encoding transcription factors can determine the transcription rates of target genes like it is known for the transcription factor Gcn4p of the general control of amino acid biosynthesis itself (Hinnebusch and Natarajan, 2002).

The Cpc2p homologue in mammals RACK1 physically interacts with signal transduction proteins including activated kinase C and Src (Chang *et al.*, 2002; Ron *et al.*, 1994). It is supposed that RACK1 acts as a versatile protein platform for signal transduction pathways, finally affecting the translation of certain groups of mRNAs (Nilsson *et al.*, 2004). In this study we show that yeast cells without Cpc2p do not express the *FLO11* gene, thereby preventing cellular differentiation processes such as diploid pseudohyphal growth or invasive/adhesive growth upon signals such as nitrogen limitation, glucose limitation, and amino acid limitation respectively. Similar to the role of RACK1 in mammals, namely to regulate processes that involve contact with the extra-cellular matrix such as cell spreading, the establishment of focal adhesions and cell-cell contact

(Hermanto *et al.*, 2002; McCahill *et al.*, 2002; Nilsson *et al.*, 2004), also in yeast Cpc2p is required for extra-cellular cell-cell or cell-environment interactions that depend on *FLO11* expression. The impact of Cpc2p on cellular differentiation processes was also shown for its homologous proteins in *Aspergillus nidulans* and *Schizosaccharomyces pombe* (Hoffmann *et al.*, 2000; Jeong *et al.*, 2004).

By function, Cpc2p might be required for the appropriate synthesis of transcription factor(s) required for activation of *FLO11* transcription. Either the translation rate of certain transcription factor mRNAs or an important processing step of its nascent polypeptide/protein might account for a possible *FLO11* transcription factor deficiency. Overexpression of the *FLO11* transcription factor Tec1p from the *GAL1-10* promoter does not restore *FLO11* transcription, underlying a fundamental and putative specific role of Cpc2p for *FLO11* expression. Assuming a possible role as a platform protein within signal transduction pathways, Cpc2p might be necessary for phosphorylation steps within cascades that finally lead to active transcription factors of *FLO11*.

In contrast to this observed repression of *FLO11*, deletion of *CPC2* generally results in the de-repression of a variety of proteins, and purified Cpc2p-deficient ribosomes have been characterized to have an increased translational activity *in vivo* compared to wild-type ribosomes (Gerbasi *et al.*, 2004). The proposed function of Cpc2p as repressor of gene expression might only reflect a part of the whole picture, as we have shown here that it is also essential for *FLO11* de-repression. However, de-repression of a *FLO11* specific repressor protein in *cpc2* deletion cells might also account for *FLO11* repression.

A further theoretical explanation for the absence of *FLO11* transcripts in *cpc2* deletion cells might be a rapid decay of *FLO11* mRNA as a consequence of a possibly prevented translation. Correlations between the translation rate and mRNA stability have occasionally been drawn. Inhibition of translation of *PGK1* mRNAs by mutation of either the 5'UTR or the region surrounding the initiation codon accelerates its deadenylation and decay (LaGrandeur and Parker, 1999; Muhlrad *et al.*, 1995). mRNA deadenylation and decay has also been shown to be favored by mutations in translation initiation factors (Schwartz and Parker, 1999). The translation factor 1A (eEF1A) and the RNA-binding protein Scp160p are located in close proximity to Cpc2p at the ribosome (Baum *et al.*, 2004). The C-terminus of Scp160p is essential for ribosome binding, and this interaction depends on Cpc2p. It has been suggested that Scp160p connects specific mRNAs, ribosomes, and a translation factor with an adaptor for signalling molecules (Baum *et al.*,

2004). These interactions might regulate the translation activity of ribosomes programmed with specific mRNAs like the *FLO11* mRNA. Loss of such interactions by deletion of *CPC2* might result in abolished translation and rapid mRNA decay.

Taken together, our study demonstrates that amino acid starvation initiates significant proteome changes resulting in severely increased spot intensities for proteins whose mRNA levels are not elevated under these growth conditions. One of those proteins is Cpc2p which we found is indispensable for *FLO11* expression, and therefore, for *FLO11*-dependent differential processes such as adhesive growth and pseudohyphal development.

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Chapter IV

The yeast *CPC2/ASC1* gene is regulated by the transcription factors Fhl1p and Ifh1p

Abstract

CPC2/ASC1 is one of the most abundantly transcribed genes in *Saccharomyces cerevisiae*. It encodes a ribosome-associated Gβ-like WD protein, which is highly conserved from yeast to man. Here, we show that *CPC2* transcription depends on the carbon source and is induced during utilization of the fermentable sugar glucose. *CPC2* promoter deletion and insertion analyses identified two upstream activation sequence elements for *CPC2*, which are required for basal expression and regulation. One of these upstream activation sequence elements has an ATGTACGATGT motif, which has previously been described as a putative binding site for the forkhead-like transcription factor Fhl1p. Deletion of *FHL1* reduces *CPC2* transcription significantly in presence of glucose, but has no effect when the non-fermentable carbon source ethanol is provided. Increased amounts of the Fhl1p co-regulator Ifh1p induce *CPC2* transcription even when ethanol is utilized. These data suggest that the interaction between Fhl1p and Ifh1p is critical for the regulation of *CPC2* transcription during utilization of different carbon sources.

Introduction

Ribosome biogenesis is a major consumer of cell energy, and therefore, tightly controlled in response to the environmental conditions and the physiological state of the cell. The number of ribosomes is adjusted to the overall protein synthetic capacity of the cell and requires a co-ordinate regulation of all ribosome components.

In *Saccharomyces cerevisiae* transcription of 137 ribosomal protein genes is co-ordinately regulated under different environmental growth conditions (Warner, 1999). This co-regulation is controlled by the conserved RAS/protein kinase A, TOR and protein kinase C signalling pathways as response to the growth conditions (Warner, 1999). During the nutritional up-shift from non-fermentable carbon sources as ethanol to fermentable carbon sources as glucose transcription of ribosomal protein genes is strongly induced (Herruer *et al.*, 1987; Kief and Warner, 1981; Kraakman *et al.*, 1993). In contrast, several environmental insults such as heat shock, amino acid starvation, nitrogen limitation or osmotic stress lead to down-regulation of ribosomal protein genes (Causton *et al.*, 2001; Gasch *et al.*, 2000; Natarajan *et al.*, 2001).

The *CPC2* (also known as *ASC1*) gene of *S. cerevisiae* encodes a highly conserved Gβ-like WD protein of 319 amino acids (Chantrel *et al.*, 1998; Hoffmann *et al.*, 1999) and is a homologue of the *cpc-2* gene of *Neurospora crassa* (Müller *et al.*, 1995), *CPC2* of *Schizosaccharomyces pombe* (McLeod *et al.*, 2000) and of the human RACK1 encoding a receptor of activated protein kinase C (Ron *et al.*, 1994), respectively. The open reading frame of *CPC2* is interrupted by an intron between the corresponding amino acids 179 and 180. This intron is located close to the 3'-end, which is unusual in comparison to other introns in yeast. It has been characterized as containing the U24 small nucleolar RNA (*SNR24*) coding region, which is required for site-specific 2'-*o*-methylation of rRNA (Kiss-Laszlo *et al.*, 1996; Qu *et al.*, 1995). Genome-wide expression analyses described the *CPC2* open reading frame as a highly transcribed gene (Velculescu *et al.*, 1997), finally resulting in an estimated 330 000 Cpc2p molecules per cell (Ghaemmaghami *et al.*, 2003). This high transcription rate was also observed when cells were starved for amino acids (Hoffmann *et al.*, 1999).

Polysome profile analyses and mass-spectrometry identified an association of Cpc2p to the 40S-ribosomal subunit (Chantrel *et al.*, 1998; Gerbasi *et al.*, 2004; Link *et al.*, 1999). Thereby, Cpc2p seems to be present at a 1:1 ratio with ribosomal proteins. In

addition to this ribosome bound form, a non-ribosome bound form of Cpc2p was identified (Baum *et al.*, 2004). In cells in a stationary growth phase, the non-ribosome bound form of Cpc2p seems to increase suggesting that association of Cpc2p to the 40S-ribosomes probably depends on the yeast cell activity. Recent experiments showed an increased translation efficiency of specific mRNAs in the absence of Cpc2p. These data suggest a regulatory role for Cpc2p in translation (Gerbasi *et al.*, 2004). A possible role of Cpc2p in translation has also been suggested for the corresponding proteins Cpc2 in *Schizosaccharomyces pombe* (Shor *et al.*, 2003) and the human RACK1 (Ceci *et al.*, 2003). In human cells, RACK1 acts as signalling platform for several signalling molecules and recruits activated protein kinase C to the ribosome to regulate translation in response to stimuli (Ceci *et al.*, 2003; Nilsson *et al.*, 2004).

Genome-wide chromatin immunoprecipitation (ChIP) analyses revealed that the forkhead-like transcription factor Fhl1p binds to nearly all promoters of yeast ribosomal protein genes (Lee *et al.*, 2002). Fhl1p was original identified as a multicopy suppressor of RNA polymerase III mutations (Hermann-Le Denmat *et al.*, 1994). It has a domain similar to the forkhead DNA-binding domain found in the developmental forkhead protein of *Drosophila melanogaster* and in the HNF-3 family of hepatocyte mammalian transcription factors. Loss of *FHL1* results in a slow growth phenotype and in a defect in 35S-rRNA processing. Overexpression of the essential gene *IFH1* (interacting with forkhead) suppresses the slow growth phenotype of *fhl1* deletion yeast cells (Cherel and Thuriaux, 1995). Recent studies found that both Fhl1p and Ifh1p are required for a tight co-regulation of ribosomal protein genes (Lee *et al.*, 2002; Martin *et al.*, 2004; Rudra *et al.*, 2005; Schawalder *et al.*, 2004; Wade *et al.*, 2004). It was shown that Ifh1p is recruited through the forkhead-associated domain of Fhl1p to ribosomal protein gene promoters to induce transcription. The level of Ifh1p association to ribosomal gene promoters depends on environmental conditions and thus it is one key regulatory step for expression of ribosomal protein genes. Beside ribosomal protein genes, also translation factors or other ribosome-associated proteins require a distinct regulation in response to environmental stimuli to ensure an efficient translation of mRNAs.

In this work we show that transcription of *CPC2* is stimulated by the fermentable carbon source glucose. The two key regulators of ribosomal proteins Fh1p and Ifh1p are involved in regulation of this transcriptional induction. These data suggest that *CPC2* and

ribosomal protein genes are co-ordinately regulated under utilization of different carbon sources.

Materials and Methods

Yeast strains and growth conditions

Saccharomyces cerevisiae strains used in this work are listed in Table 8 and are derivates of S288c. For construction of yeast strains RH3094 to RH3101 and RH3269 to RH3274, each carrying a single integration of a *CYC1-lacZ* reporter, plasmids pME2598 to pME2605 were linearized by *Stu*I and chromosomally integrated at the *URA3* locus of RH1168, YPH500 or MW667, respectively. Yeast strains RH3102, RH3261 and RH3262 carrying single *CPC2-lacZ::URA3* reporter cassettes, were constructed by integration of plasmid pME2614 at the *URA3* locus. Yeast strains RH3103 to RH13111 and RH3259 carrying individual *cpc2-lacZ* deletion constructs were obtained by *Stu*I digestion and chromosomal integration of plasmids pME2615 to pME2623 and pME2931 at the *URA3* locus. The yeast strain RH3260 (*CPC2*-GFP) was created by PCR-based C-terminal tagging of chromosomal *CPC2* ORF (Knop *et al.*, 1999). Transformations were carried out using the lithium-acetate yeast transformation method (Ito *et al.*, 1983). All integrations were confirmed by Southern hybridization analysis (Ausubel *et al.*, 1993). For measurement of *CPC2* transcript expression, yeast strains were cultivated at 30°C in liquid synthetic minimal medium (YNB) containing appropriate supplements, diluted into fresh YNB medium with 2% glucose or 2% ethanol as a carbon source and cultivated for 4 h before assaying enzymatic activities, isolation of total RNAs and fluorescence microscopy, respectively. Functionality of the *CPC2*-GFP fusion construct was tested at 15°C on YEPD medium with 2% agar.

Plasmids

All constructed plasmids are listed in Table 9. Plasmids pME2598 to pME2605 were obtained by amplification and integration of individual 75 to 150 base pairs of *CPC2* promoter fragments into pLG669Z (Guarente and Ptashne, 1981) using a *Xho*I restriction site introduced at the 5'-end of the PCR primers. For construction of *cpc2-lacZ* reporter plasmids pME2614 to pME2623 and pME2931, 600 base pairs upstream of the *CPC2* translation start site were amplified and cloned as a *Xho*I fragment into pBluescript II SK+/-. A deletion set of the *CPC2* promoter was generated by whole vector PCR excluding individual *CPC2* promoter sequences of 10, 75 or 150 base pairs. After religation by using an introduced *Bgl*II restriction site, individual *cpc2-lacZ* deletion

constructs were amplified and cloned into YIp356R (Myers *et al.*, 1986) using the *Bam*HI/*Kpn*I restriction sites introduced at the 5'-end of PCR primers. The plasmid pME2932 was constructed by amplification of the *IFH1* open reading frame (ORF) using primers containing *Spe*I/*Xho*I restriction sites at the 5'-end and integration into pRS425prom. (Mumberg *et al.*, 1994).

Table 8: *S. cerevisiae* strains used in this work.

Strain	Genotype	Reference
RH1168	*MATa, ura3-52, leu2-3*	Our collection
RH2419	*MATa, ura3-52, leu2-3, trp1, cpc2::LEU2*	(Hoffmann *et al.*, 1999)
RH3094 to RH3100	*MATa, ura3-52, leu2-3, URA3::*p*CPC2-CYC1-lacZ*	This work
RH3101	*MATa, ura3-52, leu2-3, URA3::CYC1-lacZ*	This work
RH3102	*MATa, ura3-52, leu2-3, URA3::CPC2-lacZ*	This work
RH3103 to RH3111	*MATa, ura3-52, leu2-3, URA3::*p*cpc2-lacZ*	This work
RH3112	*MATa, ura3-52, leu2-3, URA3::lacZ*	This work
RH3259	*MATa, ura3-52, leu2-3, URA3::*p*cpc2-lacZ*	This work
RH3260	*MATa, ura3-52, leu2-3, CPC2-GFP::KanR*	This work
YPH500	*MATα, ura3-52, leu2-1, ade2-101, trp1-Δ1, lys2-801, his3-Δ200*	(Sikorski and Hieter, 1989)
MW667	*MATα, ura3-52, ade2-101, trp1-Δ1, lys2-801, his3-Δ200, fhl1-Δ1::HIS3*	(Hermann-Le Denmat *et al.*, 1994)
RH3261	*MATα, ura3-52, ade2-101, trp1-Δ1, lys2-801, his3-Δ200, fhl1-Δ1::HIS3, fhl1::HIS3, URA3::CPC2-lacZ*	This work
RH3262	*MATα, ura3-52, leu2-1, ade2-101, trp1-Δ1, lys2-801, his3-Δ200, URA3::CPC2-lacZ*	This work
RH3269	*MATα, ura3-52, leu2-1, ade2-101, trp1-Δ1, lys2-801, his3-Δ200 URA3::pCPC2*$_{-375/-225}$*- CYC1-lacZ*	This work
RH3270	*MATα, ura3-52, leu2-1, ade2-101, trp1-Δ1, lys2-801, his3-Δ200 URA3::pCPC2*$_{-300/-150}$*- CYC1-lacZ*	This work
RH3271	*MATα, ura3-52, leu2-1, ade2-101, trp1-Δ1, lys2-801, his3-Δ200, URA3:: CYC1-lacZ*	This work
RH3272	*MATα, ura3-52, ade2-101, trp1-Δ1, lys2-801, his3-Δ200, fhl1-Δ1::HIS3, URA3::pCPC2*$_{-375/-225}$*- CYC1-lacZ*	This work
RH3273	*MATα, ura3-52, ade2-101, trp1-Δ1, lys2-801, his3-Δ200, fhl1-Δ1::HIS3, URA3::pCPC2*$_{-300/-150}$*- CYC1-lacZ*	This work
RH3274	*MATα, ura3-52, ade2-101, trp1-Δ1, lys2-801, his3-Δ200, fhl1-Δ1::HIS3, URA3::CYC1-lacZ*	This work

Table 9: Plasmids used in this work.

Plasmid	Description	Reference
pLI4	10 kb vector, *CYC1-lacZ, URA3, bla*	Guarente and Ptashne, 1981
pME2598 to pME2604	75 or 150 bp *CPC2* promoter sequence elements cloned into pLI4	This work
YIp356R	6.9 kb vector, MCS, *lacZ, URA3, bla*	Myers *et al*., 1986
pME2614	600 bp *CPC2* promoter fragment in YIp356R	This work
pME2615 to pME2623	75 or 150 bp *CPC2* promoter deletions in pME2614	This work
pME2931	10 bp *CPC2* promoter deletion in pME2614	This work
pRS425prom.	6.4 kb vector, *MET25*prom, MCS, *CYC1*term, *LEU2*, *bla*, 2μm	Mumberg *et al*., 1994
pME2932	*IFH1* ORF cloned into pRS425prom.	This work

Northern hybridization analysis

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

β-galactosidase assay

Assays were performed with yeast extracts derived from cultures grown in liquid medium. Specific β-galactosidase activities were normalized to total protein contents (Bradford, 1976) in each extract and equalized (OD_{415} x 1.7)/(0.0045 x protein concentration x extract volume x time) (Rose and Botstein, 1983)

GFP fluorescence microscopy

The *S. cerevisae* strain RH3260 was grown in liquid synthetic minimal medium (YNB + Ura, Leu) with either 2% glucose or 2% ethanol as a carbon source. 1 ml cells of the cultures were harvested by centrifugation and immediately viewed *in vivo* on a Zeiss Axiovert microscope by either differential interference contrast microscopy (DIC) or fluorescence microscopy using a GFP filter set (AHF Analysentechnik AG, Tübingen, Germany). Cells were photographed using a Xillix Microimager digital camera and the Improvision Openlab software (Improvision, Coventry, UK).

Results

The *CPC2* expression is two-fold increased when glucose as fermentable carbon source is used instead of ethanol as non-fermentable carbon source

The *CPC2* gene of *S. cerevisiae* encodes a highly conserved 40S-ribosomal subunit-associated protein. Expression analyses of the whole yeast genome revealed for *CPC2* a high transcription rate (Velculescu *et al.*, 1997), finally resulting in an estimated 330 000 Cpc2p molecules per cell (Ghaemmaghami *et al.*, 2003). In spite of this high expression, *CPC2* is not essential under the analysed conditions. Therefore we asked whether this gene is constitutively expressed under every condition or distinctly regulated as it has been described for ribosomal protein genes. For instance, expression of ribosomal protein genes is co-ordinately induced during the switch from the non-fermentable carbon source ethanol to the fermentable carbon source glucose (Herruer *et al.*, 1987; Kief and Warner, 1981; Kraakman *et al.*, 1993).

Northern hybridization experiments were performed to analyse the amount of *CPC2* mRNA in cells when growing with glucose or with ethanol as carbon source. In addition, the transcript levels of the *RPS26A/B* isogenes, which encode both ribosomal proteins of the 40S-ribosomal subunit, were determined to compare transcription of *CPC2* and ribosomal protein genes under these conditions. Figure 15A shows that transcription of *CPC2* is induced by a factor of two under utilization of the fermentable carbon source glucose when compared to the non-fermentable carbon source ethanol. The mRNA level of the *RPS26A/B* isogenes is 2.5-fold induced in presence of glucose (Figure 15A), which suggests a co-regulation of *CPC2* and ribosomal protein genes during utilization of the fermentable carbon source glucose.

Specific β-galactosidase activity assays with the *CPC2–lacZ* reporter strain RH3102 were performed to verify glucose-dependent induction. As shown for the *CPC2* mRNA, specific β-galactosidase activity of the *CPC2-lacZ* reporter increases by a factor of about two in presence of the fermentable carbon source glucose (Figure 15B). These data confirm that *CPC2* is predominantly induced on a transcriptional level in response to the fermentable carbon source glucose.

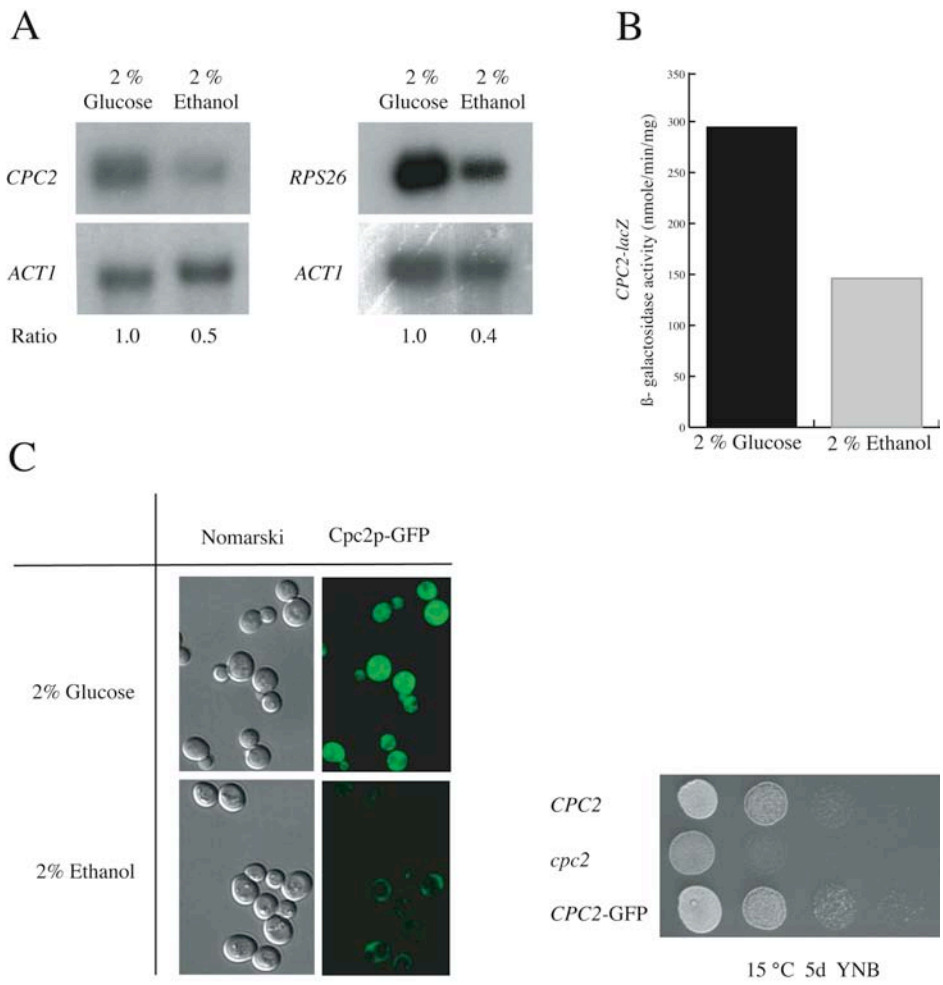

Figure 15: *CPC2* expression in *S. cerevisiae* under utilization of fermentable glucose or non-fermentable ethanol as carbon source.
The yeast strains RH1168 (*CPC2*), RH3102 (*CPC2*; *URA3*::*CPC2-lacZ*) or RH3260 (*CPC2*-GFP) were cultivated on YNB medium to mid-log phase at 30°C. Cells were sedimented and shifted on YNB medium with 2% glucose or with 2% ethanol for 4 h at 30°C, respectively. **A** 20 µg of total RNA of RH1168 (wt) under glucose or ethanol conditions were used for northern hybridization analyses. Numbers indicate relative transcript levels of *CPC2* or *RPS26* in comparison to the internal standard *ACT1*. **B** Expression of the *CPC2-lacZ* reporter construct in the yeast strain RH3102 (*CPC2–lacZ*) was measured after cultivation in glucose (black bars) or ethanol (grey bars). Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. Bars depict means of at least three independent measurements with a standard deviation not exceeding 20%. **C** Functional Cpc2-GFP fusion protein was visualized under glucose or ethanol conditions by fluorescence microscopy (left). Functionality of the Cpc2-GFP fusion protein was verified by comparing the low temperature growth defect of RH2419 (*cpc2*) to the yeast strains RH1168 (*CPC2*) or RH3260 (*CPC2*-GFP) on YPD plates at 15°C for 5 days (right).

The GFP open reading frame was chromosomally fused to *CPC2* to localize Cpc2p *in vivo*. The functionality of the Cpc2p-GFP fusion protein was verified by comparison of the growth of RH1168 (*CPC2*), RH2419 (*cpc2*) or RH3260 (*CPC2*-GFP) at 15°C for five days (Figure 15C). Cells of the *cpc2* deletion strain RH2419 (*cpc2*) produce a cold sensitivity growth phenotype, whereas cells expressing the Cpc2-GFP fusion protein grow similarly as cells with the wild-type *CPC2* allele. Fluorescence microscopy studies show the cytoplasmatic localization and strong expression of the Cpc2p-GFP fusion protein under glucose conditions (Figure 15C). Growth in ethanol results in weak Cpc2p-GFP expression with the same localization in the cytoplasm.

These data show that expression of *CPC2* depends on the carbon source. Utilization of a fermentable sugar as glucose induces *CPC2* transcription, which is presumably reflected by an increased amount of Cpc2 protein in the yeast cell.

The yeast *CPC2* promoter comprises two UAS elements, which are required for a distinct transcriptional regulation

A *CPC2* promoter deletion set was cloned in front of a *lacZ* reporter gene to analyse whether the difference in *CPC2* expression between cells utilizing glucose or ethanol depends on a distinct transcriptional regulation. The set of the *cpc2–lacZ* promoter deletion constructs spans 600 base pairs upstream of the *CPC2* initiation codon. All constructs have deletions of 75 or 150 base pairs in the *CPC2* promoter and overlap at a length of 75 base pairs (Figure 16A). The *CPC2-lacZ* fusions carrying promoter deletions were chromosomally integrated at the *URA3* locus to ensure a single copy per cell. A deletion was defined to contain an upstream activation sequence (UAS) when *CPC2* expression was reduced by a factor of two and more. An upstream repression sequence (URS) corresponds to at least two-fold enhanced expression.

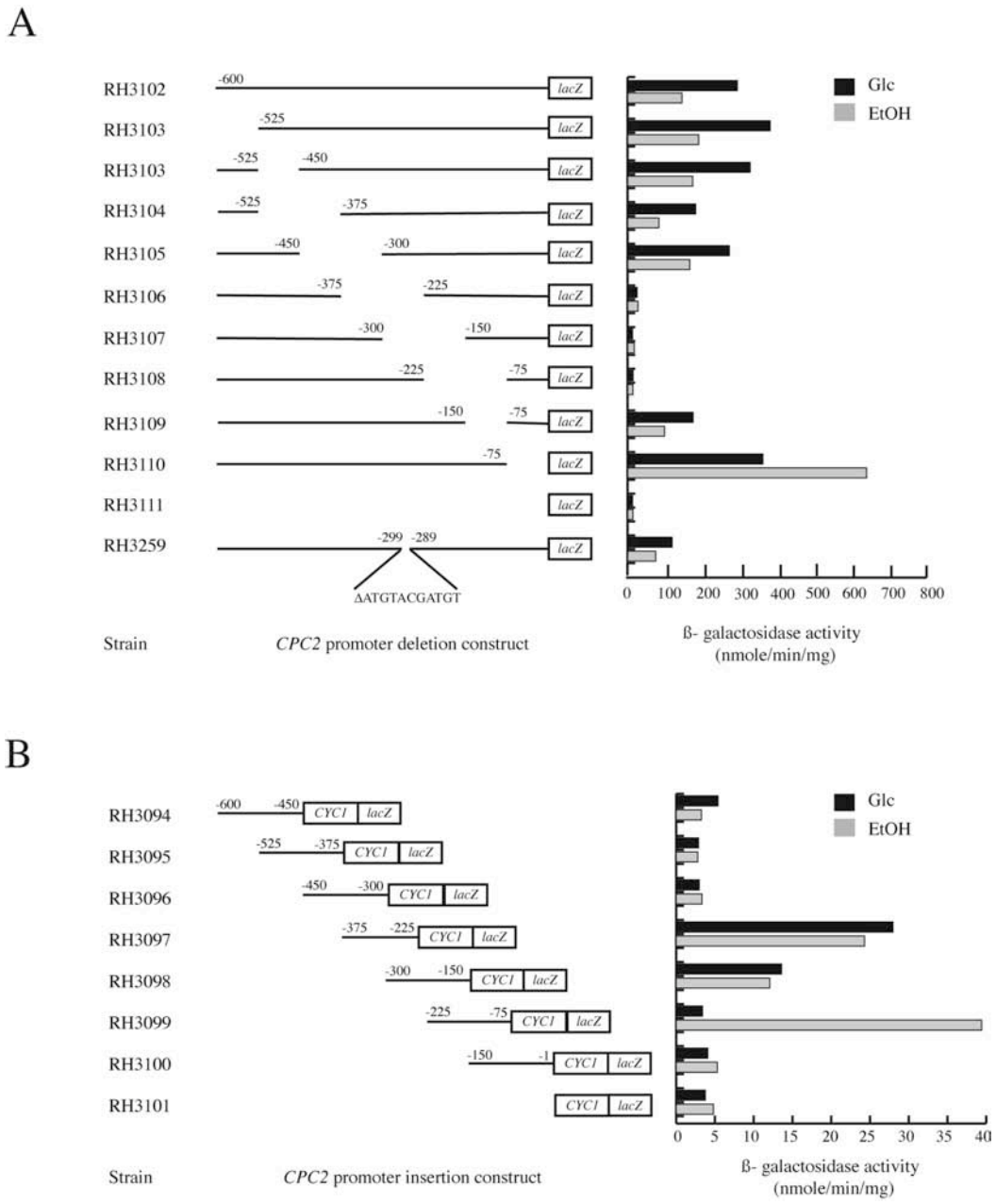

Figure 16: Expression of a set of deletion or insertion *CPC2-lacZ* reporter constructs in yeast grown under glucose and ethanol conditions.
A Specific β-galactosidase activities were determined in yeast strains RH3102 to RH3110 and RH3259 grown under glucose (black bars) and ethanol (grey bars) conditions, respectively. Each yeast strain carries an individual p*cpc2-lacZ* promoter deletion construct in addition to a *CPC2* wild-type gene. Deleted segments in the individual constructs are numbered with respect to the translational start site of *CPC2* at position +1. Yeast strain RH3111 served as negative control carrying no *CPC2* promoter element upstream of the *lacZ* reporter. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. Bars depict means of at least three independent measurements with a standard deviation not exceeding 20%. **B** Expression of individual p*CPC2-CYC1-lacZ* insertion constructs in the yeast strains RH3094 to RH3100 was measured under glucose

(black bars) or ethanol (grey bars) conditions, respectively. Boundaries of insertion are indicated with respect to the translational start site of *CPC2* at position +1. RH3101 was used as negative control strain carrying no insert upstream of the basal *CYC1* promoter of the *CYC1-lacZ* fusion reporter. Bars indicate means of specific β-galactosidase activities in actual units nanomoles per minute per milligram of least three independent measurements. Standard deviations did not exceed 20%.

Figure 16A shows that deletions of the regions -600 to -300 relative to the ATG start codon did not significantly affect specific β-galactosidase activities under glucose or ethanol conditions. This suggests that the *CPC2* promoter is localized in the region -300 to -1. Deletions of the regions -300 to -225 and -225 to -150 reduced the specific β-galactosidase activity to approximate 10% of the full-length *CPC2* promoter activity and abolished the carbon source-dependent *CPC2* regulation. Therefore the region -300 to -150 is required for basal *CPC2* expression and regulation. At least two UAS elements seem to be present in the *CPC2* promoter. Deletion of the region -150 to -75 had a weak influence on basal activity, but did not affect regulation. A deletion of the first 75 base pairs upstream of the *CPC2* translational start site increased the specific β-galactosidase activity by a factor of four when ethanol was utilized (Figure 16A). This data suggest a putative URS element within the *CPC2* promoter for the ethanol-dependent expression.

The β-galactosidase assays of the *CPC2* promoter deletion constructs fused to *lacZ* showed that the *CPC2* promoter comprises the first 300 base pairs upstream of the translational start site. Within this promoter two regions including UAS elements are localized at the position -300 to -225 (UAS_1) and -225 to -150 (UAS_2). Both UAS containing regions elements are necessary for basal *CPC2* expression and in addition for regulation of the promoter.

The *CPC2* promoter -300 to -150 region stimulates activity of the basal yeast *CYC1* promoter

We tested whether single fragments of the *CPC2* promoter are sufficient to stimulate or to repress activity of a basal yeast promoter. *CPC2* promoter fragments of a size of 150 base pairs that overlap by 75 base pairs were cloned upstream of the basal *CYC1* promoter, which was fused to the *lacZ* reporter gene (Figure 16B). All constructs were chromosomally integrated at the *URA3* locus. As shown for the *CPC2* promoter deletion

set, insertions carrying fragments of the region -600 to -300 have no effect on the expression of the *CYC1-lacZ* reporter. These data confirm our observation that the *CPC2* promoter ranges from -300 to -1. Fragments carrying the assumed two UAS elements (-375 to -225, -300 to -150 and -225 to -75) are able to stimulate expression of the *CYC1-lacZ* fusion gene, which support the existence of at least two UAS in the *CPC2* promoter region (Figure 16B). Interestingly, insertion of the *CPC2* promoter region -225 to -75 stimulates only the ethanol-dependent expression suggesting a specific role for this UAS element under ethanol conditions. (Figure 16B). The fragment carrying the putative URS has no effect on the activity of the basal *CYC1* promoter (Figure 16B).

Measurements of the *CPC2* promoter insertion constructs confirmed the existence of at least two UAS elements for the *CPC2* promoter. The existence of an ethanol-dependent URS element could not be confirmed. The UAS_2 element of the region -225 to -150 seems to be specific for an efficient ethanol-dependent expression.

Transcriptional induction of *CPC2* depends on the fork head transcription factor Fhl1p.

Sequence analysis revealed within the UAS_1 region ranging from -300 to -225 a conserved sequence motif at position -299 to -289. This sequence motif consists of the nucleotides ATGTACGATGT and was proposed in a genome sequence comparison of different *Saccharomyces* species as a putative binding site for the transcription factor Fhl1p (Cliften *et al.*, 2003; Kellis *et al.*, 2003). *FHL1* was originally identified as a suppressor of RNA polymerase III mutations (Hermann-Le Denmat *et al.*, 1994). Loss of *FHL1* results in a slow growth phenotype and in a defect in 35S-rRNA processing. Recent studies revealed a central role for Fhl1p in the regulation of transcription of ribosomal protein genes (Lee *et al.*, 2002; Martin *et al.*, 2004; Rudra *et al.*, 2005; Schawalder *et al.*, 2004; Wade *et al.*, 2004).

A *fhl1* deletion strain (MW667) was tested under glucose and ethanol conditions in Northern hybridization experiments to analyse the impact of Fhl1p on *CPC2* transcription. Figure 17A shows, that deletion of *FHL1* reduces *CPC2* transcription by a factor of about three under utilization of glucose. Under ethanol conditions the level of *CPC2* mRNA in

fhl1 deletion cells is similar to those in wild-type cells, which suggests that Fhl1p is not involved in basal transcription of *CPC2*.

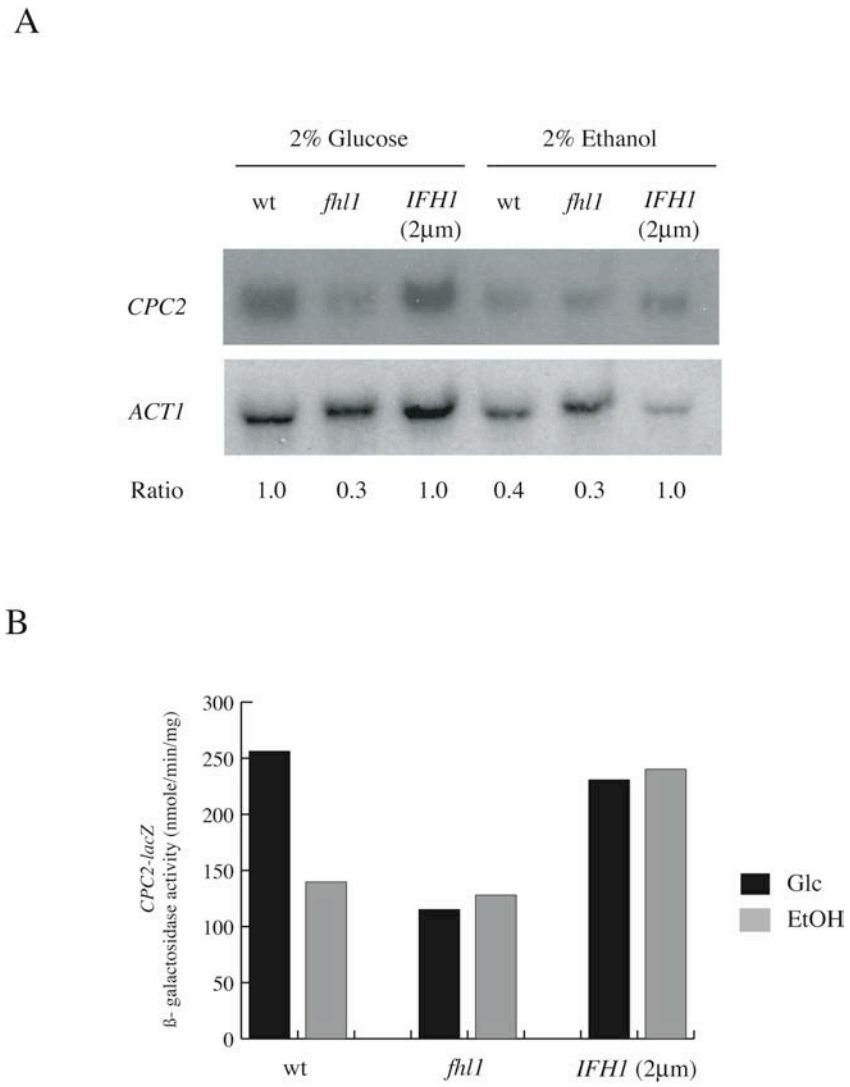

Figure 17: *CPC2* expression in *fhl1* deletion and *IFH1* overexpression yeast strains under utilization of glucose or ethanol as carbon source.
A Yeast strains YPH500 (wt), MW667 (*fhl1*) or YPH500 (*IFH1* 2µm) were cultivated on YNB medium to mid-log phase at 30°C. Cells were sedimented and shifted on YNB medium with 2% glucose or with 2% ethanol for 4 h at 30°C, respectively. Aliquots of total RNA (20-30 µg) from these cultures were used for Northern hybridization analysis. Numbers indicate relative transcript levels of *CPC2* to the internal standard *ACT1*. **B** Specific β-galactosidase activities of the p*CPC2-lacZ* reporter was assayed in yeast strains RH3261 (wt), RH3262 (*fhl1*) and RH3261 (*IFH1* 2µm) in presence of 2% glucose (black bars) and 2% ethanol (grey bars), respectively. Bars indicate means of specific β-galactosidase activities in nanomoles per minute per milligram of least three independent measurements. Standard deviations did not exceed 20%.

Measurements of specific β-galactosidase activities of a *CPC2-lacZ* reporter in *fhl1* deletion cells confirmed requirement of Fhl1p for *CPC2* expression under glucose conditions (Figure 17B). Accordingly, cells carrying the *fhl1* deletion showed a two-fold reduced specific β-galactosidase activity in presence of glucose, whereas the basal expression was not impaired.

Northern hybridization experiments and β-galactosidase assays suggest that the regulator of ribosomal protein genes Fhl1p is required for the glucose-dependent transcriptional induction of *CPC2*. The basal *CPC2* expression under ethanol conditions, however, does not require a functional *FHL1* gene.

The ATGTACGATGT motif within the *CPC2* promoter region is required for efficient transcription of *CPC2* under glucose and ethanol conditions

To analyse whether deletion of the putative Fhl1p binding site at position -299 to -289 also influences glucose-dependent transcriptional induction, the specific β-galactosidase activity of a *cpc2-lacZ* reporter with deleted ATGTACGATGT motif was assayed. Deletion of ATGTACGATGT reduces *CPC2* expression by a factor of about 2.5 in presence of glucose (Figure 16A). Under ethanol conditions deletion of ATGTACGATGT results in a two-fold reduced expression. Since *FHL1* is not required for basal expression of *CPC2*, this suggests that an additional factor binds to the ATGTACGATGT motif when ethanol as non-fermentable carbon source is utilized.

We further analysed whether *CPC2* promoter fragments carrying the ATGTACGATGT motif require Fhl1p for stimulation of the basal yeast *CYC1* promoter. Therefore, *CPC2* promoter insertion constructs were integrated into the *fhl1* deletion strain and the specific β-galactosidase activities of these constructs were measured under glucose and ethanol conditions. Figure 18 shows that fragment -375 to -225 enhanced specific β-galactosidase activity of the *CYC1-lacZ* reporter in *fhl1* deletion cells. This further supports that an additional factor is able to bind to this region and to affect *CPC2* expression. The *CPC2* promoter insertion construct -300 to -150 requires Fhl1p for activation of the *CYC1-lacZ* reporter under both glucose and ethanol conditions (Figure 18).

These data suggest that the ATGTACGATGT motif at position -299 to -289 acts as a specific activation site in combination with Fhl1p as well as a basal element independently of Fhl1p.

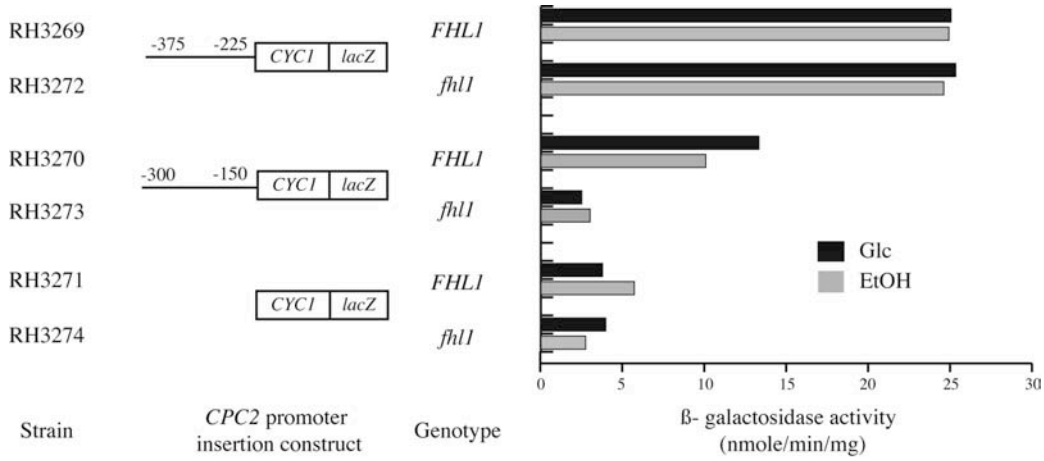

Figure 18: Expression of *CPC2* promoter insertion constructs in yeast *fhl1* deletion cells under glucose and ethanol conditions.
Expression of p*CPC2-CYC1-lacZ* insertion constructs was assayed in the wild-type strains RH3269 and RH3270 or in the *fhl1* deletion strains RH3272 and RH3273 under glucose (black bars) or ethanol (grey bars) conditions, respectively. Boundaries of insertion are indicated with respect to the translational start site of *CPC2* at position +1. RH3271 and RH3274 were used as negative control strains carrying no insert upstream of the basal *CYC1* promoter of the *CYC1-lacZ* fusion reporter. Bars indicate means of specific β-galactosidase activities in actual units nanomoles per minute per milligram of least three independent measurements. Standard deviations did not exceed 20%.

Overexpression of *IFH1* induces *CPC2* transcription in absence of glucose

The essential protein Ifh1p was first identified as a weak multicopy suppressor of a *fhl1* deletion (Cherel and Thuriaux, 1995). Recent studies show that Ifh1p is recruited through Fhl1p to promoters to activate transcription of ribosomal protein genes (Martin *et al.*, 2004; Rudra *et al.*, 2005; Schawalder *et al.*, 2004; Wade *et al.*, 2004). Due to the fact that *IFH1* is essential for cell growth, we transformed yeast strains YPH500 (wt) and MW666 (*fhl1*) with plasmid pME2932 (*IFH1*; 2μm; see Table 9) and tested an *IFH1* overexpression in a Northern hybridization experiment for its impact on *CPC2* transcription.

Figure 17A shows that *IFH1* overexpression does not affect *CPC2* transcription under glucose conditions. In contrast, overexpression of *IFH1* in cells growing with ethanol as carbon source stimulates transcription of *CPC2* to the level under glucose conditions.

Measurements of specific β-galactosidase activities confirmed the Ifh1p-dependent induction of *CPC2* under utilization of the non-fermentable carbon source ethanol (Figure 17B). As shown for the *CPC2* mRNA, overexpression of *IFH1* induces the specific β-galactosidase activity of the *CPC2-lacZ* reporter under ethanol, but does not affect expression under glucose conditions. These data suggest that the amount of Ifh1p is important for transcriptional induction of *CPC2*.

To summarize, transcription of *CPC2* is induced by the fermentable carbon source glucose. Two key regulators of ribosomal protein genes, Fhl1p and Ifh1p, are involved in regulation of this process suggesting a putative co-ordinate regulation of *CPC2* and ribosomal protein genes.

Discussion

The *CPC2* gene has been characterized as abundantly transcribed at levels equivalent to those of ribosomal protein genes (Velculescu *et al*., 1997). The high transcription rate results in approximately 330 000 Cpc2 molecules per log-phase cell, which is in the estimated range of 4 500 to 602 000 molecules of ribosomal proteins per log-phase cell (Ghaemmaghami *et al.*, 2003). In this work, we show that strong transcriptional induction of *CPC2* depends on the carbon source. Under utilization of the fermentable carbon source glucose transcription of *CPC2* is two-fold induced when compared to the non-fermentable carbon source ethanol. This is nearly the same factor of induction as we determined for the *RPS26* isogenes as control of genes for the small subunit of the ribosomes. Measurements of the macromolecular composition of *S. cerevisiae* revealed that cells grown on glucose have 2.5-fold more ribosomes than those grown on ethanol (Kief and Warner, 1981). A synchronized transcriptional regulation of *CPC2* and ribosomal protein genes has also been identified by transcriptional profiling experiments under different environmental growth conditions (Gasch *et al.*, 2000). All these same characteristics in gene expression of *CPC2* and ribosomal protein genes suggest that Cpc2p is a ribosomal component during utilization of the fermentable carbon source glucose. In contrast to many other genes encoding ribosomal proteins, *CPC2* is not present as a gene pair in the yeast genome.

This study also demonstrates that the transcription factor Fhl1p is required for transcriptional induction of *CPC2*. Increased amounts of the transcriptional activator Ifh1p induce *CPC2* transcription also during utilization of the non-fermentable carbon source ethanol. The identification of these two key regulators of ribosomal protein genes suggests for *CPC2* a similar regulatory mechanism as it has been described for ribosomal protein genes (Lee *et al.*, 2002; Martin *et al.*, 2004; Rudra *et al.*, 2005; Schawalder *et al.*, 2004; Wade *et al.*, 2004). Fhl1p presumably binds to the ATGTACGATGT motif at position -299 to -289 within the *CPC2* promoter region (Figure 19). This sequence motif has previously been characterized by a genome sequence comparison of different *Saccharomyces* species as a putative binding site for the transcription factor Fhl1p (Cliften *et al.*, 2003; Kellis *et al.*, 2003). Deletion of the sequence ATGTACGATGT or of *FHL1* reduces transcription of *CPC2* by a factor of about two and half. These data hint to an interaction of Fhl1p to the ATGTACGATGT motif. Genome-wide chromatin immunoprecipitation analyses also confirmed association of Fhl1p with the *CPC2*

promoter region (Lee *et al.*, 2002). The association of Fhl1p at the *CPC2* promoter probably requires additional factors. It was shown for several ribosomal protein genes, that in spite of association with the promoter in chromatin immunoprecipitation experiments no binding of purified Fhl1p was detected *in vitro* by band shift experiments (Rudra *et al.*, 2005). These data suggest that Fhl1p requires other factors or specific chromatin structures to bind gene promoters. Some of these factors might be the RSC chromatin remodelling complex (Angus-Hill *et al.*, 2001), the protein acetylase Esa1p (Reid *et al.*, 2000) or the multifunctional protein Rap1p (Morse, 2000; Pina *et al.*, 2003), which have all been described to be involved in regulation of ribosomal protein genes. Interestingly, binding of Rap1p to the *CPC2* promoter was also identified by genome-wide chromatin immunoprecipitation analyses (Lee *et al.*, 2002).

We assume that Fhl1p recruits Ifh1p via its forkhead-associated domain to the *CPC2* promoter when glucose is used as a carbon source (Figure 19). Thereby, the level of Ifh1p association determines transcriptional induction of *CPC2*. Recent studies with the inhibitor of the TOR pathway rapamycin identified the transcriptional co-repressor Crf1p as an additional factor for regulation of ribosomal protein genes (Martin *et al.*, 2004). In presence of rapamycin, Crf1p enters the nucleus and binds instead of Ifh1p to the forkhead-associated domain of Fhl1p to repress transcription of ribosomal protein genes. Overexpression of Ifh1p suppresses this down-regulation. The fact that also glucose limitation results in a nuclear accumulation of Crf1p suggests that Crf1p is probably involved in carbon source-dependent regulation of *CPC2*.

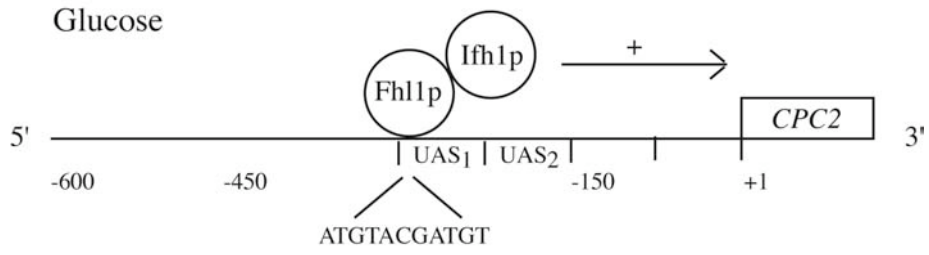

Figure 19: Scheme for transcriptional induction of *CPC2* in yeast under utilization of the fermentable carbon source glucose.
Two upstream activation sequence elements (UAS_1 and UAS_2) are shown for the *CPC2* promoter region. Numbers indicate positions relative to the translation start site of *CPC2*, which is marked as +1. The ATGTACGATGT motif represents the presumed binding site for Fhl1p. Fhl1p co-activator Ifh1p stimulates *CPC2* transcription only in the presence of glucose.

The increased *CPC2* mRNA level under glucose conditions is also a hint for a transcriptional induction of the highly conserved small nucleolar RNA (*SNR24*), which is located on the *CPC2* intron, and therefore, putatively co-regulated with *CPC2*. *SNR24* is required for site-specific 2'-*o*-methylation of rRNA and is present from yeast to human (Kiss-Laszlo *et al.*, 1996; Qu *et al.*, 1995). Induction of *SNR24* might be important for controlling of pre-RNA folding during ribosome biogenesis.

Beside the ribosome bound form, also a non-ribosome bound form of Cpc2p has been identified by previous studies (Baum *et al.*, 2004). In cells of a stationary culture the non-ribosome bound form of Cpc2p increases, which seems to be dependent on high Cpc2p levels under such conditions. In *Schizosaccharomyces pombe* or humans also a non-ribosome bound form of Cpc2 or RACK1 has been characterized (Ceci *et al.*, 2003; Shor *et al.*, 2003). These data suggest that Cpc2p, beside its function at the ribosomes, also might have additional functions in yeast cells as it has been reported for the homologue proteins of *S. pombe* and mammals. In the fission yeast, Cpc2 is involved in regulation of the cell cycle, actin cytoskeleton organisation or sexual differentiation (Jeong *et al.*, 2004; McLeod *et al.*, 2000; Won *et al.*, 2001). The human RACK1 regulates several processes including cell spreading, recruitement of other proteins to focal adhesions or cell-cell contact (McCahill *et al.*, 2002; Nilsson *et al.*, 2004). Tandem affinity purification (TAP) experiments in *S. cerevisiae* also support the suggestion of a multifunctional Cpc2p. Cpc2p was co-purified with eleven different multi protein complexes with functions in protein synthesis and turnover, transcription/DNA maintenance/chromatin structure, RNA-metabolism, membrane biogenesis and transport (Gavin *et al.*, 2002). Most of these complexes are predominantly localized in the cytoplasm and at the endoplasmatic reticulum, but two complexes involved in transcription/DNA maintenance/chromatin structure and in the RNA metabolism have been reported to be localized exclusively in the nucleus. A nuclear localized Cpc2p affects possibly indirectly translation by controlling of ribosome assembly or transport of mRNAs into the cytoplasm.

To summarize, in this work we demonstrate that transcriptional induction of *CPC2* depends on the fermentable carbon source glucose. The two key players for a co-ordinated regulation of ribosomal protein genes Fhl1p and Ifh1p are involved in this process. These data suggest that Cpc2p is a ribosomal component and co-regulated to other ribosomal proteins during utilization of different carbon sources.

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Chapter V

Conclusions and perspectives

Cell-cell or cell-surface adhesion represents one of the most prominent determinants of fungal pathogenesis. In the last years, an increase in hospital-acquired fungal infections was observed. This increase has been attributed to the ability of fungi to adhere not only to human tissues, but also to plastic devices such as catheters, prosthetic heart values or cardiac pacemakers (Cormack *et al.*, 1999; Douglas, 2003; Sundstrom, 2002). The most common fungal pathogens of humans are *Candida albicans* and *Candia glabrata*. *Candidia* ssp. can develop multicellular structures such as biofilms in an adhesion-dependent manner, which are extremely resistant to antifungal drugs and act as a source of reinfections (Baillie and Douglas, 2000; Chandra *et al.*, 2001; Douglas, 2003; Lamfon *et al.*, 2004; Ramage *et al.*, 2005). The better understanding of regulatory mechanisms and specific expression programs during adhesive growth of fungal cells is one prerequisite for the development of more effective drugs and therapies against fungal infections.

The non-pathogenic fungus *Saccharomyces cerevisiae* is an appropriate model organism for adherence-dependent fungal infections. Many signalling pathways and proteins in *S. cerevisiae* are highly conserved throughout the fungi. The access to a sequenced genome and the easy handling of yeast cells in the laboratory allow the application of genome-wide expression studies. Adhesion of Σ1278b yeast cells can easily be induced by conditions of amino acid limitation (Braus *et al.*, 2003). This specific adhesive growth phenotype requires the expression of the cell surface adhesin Flo11p and of Gcn4p, the transcriptional activator of the general control of amino acid biosynthesis. Until now, not much was known about gene expression during amino acid starvation-induced adhesive growth.

Gene expression under adhesion-inducing conditions

In this work, numerous novel Gcn4p-dependent and Gcn4p-independent regulated genes were identified in Σ1278b yeast cells under adhesion-inducing conditions (Chapter II). A comparison with the transcriptional profiles of amino acid-starved and non-adherent

S288c yeast cells (Natarajan *et al.*, 2001) suggests that adhesion is regulated by a complex and Σ1278b specific transcriptional response to amino acid starvation. Many of these genes encode proteins involved in signalling pathways, cell wall structure or maintenance, and therefore these genes are interesting candidates to study regulation of amino acid starvation-induced adhesive growth. In addition, also regulated proteins were identified in amino acid-starved and adherent yeast cells (Chapter III). Despite of a reduced overall translation efficiency upon amino acid limitation, seven protein spots showed a highly increased intensity. Comparisons with the respective transcriptome data revealed posttranscriptional regulatory events for six of these proteins during adhesive growth. This posttranscriptional regulation can occur at the level of translation, protein stability or modification. Thereby, the translational control seems to be an important regulatory mechanism, because two of the novel identified regulated proteins, namely Cpc2p and Efb1p, have previously been described to be involved in translation (Gerbasi *et al.*, 2004; Kinzy and Woolford, 1995).

Ribosomal proteins and adhesion

The *CPC2* gene (also known as *ASC1*) encodes a highly conserved Gβ-like ribosome-associated WD-repeat protein (Chantrel *et al.*, 1998; Hoffmann *et al.*, 1999) and is a homologue of the *cpc-2* gene of *Neurospora crassa* (Müller *et al.*, 1995), *CPC2* of *Schizosaccharomyces pombe* (McLeod *et al.*, 2000) and of the human *RACK1* encoding a receptor of activated protein kinase C (Ron *et al.*, 1994), respectively. This work shows that *CPC2* behaves in several aspects like a 'true' ribosomal protein gene (Chapter IV). The gene is transcriptionally controlled by the carbon source and the two key regulators of ribosomal protein genes Fhl1p and Ifh1p (Lee *et al.*, 2002; Martin *et al.*, 2004; Rudra *et al.*, 2005; Schawalder *et al.*, 2004; Wade *et al.*, 2004) are involved in this process.

Deletion of *CPC2* in yeast results in an adhesion deficient phenotype (Chapter III). Surprisingly, basal and amino acid starvation-induced activation of *FLO11* transcription require also a functional *CPC2* gene. Because of its function as a ribosomal protein, Cpc2p might be involved in the appropriate synthesis of a transcription factor, which is essential for *FLO11* transcription. Either the translation rate of the transcription factor mRNAs or an

important processing step of its nascent polypeptide/protein might be the reason for a putative *FLO11* transcription factor deficiency.

Recent analyses of the *RPS26A/B* isogenes support the impact of a translational control on adhesive growth (Strittmatter *et al.*, 2005). Both genes encode two almost identical proteins of the 40S-ribosomal subunit, which differ only by two amino acid residues. *RPS26A* but not *RPS26B* is required for the *FLO11*-mediated adhesive growth phenotypes of haploid invasive growth and diploid pseudohyphal formation. Deletion of *RPL16A/B*, which code for proteins of the 60S-ribosomal subunit, has no effect on adhesive growth. Interestingly, in contrast to the *FLO11* mRNA, the specific β-galactosidase activity of a *FLO11-lacZ* reporter is not present in haploid *rps26A* deletion strains. These data suggest that in addition to the previously known transcriptional regulation of *FLO11*, also a direct or indirect translational regulation takes place.

Evidences for a translational control of *FLO11* expression

It is not clear how specific ribosomal proteins influence *FLO11* expression or its transcriptional regulators, or if their deletion results in a general translational imbalance during adhesive growth. A recent study suggests that *FLO11* itself could be controlled on the level of translation. In spite of reduced amounts of *FLO11* mRNA in a *rsc1* deletion strain, cells are able to grow adhesively upon amino acid limitation in a *FLO11*-dependent manner (Fischer *et al*., 2005).

One putative mechanism for a translational regulation of *FLO11* could be the presence of a putative upstream open reading frame (uORF) in the promoter region. Typically, the presence of uORFs in the 5'leader sequence negatively influences translation of a gene, but under amino acid starvation ribosomes bypass, *e.g*., the uORF4 of the *GCN4* mRNA, which results in a translational de-repression of this gene (Hinnebusch, 1997). The putative uORF of *FLO11* is located at position -61 relative to translational start site (Vivier *et al.*, 1999). Interestingly, the presence of a putative uORF at position -61 has also been described for the *STA1-3* genes (Figure 20). The *STA1-3* genes of *S. cerevisiae* encode extracellular glucoamylase isozymes for utilization of starch as a carbon source (Pretorius *et al.*, 1991; Vivier *et al.*, 1997). All three members of the *STA* gene family are located in subtelomeric positions, similar to the *FLO* genes. The 5'upstream region of

STA1-3 is almost identical to that of *FLO11*. The homology extends over more than 3,5 kb upstream of the ATG start codon. The only significant differences between the *STA2* and *FLO11* promoter are two insertions of 20 and 64 bp in the *FLO11* promoter, which are absent in the *STA2* promoter. For the *STA1-3* genes three different transcription initiation sites downstream (-13, -23 and -26) and one initiation site upstream (-100) of the putative uORF have been found (Figure 20) (Vivier *et al.*, 1999). The high homology between the *FLO11* and the *STA1-3* promoters suggests also for the *FLO11* the use of different transcription initiation sites, which might cause a different translation efficiency of *FLO11* mRNAs.

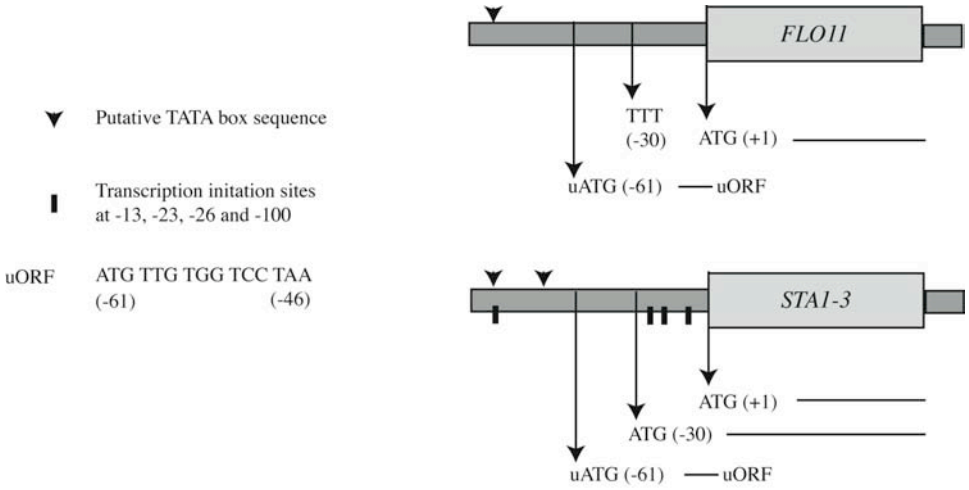

Figure 20: Structural features of the *FLO11* and *STA1-3* gene promoters important for translation initiation (adapted from Vivier *et al.*, 1999).
The promoter region and first 96 bp of the *FLO11* gene are 97% identical to those of the *STA1-3* genes. The *STA1-3* genes contain two in-frame ATG codons. Both of these codons have been shown to be functional translation initiation sites. The translation initiation site at -30 is mutated to TTT in the *FLO11* promoter. A putative four amino acid uORF is present upstream of the *FLO11* and *STA1-3* genes. The *STA1-3* genes have two putative TATA box sequence elements at position -75 to -70 and -100 to -94, whereas *FLO11* has only the putative TATA element at position -100 to -94. Four transcription initiation sites were mapped for the *STA1-3* genes at position -13, -23, -26 and -100.

In summary, in this work numerous novel transcriptional and posttranscriptional regulated genes were identified in *S. cerevisiae* during amino acid starvation-induced adhesive growth. These data show the complexity of gene regulation in adherent yeast cells. The identification and characterization of Cpc2p as an essential ribosomal protein for adhesive growth gives new insights in the regulation of this phenotype. The high conservation of Cpc2p throughout the eukaryotes suggests a similar role for its homologous proteins in human pathogenic fungi.

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Chapter VI

Supplement

Supplementary Table 1: Complete list of differentially expressed genes identified in the *GCN4*/*gcn4* comparision

ORF	Ratio	p-value	Gene	Function[A]
YLR265C	0.225357	0.00108212	*NEJ1*	DNA repair
YNL120C	0.244208	0.00316246		Biological_process unknown
YDR133C	0.279818	0.000819896		Biological_process unknown
YJL179W	0.312091	0.00289419	*PFD1*	Protein folding
YOR368W	0.355959	0.00575163	*RAD17*	Meiotic recombination
YFL035C-B	0.359932	0.00157662		
YPR102C	0.383986	0.00983651	*RPL11A*	Protein biosynthesis
YJL194W	0.388294	0.00217912	*CDC6*	Pre-replicative complex formation and maintenance
YOL124C	0.390577	0.00343278		Biological_process unknown
YNR003C	0.394082	0.00978944	*RPC34*	Transcription from Pol III promoter
YOR182C	0.426926	0.00566862	*RPS30B*	Protein biosynthesis
YNR044W	0.435838	0.00560923	*AGA1*	Agglutination during conjugation with cellular fusion
YJL098W	0.438815	0.00275217	*SAP185*	G1/S transition of mitotic cell cycle
YJR094W-A	0.445885	0.00655024	*RPL43B*	Protein biosynthesis
YLR457C	0.449348	0.000436914	*NBP1*	Biological_process unknown
YOR369C	0.479007	0.00976005	*RPS12*	Protein biosynthesis
YPL068C	0.480669	0.00934901		Biological_process unknown
YBL054W	0.480888	0.00474332		Biological_process unknown
YIR020C	0.489484	0.00122073		Biological_process unknown
YLR195C	2.04262	0.00588125	*NMT1*	N-terminal peptidyl-glycine N-myristoylation
YLR194C	2.05465	0.00306418		Biological_process unknown
YNL134C	2.10526	0.00257766		Biological_process unknown
YLR257W	2.15176	0.00791238		Biological_process unknown
YNL055C	2.24848	0.00556952	*POR1*	Aerobic respiration
YHR138C	2.28057	0.00396375		Vacuole fusion (non-autophagic)
YJL034W	2.31918	0.00477418	*KAR2*	Karyogamy during conjugation with cellular fusion
YHR104W	2.35814	0.003412	*GRE3*	Response to stress
YGL121C	2.41674	0.000604676	*GPG1*	Signal transduction
YBL064C	2.42861	0.00213167		Regulation of redox homeostasis
YDL022W	2.46291	0.00067565	*GPD1*	Intracellular accumulation of glycerol
YGR023W	2.49603	0.00878305	*MTL1*	Cell wall organization and biogenesis
YGL117W	2.5336	0.00799663		Biological_process unknown
YKR076W	2.53854	0.00819954	*ECM4*	Cell wall organization and biogenesis
YOR382W	2.54577	0.00924646	*FIT2*	Siderochrome transport
YJR155W	2.61133	0.00830897	*AAD10*	Aldehyde metabolism
YGL037C	2.61925	0.00141761	*PNC1*	Chromatin silencing at telomere
YLR121C	2.76637	0.000888835	*YPS3*	Protein metabolism
YDR007W	2.84816	0.000169201	*TRP1*	Tryptophan biosynthesis
YDR008C	2.87043	9.67E-005		Biological_process unknown
YGR161C	2.91207	0.00221207		Biological_process unknown
YDR171W	3.06564	0.00839897	*HSP42*	Response to stress

Supplementary Table 1: Continued

ORF	Ratio	p-value	Gene	Function[A]
YMR173W-A	3.37488	0.00323896		Biological_process unknown
YCL040W	3.45521	0.00355931	*GLK1*	Carbohydrate metabolism
YBR214W	3.48252	0.000255192	*SDS24*	Meiosis
YDR380W	3.58409	0.0054351	*ARO10*	Leucine catabolism
YLR004C	3.63323	0.00152721		Biological_process unknown
YMR173W	4.03388	0.00577883	*DDR48*	DNA repair
YNR058W	4.05204	0.0010782	*BIO3*	Biotin biosynthesis
YOL058W	4.05454	0.000155874	*ARG1*	Arginine biosynthesis
YLR327C	4.33345	0.000375144		Biological_process unknown
YDL124W	4.75771	0.00233281		Biological_process unknown
YOL055C	4.83163	0.000137827	*THI20*	Thiamin biosynthesis
YCR030C	4.88022	0.00934348	*SYP1*	Biological_process unknown
YLR327C	4.33345	0.000375144		Biological_process unknown
YDL124W	4.75771	0.00233281		Biological_process unknown
YOL055C	4.83163	0.000137827	*THI20*	Thiamin biosynthesis
YCR030C	4.88022	0.00934348	*SYP1*	Biological_process unknown
YDL124W	4.75771	0.00233281		Biological_process unknown
YOL055C	4.83163	0.000137827	*THI20*	Thiamin biosynthesis
YCR030C	4.88022	0.00934348	*SYP1*	Biological_process unknown

[A] according to the *Saccharomyces* Genome Database (http://www.yeastgenome.org)

Supplementary Table 2: Complete list of genes regulated by exposure of *GCN4* cells to 10 mM 3AT for 8 h

ORF	Ratio	p-value	Gene	Function[A]
YDL101C	0.311473	0.00110371	*DUN1*	Protein amino acid phosphorylation
YBR182C	0.334742	0.00379314	*SMP1*	Transcription
YDR371W	0.339737	0.00118116	*CTS2*	Biological_process unknown
YLR121C	0.350998	0.00094402	*YPS3*	Protein metabolism
YHR094C	0.399395	0.000490556	*HXT1*	Hexose transport
YDR357C	0.404395	0.00330243		Biological_process unknown
YLR322W	0.481938	0.00690435	*VPS65*	Protein-vacuolar targeting
YJL216C	2.03287	0.00342101		Biological_process unknown
YAR015W	2.03847	0.00135427	*ADE1*	Purine base metabolism
YBR147W	2.05468	0.00103681		Biological_process unknown
YJR025C	2.07948	0.00780693	*BNA1*	Nicotinamide adenine dinucleotide biosynthesis
YPL014W	2.08018	0.000343839		Biological_process unknown
YCL020W	2.08544	0.0043234		
YDL048C	2.09692	0.00566712	*STP4*	Biological_process unknown
YER166W	2.11309	0.0070597	*DNF1*	Intracellular protein transport
YNL011C	2.11989	0.00815365		Biological_process unknown
YBR083W	2.14699	0.00172363	*TEC1*	Pseudohyphal growth
YNR044W	2.16344	0.00883538	*AGA1*	Agglutination during conjugation with cellular fusion
YHR029C	2.16496	0.00218768		Biological_process unknown
YML052W	2.23071	0.00135418	*SUR7*	Sporulation (sensu *Saccharomyces*)
YCL030C	2.25287	0.00129061	*HIS4*	Histidine biosynthesis

Supplementary Table 2: Continued

ORF	Ratio	p-value	Gene	Function[A]
YDL194W	2.27843	0.00351271	*SNF3*	Signal transduction
YLR004C	2.28279	0.00247849		Biological_process unknown
YHL028W	2.29908	0.00981759	*WSC4*	Cell wall organization and biogenesis
YBR047W	2.33808	0.00500959		Biological_process unknown
YJR155W	2.3899	0.00751025	*AAD10*	Aldehyde metabolism
YER172C	2.39296	0.000168408	*BRR2*	Lariat formation
YPL252C	2.39856	6.13E-005	*YAH1*	Heme a biosynthesis
YOR108W	2.44192	0.00790295		Biological_process unknown
YPL251W	2.47214	0.0040274		Biological_process unknown
YNL006W	2.65376	0.00905728	*LST8*	Transport
YBL005W-A	2.67574	0.000208982		
YAR010C	2.75255	0.000119433		
YMR321C	2.76177	0.00183525		Biological_process unknown
YKR058W	2.78335	0.000146137	*GLG1*	Glycogen biosynthesis
YIL119C	2.83357	0.00198193	*RPI1*	Thiamin biosynthesis
YBR012W-A	2.83897	0.0024247		
YER175C	2.84091	0.000108432	*TMT1*	Biological_process unknown
YGL117W	3.01636	0.00143982		Biological_process unknown
YMR097C	3.0411	0.00712673	*MTG1*	Biological_process unknown
YJR028W	3.06776	0.00128319		
YIR035C	3.10729	0.00944764		Biological_process unknown
YBR012W-B	3.24292	4.34E-005		
YMR046C	3.30503	0.000172488		
YNR074C	3.31388	0.00433017		Response to singlet oxygen
YDR277C	3.33117	0.000716324	*MTH1*	Signal transduction
YMR051C	3.33132	0.00136048		
YDR019C	3.34609	2.41E-006	*GCV1*	One-carbon compound metabolism
YNL337W	3.48764	0.00300121		Biological_process unknown
YMR094W	3.84309	0.00151937	*CTF13*	Mitosis
YOR171C	4.44167	0.0043145	*LCB4*	Sphingolipid metabolism
YMR096W	5.04061	7.35E-005	*SNZ1*	Thiamin biosynthesis
YMR195W	5.08402	0.000541431	*ICY1*	Biological_process unknown
YBR288C	5.6094	0.000829875	*APM3*	Golgi to vacuole transport
YDR133C	7.61247	6.81E-005		Biological_process unknown

[A] according to the *Saccharomyces* Genome Database (http://www.yeastgenome.org)

Supplementary Table 3: Complete list of genes regulated by exposure of *gcn4* cells to 10 mM 3AT for 8 h

ORF	Ratio	p-value	Gene	Function[A]
YBL003C	0.182626	4.04E-005	*HTA2*	Chromatin assembly/disassembly
YJR073C	0.21005	0.00152236	*OPI3*	Phosphatidylcholine biosynthesis
YNL300W	0.295274	0.000363691		Biological_process unknown
YNL208W	0.315998	0.000336705		Biological_process unknown

Supplementary Table 3: Continued

ORF	Ratio	p-value	Gene	Function[A]
YJL088W	0.382875	0.00751747	*ARG3*	Arginine biosynthesis
YFL035C-B	0.401795	2.13E-005		
YOR248W	0.425147	9.53E-005		Biological_process unknown
YOR342C	0.442803	0.00259375		Biological_process unknown
YLR194C	0.457416	5.63E-005		Biological_process unknown
YAR068W	0.46749	0.000187486		Biological_process unknown
YDL182W	0.490087	1.80E-005	*LYS20*	Lysine biosynthesis
YOR247W	0.490765	0.00248619	*SRL1*	Nucleobase
YDR109C	2.00663	0.00154693		Biological_process unknown
YCL034W	2.01973	0.00175699	*LSB5*	Endocytosis
YLR056W	2.02189	0.00126268	*ERG3*	Ergosterol biosynthesis
YFR041C	2.02293	0.0040308		Biological_process unknown
YDR143C	2.03025	0.00877802	*SAN1*	Establishment and/or maintenance of chromatin Architecture
YHR111W	2.04364	0.00244068	*UBA4*	Protein modification
YER044C	2.05128	0.00497428	*ERG28*	Ergosterol biosynthesis
YMR077C	2.0591	0.00388398	*VPS20*	Late endosome to vacuole transport
YHR199C	2.06788	0.00915206		Biological_process unknown
YBR096W	2.06804	0.00690571		Biological_process unknown
YPL245W	2.06834	0.00470656		Biological_process unknown
YMR132C	2.06867	0.00895096		Biological_process unknown
YOR258W	2.0822	0.00110146	*HNT3*	Biological_process unknown
YMR255W	2.09344	0.00327157	*GFD1*	mRNA-nucleus export
YDR427W	2.1005	0.00172505	*RPN9*	Ubiquitin-dependent protein catabolism
YFR030W	2.11139	0.00209618	*MET10*	Sulfate assimilation
YGR038W	2.12148	0.00930847	*ORM1*	Biological_process unknown
YOR064C	2.12965	0.000953509	*YNG1*	Histone acetylation
YDL086W	2.14483	0.00793916		Biological_process unknown
YGL207W	2.1451	0.00568986	*SPT16*	Chromatin modeling
YDL108W	2.1553	9.67E-005	*KIN28*	Protein amino acid phosphorylation
YDL165W	2.16443	0.000683438	*CDC36*	Regulation of transcription from Pol II promoter
YBR279W	2.1981	0.00567096	*PAF1*	RNA elongation from Pol II promoter
YHR063C	2.22623	0.00281569	*PAN5*	Biological_process unknown
YBR233W	2.25536	0.00688787	*PBP2*	Biological_process unknown
YGR133W	2.25631	0.00629501	*PEX4*	Polyubiquitination
YKL210W	2.27122	0.00333145	*UBA1*	Ubiquitin cycle
YAL021C	2.27362	0.00848796	*CCR4*	Regulation of transcription from Pol II promoter
YCR023C	2.275	0.00523612		Biological_process unknown
YMR097C	2.27991	0.00320568	*MTG1*	Biological_process unknown
YOR007C	2.28008	0.000791174	*SGT2*	Biological_process unknown
YER100W	2.30717	0.000318959	*UBC6*	Polyubiquitination
YER168C	2.3076	0.00150259	*CCA1*	tRNA modification
YKR001C	2.33783	0.00720678	*VPS1*	Protein-vacuolar targeting
YJL101C	2.36074	0.00883353	*GSH1*	Glutathione biosynthesis
YDL007W	2.37503	0.00717127	*RPT2*	Ubiquitin-dependent protein catabolism
YIR008C	2.37782	0.00190422	*PRI1*	DNA replication initiation
YDL175C	2.44482	5.07E-005	*AIR2*	mRNA-nucleus export
YGR117C	2.45702	0.00891662		Biological_process unknown
YAL012W	2.47439	0.00778029	*CYS3*	Sulfur amino acid metabolism
YMR208W	2.48049	0.00295183	*ERG12*	Ergosterol biosynthesis
YNL335W	2.57	0.00501849		Biological_process unknown
YCL075W	2.63135	0.00534249		
YLR225C	2.63922	0.00639849		Biological_process unknown
YER108C	2.64598	0.00669128		
YDL142C	2.66496	0.00170841	*CRD1*	Lipid biosynthesis

Supplementary Table 3: Continued

ORF	Ratio	p-value	Gene	Function[A]
YJR046W	2.73471	0.00392349	*TAH11*	DNA replication licensing
YGL181W	2.74177	1.43E-006	*GTS1*	Sporulation (sensu *Saccharomyces*)
YNL064C	2.76705	4.59E-006	*YDJ1*	Protein-mitochondrial targeting
YDR078C	2.772	0.0025194	*SHU2*	Biological_process unknown
YLR363C	2.77299	0.00927219	*NMD4*	mRNA catabolism
YKR066C	2.78154	0.0029923	*CCP1*	Response to oxidative stress
YGL252C	2.79397	0.00549371	*RTG2*	Intracellular signaling cascade
YLR228C	2.8078	0.0042382	*ECM22*	Sterol biosynthesis
YLL062C	2.84243	0.000833238	*MHT1*	Sulfur amino acid metabolism
YKL040C	2.84344	0.00331596	*NFU1*	Iron homeostasis
YHR055C	2.85818	3.45E-005	*CUP1-2*	Response to copper
YDR179W-A	2.86017	0.00204209		Biological_process unknown
YER147C	2.89469	0.00799994	*SCC4*	Sister chromatid cohesion
YGL001C	2.92514	0.00194005	*ERG26*	Ergosterol biosynthesis
YHR053C	2.97788	2.32E-007	*CUP1-1*	Response to copper
YOL063C	2.99143	0.00967502	*MOR1*	Biological_process unknown
YJR062C	3.00241	0.00505331	*NTA1*	Protein catabolism
YHL039W	3.01399	0.00373382		Biological_process unknown
YIL097W	3.03311	0.00806084	*FYV10*	Biological_process unknown
YOR259C	3.04994	0.00593763	*RPT4*	Ubiquitin-dependent protein catabolism
YHR201C	3.06104	0.00725333	*PPX1*	Polyphosphate metabolism
YHR056C	3.10919	1.69E-006	*RSC30*	Regulation of transcription
YOL118C	3.15376	0.00396194		Biological_process unknown
YGR161C	3.1744	0.000279979		Biological_process unknown
YNL007C	3.18007	0.000181606	*SIS1*	Translational initiation
YDR151C	3.184	0.000300724	*CTH1*	mRNA capping
YJL034W	3.18814	0.000959507	*KAR2*	Karyogamy during conjugation with cellular fusion
YKL041W	3.20956	0.00510692	*VPS24*	Late endosome to vacuole transport
YDL170W	3.21506	0.00747484	*UGA3*	Regulation of transcription from Pol II promoter
YHR122W	3.36657	0.00133777		Biological_process unknown
YPL259C	3.36897	0.0071719	*APM1*	Vesicle-mediated transport
YHR061C	3.36944	0.00497902	*GIC1*	Establishment of cell polarity
YHL044W	3.38568	0.0013745		Biological_process unknown
YIR043C	3.49497	0.00195965		Biological_process unknown
YOL032W	3.49782	0.000627109		Biological_process unknown
YCR038C	3.53476	0.00497519	*BUD5*	Pseudohyphal growth
YLL007C	3.54409	0.000890761		Biological_process unknown
YGL169W	3.68935	0.0040802	*SUA5*	Cell growth and/or maintenance
YGL222C	3.73347	0.00219773	*EDC1*	Deadenylation-dependent decapping
YGL091C	3.73882	0.000566137	*NBP35*	Biological_process unknown
YDR013W	3.7448	0.000545676		Biological_process unknown
YML130C	3.81472	0.000585504	*ERO1*	Protein folding
YGR127W	3.83092	0.00121577		Biological_process unknown
YDR214W	3.89051	1.27E-005	*AHA1*	Biological_process unknown
YMR100W	3.95344	0.0080594	*MUB1*	Regulation of budding
YDR235W	3.97898	0.00169728	*PRP42*	mRNA splicing
YPL106C	4.01546	0.00590148	*SSE1*	Protein folding
YBR101C	4.02677	6.73E-005		Biological_process unknown
YIL068C	4.03705	0.000473251	*SEC6*	Establishment of cell polarity
YER141W	4.06779	0.00799037	*COX15*	Cytochrome c oxidase biogenesis
YPL240C	4.10996	0.00090393	*HSP82*	Response to stress
YLR216C	4.16026	0.00020055	*CPR6*	Protein folding
YJR148W	4.16983	0.00524192	*BAT2*	Branched chain family amino acid biosynthesis
YFL016C	4.1798	0.000191627	*MDJ1*	Protein folding
YHR180W	4.26326	0.000700663		Biological_process unknown

Supplementary Table 3: Continued

ORF	Ratio	p-value	Gene	Function[A]
YLL024C	4.39626	0.00178865	*SSA2*	Protein folding
YDR436W	4.57197	0.00109614	*PPZ2*	Sodium ion homeostasis
YOR027W	4.61424	0.000860791	*STI1*	Protein folding
YIL117C	4.70807	0.00319425	*PRM5*	Conjugation with cellular fusion
YIR030C	4.83687	0.00969295	*DCG1*	Nitrogen metabolism
YAL005C	5.00009	0.00477549	*SSA1*	Protein folding
YER103W	5.08662	0.000531118	*SSA4*	Response to stress
YDR171W	5.37559	0.000542354	*HSP42*	Response to stress
YGR177C	5.74504	0.00126859	*ATF2*	Steroid metabolism
YDL020C	5.87133	0.000537056	*RPN4*	Ubiquitin-dependent protein catabolism
YLL026W	5.93775	0.00160423	*HSP104*	Response to stress
YML016C	6.13492	0.00683787	*PPZ1*	Sodium ion homeostasis
YIR031C	9.32873	0.00452769	*DAL7*	Allantoin catabolism
YEL039C	10.5895	8.61E-005	*CYC7*	Electron transport
YDR258C	12.9859	0.00048149	*HSP78*	Response to stress
YGR142W	13.9449	2.94E-005	*BTN2*	Regulation of pH

[A] according to the *Saccharomyces* Genome Database (http://www.yeastgenome.org)

Supplementary Table 4: All genes that are differentially expressed in *GCN4* versus *gcn4* cells exposed to 10 mM 3AT for 8 h

ORF	Ratio	p-value	Gene	Function[A]
YEL013W	0.0896997	0.00248033	*VAC8*	Protein-vacuolar targeting
YGL101W	0.0970159	0.00010889		Biological_process unknown
YLR217W	0.104828	0.00964767		Biological_process unknown
YGR142W	0.1157	0.00125633	*BTN2*	Regulation of pH
YDL006W	0.116706	0.00465003	*PTC1*	Response to osmotic stress
YDR235W	0.119699	0.00290845	*PRP42*	mRNA splicing
YFL042C	0.125147	0.00157737		Biological_process unknown
YOL016C	0.140446	0.000984843	*CMK2*	Protein amino acid phosphorylation
YGR179C	0.141147	0.00904677	*OKP1*	Chromosome segregation
YER099C	0.143411	0.00665623	*PRS2*	Histidine biosynthesis
YGR177C	0.155551	0.0014594	*ATF2*	Steroid metabolism
YJL009W	0.171289	0.00466355		Biological_process unknown
YGL213C	0.174914	0.00076012	*SKI8*	mRNA catabolism
YHR119W	0.180115	0.00401944	*SET1*	Chromatin silencing at telomere
YDR371W	0.185143	0.000284425	*CTS2*	Biological_process unknown
YJR003C	0.187918	0.0074431		Biological_process unknown
YLR417W	0.190253	0.00732256	*VPS36*	Protein-vacuolar targeting
YHR003C	0.190286	0.00333384		Biological_process unknown
YGL005C	0.196025	0.00636288	*COG7*	Intra-Golgi transport
YGR168C	0.202188	0.0086199		Biological_process unknown
YHR065C	0.205405	0.00192915	*RRP3*	mRNA splicing
YOR153W	0.209335	0.00911328	*PDR5*	Response to drug
YDL133W	0.212106	0.00422489		Biological_process unknown

Supplementary Table 4: Continued

ORF	Ratio	p-value	Gene	Function[A]
YHR061C	0.216834	2.57E-005	*GIC1*	Establishment of cell polarity (sensu *Saccharomyces*)
YMR135C	0.218968	0.00360802	*DCR1*	Biological_process unknown
YHL039W	0.221794	0.000996922		Biological_process unknown
YDR258C	0.223668	0.00304941	*HSP78*	Response to stress
YER103W	0.227105	0.00449325	*SSA4*	Response to stress
YGL238W	0.228375	1.05E-005	*CSE1*	Protein-nucleus export
YER035W	0.230811	0.0055345	*EDC2*	Deadenylation-dependent decapping
YHR051W	0.231716	0.000998637	*COX6*	Aerobic respiration
YDR043C	0.238405	0.0061314	*NRG1*	Regulation of transcription from Pol II promoter
YBR101C	0.247473	8.16E-005		Biological_process unknown
YGR284C	0.25555	0.0097055	*ERV29*	ER to Golgi transport
YGL222C	0.256164	0.000202044	*EDC1*	Deadenylation-dependent decapping
YLL019C	0.26301	0.0061229	*KNS1*	Protein amino acid phosphorylation
YHR201C	0.266909	0.000459938	*PPX1*	Polyphosphate metabolism
YDR020C	0.271886	0.00181456		Biological_process unknown
YDR194C	0.272613	0.0076296	*MSS116*	RNA splicing
YLL026W	0.275277	0.00417916	*HSP104*	Response to stress
YBL011W	0.277208	0.00896226	*SCT1*	Phospholipid biosynthesis
YGR050C	0.27903	0.00260502		Biological_process unknown
YFL002C	0.279385	0.00123389	*SPB4*	35S primary transcript processing
YGL110C	0.280081	0.00163946	*CUE3*	Biological_process unknown
YCL037C	0.280742	0.00749607	*SRO9*	Protein biosynthesis
YER141W	0.281183	0.00784223	*COX15*	Cytochrome c oxidase biogenesis
YDR306C	0.284467	0.00953999		Biological_process unknown
YGR127W	0.28708	0.00442882		Biological_process unknown
YDR370C	0.287575	0.000116358		Biological_process unknown
YLL024C	0.289789	0.00559819	*SSA2*	Protein folding
YCL011C	0.29098	0.00118908	*GBP2*	Biological_process unknown
YHR041C	0.293556	0.00612071	*SRB2*	Transcription from Pol II promoter
YKL008C	0.295402	0.00413282	*LAC1*	Aging
YBR293W	0.298609	0.00562097		Biological_process unknown
YGR125W	0.299311	0.00879778		Biological_process unknown
YGR013W	0.299666	0.000541304	*SNU71*	mRNA splicing
YFL062W	0.300873	0.00266677	*COS4*	Biological_process unknown
YIR005W	0.302386	0.00366156	*IST3*	Spliceosome assembly
YBR274W	0.304242	0.000531114	*CHK1*	Cell cycle
YER155C	0.307165	0.00639349	*BEM2*	Cell wall organization and biogenesis
YMR154C	0.31271	0.00301347	*RIM13*	Protein processing
YOR095C	0.316637	0.00415446	*RKI1*	Pentose-phosphate shunt
YGL195W	0.317785	0.000552945	*GCN1*	Regulation of translational elongation
YLR216C	0.318424	0.000249636	*CPR6*	Protein folding
YMR212C	0.321155	0.00195972	*EFR3*	Cellular morphogenesis
YPL107W	0.321635	0.00131342		Biological_process unknown
YJR148W	0.324798	0.00196076	*BAT2*	Branched chain family amino acid biosynthesis
YGR089W	0.326989	0.00243343		Biological_process unknown
YGR210C	0.328935	0.00773063		Biological_process unknown
YHL029C	0.329973	0.00751725		Biological_process unknown
YIL115C	0.332177	0.00131613	*NUP159*	mRNA-nucleus export
YGL078C	0.333358	0.00721719	*DBP3*	35S primary transcript processing

Supplementary Table 4: Continued

ORF	Ratio	p-value	Gene	Function[A]
YKL040C	0.333487	0.00298902	*NFU1*	Iron homeostasis
YDL148C	0.33377	0.00594552	*NOP14*	Processing of 20S pre-rRNA
YOR027W	0.335508	0.000626936	*STI1*	Protein folding
YJL004C	0.33588	0.000867871	*SYS1*	Golgi to endosome transport
YGL054C	0.336884	0.00429032	*ERV14*	ER to Golgi transport
YOR076C	0.336952	0.00745062	*SKI7*	mRNA catabolism
YNL096C	0.337109	0.000697334	*RPS7B*	Protein biosynthesis
YHR056C	0.337487	2.63E-006	*RSC30*	Regulation of transcription
YLR144C	0.33835	0.00267745	*ACF2*	Actin cytoskeleton organization and biogenesis
YER174C	0.339101	0.00473987	*GRX4*	Response to oxidative stress
YHR169W	0.340046	0.00253717	*DBP8*	35S primary transcript processing
YMR015C	0.340239	0.00369995	*ERG5*	Ergosterol biosynthesis
YHR053C	0.342126	3.98E-007	*CUP1-1*	Response to copper
YOR094W	0.34226	0.00186804	*ARF3*	Intracellular protein transport
YDR190C	0.343284	0.00699859	*RVB1*	Regulation of transcription from Pol II promoter
YER108C	0.344204	0.00955237		
YLR064W	0.3462	0.000777641		Biological_process unknown
YBR236C	0.34621	0.00486124	*ABD1*	mRNA capping
YPL240C	0.34649	0.00831054	*HSP82*	Response to stress
YLR225C	0.350284	1.29E-005		Biological_process unknown
YER024W	0.352328	0.00565482	*YAT2*	Alcohol metabolism
YLR130C	0.354232	0.00444561	*ZRT2*	Low-affinity zinc ion transport
YGL198W	0.354432	0.00972168		Biological_process unknown
YHR151C	0.354733	0.00130369		Biological_process unknown
YHR066W	0.355114	3.86E-005	*SSF1*	Ribosomal large subunit assembly and maintenance
YDR076W	0.357026	0.00348471	*RAD55*	Double-strand break repair via break-induced Replication
YMR115W	0.358725	0.00460831		Biological_process unknown
YDR021W	0.360209	0.00475866	*FAL1*	mRNA splicing
YER127W	0.360859	0.00224524	*LCP5*	rRNA modification
YCR018C	0.360952	0.000998158	*SRD1*	rRNA processing
YCR094W	0.361835	0.00339265	*CDC50*	G1 phase of mitotic cell cycle
YIL145C	0.36445	0.00389448	*PAN6*	Pantothenate biosynthesis
YIL118W	0.365044	0.00196761	*RHO3*	Establishment of cell polarity (sensu *Saccharomyces*)
YEL050C	0.367563	0.00311728	*RML2*	Protein biosynthesis
YBL097W	0.368865	0.0013743	*BRN1*	Mitotic chromosome segregation
YNL290W	0.370624	0.000942904	*RFC3*	Mismatch repair
YHR133C	0.37141	0.000711336		Biological_process unknown
YFR045W	0.372028	0.00429857		Transport
YPR060C	0.372409	0.00116551	*ARO7*	Aromatic amino acid family biosynthesis
YBR171W	0.373859	0.00523156	*SEC66*	SRP-dependent
YMR215W	0.374113	0.00300099	*GAS3*	Biological_process unknown
YLR107W	0.375112	0.00820099	*REX3*	RNA processing
YFL016C	0.375225	0.000338022	*MDJ1*	Protein folding
YHL013C	0.375309	0.00916439		Biological_process unknown
YIL114C	0.379591	0.00917329	*POR2*	Ion transport
YDL170W	0.379713	0.00911708	*UGA3*	Regulation of transcription from Pol II promoter
YGL164C	0.379735	0.00888325		Biological_process unknown
YDL077C	0.38018	0.00897013	*VAM6*	Homotypic vacuole fusion (non-autophagic)

Supplementary Table 4: Continued

ORF	Ratio	p-value	Gene	Function[A]
YDL078C	0.38072	0.00615009	*MDH3*	Fatty acid beta-oxidation
YML130C	0.384861	0.00316342	*ERO1*	Protein folding
YHR055C	0.385142	6.63E-007	*CUP1-2*	Response to copper
YHR149C	0.387061	0.00395958		Biological_process unknown
YLR050C	0.387876	0.00457147		Biological_process unknown
YNL069C	0.38854	0.00370926	*RPL16B*	Protein biosynthesis
YBR260C	0.388814	0.00214082	*RGD1*	Osmosensory signaling pathway
YHR196W	0.389516	0.000590692	*UTP9*	Processing of 20S pre-rRNA
YHR069C	0.389771	0.00207173	*RRP4*	35S primary transcript processing
YGR285C	0.389839	0.00149639	*ZUO1*	Protein folding
YCR054C	0.393364	0.00235117	*CTR86*	Biological_process unknown
YOL041C	0.394095	0.000829875	*NOP12*	rRNA metabolism
YLL062C	0.396372	0.000473078	*MHT1*	Sulfur amino acid metabolism
YGL130W	0.398401	0.00334348	*CEG1*	mRNA capping
YDR049W	0.399712	0.000327793		Biological_process unknown
YLL003W	0.401038	0.00192042	*SFI1*	G2/M transition of mitotic cell cycle
YMR208W	0.401535	0.00517293	*ERG12*	Ergosterol biosynthesis
YCL041C	0.401853	0.00931867		Biological_process unknown
YNL103W	0.402034	0.00560719	*MET4*	Positive regulation of transcription from Pol II promoter
YJL008C	0.402069	0.00792393	*CCT8*	Protein folding
YJR098C	0.406531	0.00178897		Biological_process unknown
YDR079W	0.409513	0.000790037	*PET100*	Aerobic respiration
YBR142W	0.410387	0.00065717	*MAK5*	rRNA processing
YKR053C	0.413159	0.00404731	*YSR3*	Sphingolipid biosynthesis
YLR007W	0.413356	0.00506516	*NSE1*	DNA repair
YDL010W	0.413508	0.000385204		Biological_process unknown
YDL069C	0.414159	0.00927969	*CBS1*	Protein biosynthesis
YLR003C	0.415466	0.00122484		Biological_process unknown
YGL120C	0.419695	0.00451785	*PRP43*	Lariat formation
YHR204W	0.421675	0.00435301	*MNL1*	ER-associated protein catabolism
YIR008C	0.421892	0.00326044	*PRI1*	DNA replication initiation
YDR151C	0.425222	0.00806595	*CTH1*	mRNA capping
YGR038W	0.425548	0.000940796	*ORM1*	Biological_process unknown
YJR013W	0.425712	0.00197773		Biological_process unknown
YHL036W	0.425868	0.000664507	*MUP3*	Amino acid transport
YOL032W	0.427372	0.000343769		Biological_process unknown
YER168C	0.432021	0.00151759	*CCA1*	tRNA modification
YJL033W	0.432202	0.004348	*HCA4*	35S primary transcript processing
YDR101C	0.432726	0.0051495	*ARX1*	Biological_process unknown
YMR235C	0.432777	0.00567605	*RNA1*	rRNA-nucleus export
YGR283C	0.434021	0.000801174		Biological_process unknown
YDR331W	0.437661	0.00882592	*GPI8*	Attachment of GPI anchor to protein
YGR103W	0.437863	0.000117094	*NOP7*	Ribosomal large subunit biogenesis
YDR363W	0.438746	0.00651015	*ESC2*	Chromatin silencing at HML and HMR (sensu *Saccharomyces*)
YCR055C	0.439318	0.0022347		
YKR056W	0.439626	0.00273826	*TRM2*	tRNA modification
YER157W	0.441103	0.00821573	*COG3*	ER to Golgi transport
YKL077W	0.441342	0.00383814		Biological_process unknown

Supplementary Table 4: Continued

ORF	Ratio	p-value	Gene	Function[A]
YBR096W	0.44222	0.000547537		Biological_process unknown
YDR256C	0.442513	0.00396512	*CTA1*	Oxygen and reactive oxygen species metabolism
YDL132W	0.447476	0.00549727	*CDC53*	Ubiquitin-dependent protein catabolism
YHR170W	0.447705	0.00485433	*NMD3*	Ribosomal large subunit assembly and maintenance
YML082W	0.44859	0.00314404		Biological_process unknown
YOR070C	0.450166	0.00995198	*GYP1*	Vesicle-mediated transport
YDR303C	0.453676	0.00522213	*RSC3*	Regulation of transcription
YGR163W	0.453684	0.00370521	*GTR2*	Biological_process unknown
YKL203C	0.455178	0.000427948	*TOR2*	Signal transduction
YBR164C	0.455844	0.00924788	*ARL1*	Vesicle-mediated transport
YHR034C	0.458429	0.0060748		Biological_process unknown
YGR033C	0.458708	0.00553892		Biological_process unknown
YOR004W	0.460683	0.00665536		Biological_process unknown
YGR289C	0.463105	0.00764202	*MAL11*	Alpha-glucoside transport
YLR221C	0.463825	0.000686356	*RSA3*	Biological_process unknown
YDR195W	0.464022	0.00754475	*REF2*	mRNA processing
YOR259C	0.464538	0.0072646	*RPT4*	Ubiquitin-dependent protein catabolism
YKR004C	0.465387	0.0048484	*ECM9*	Cell wall organization and biogenesis
YJL213W	0.466236	0.000327207		Biological_process unknown
YDR324C	0.467056	0.0092265	*UTP4*	Processing of 20S pre-rRNA
YBL036C	0.467111	0.0068624		Biological_process unknown
YLL020C	0.468163	0.00735591		Biological_process unknown
YFL048C	0.471038	0.00025773	*EMP47*	ER to Golgi transport
YGR145W	0.471242	0.000851635	*ENP2*	Biological_process unknown
YKL154W	0.471299	0.00894014	*SRP102*	Protein-ER targeting
YDR361C	0.471521	0.00688969	*BCP1*	Biological_process unknown
YGR095C	0.471709	0.000764242	*RRP46*	35S primary transcript processing
YNR032W	0.474949	0.00246087	*PPG1*	Glycogen metabolism
YOL144W	0.475082	0.00262779	*NOP8*	rRNA processing
YLL027W	0.475983	0.002285	*ISA1*	Iron transport
YOR262W	0.477691	0.00976378		Biological_process unknown
YLL007C	0.478717	0.00460719		Biological_process unknown
YDR214W	0.479047	0.00274744	*AHA1*	Biological_process unknown
YGL181W	0.481751	0.000133289	*GTS1*	Sporulation (sensu Saccharomyces)
YLR002C	0.483422	0.00169866	*NOC3*	rRNA processing
YLR227C	0.489928	0.000119289	*ADY4*	Sporulation
YCR016W	0.490415	0.00316646		Biological_process unknown
YDR171W	0.490531	8.97E-005	*HSP42*	Response to stress
YDL150W	0.493503	0.00989263	*RPC53*	Transcription from Pol III promoter
YKR066C	0.495747	0.00358951	*CCP1*	Response to oxidative stress
YHR020W	0.497427	0.00761653		Biological_process unknown
YEL006W	0.498749	0.00778714		Transport
YDR312W	0.499926	0.00784585	*SSF2*	Ribosomal large subunit assembly and maintenance
YOR247W	2.01222	0.000386155	*SRL1*	Nucleobase
YDR154C	2.0237	7.56E-005		Biological_process unknown
YKR080W	2.04639	0.000375498	*MTD1*	One-carbon compound metabolism
YCL018W	2.05233	0.000348227	*LEU2*	Leucine biosynthesis
YHR025W	2.05372	0.000114249	*THR1*	Methionine metabolism
YKL060C	2.05812	0.000358903	*FBA1*	Gluconeogenesis

Supplementary Table 4: Continued

ORF	Ratio	p-value	Gene	Function[A]
YCR032W	2.05951	0.00365435	*BPH1*	Response to pH
YHR049W	2.07025	0.000209557	*FSH1*	Biological_process unknown
YLR303W	2.0802	0.000385799	*MET17*	Methionine metabolism
YFL-TYA	2.086	0.00408508		
YPL271W	2.09727	1.43E-005	*ATP15*	ATP synthesis coupled proton transport
YBL005W-A	2.10063	8.63E-005		
YNR009W	2.1042	0.005928		Biological_process unknown
YBL002W	2.11497	0.000195983	*HTB2*	Chromatin assembly/disassembly
YOR248W	2.11557	2.71E-005		Biological_process unknown
YHR029C	2.13287	0.000358761		Biological_process unknown
YJR028W	2.13489	0.00103225		
YNL134C	2.13967	8.96E-005		Biological_process unknown
YER172C	2.14418	0.000358668	*BRR2*	Lariat formation
YAR015W	2.14705	0.000318693	*ADE1*	Purine base metabolism
YAL040C	2.1542	8.45E-005	*CLN3*	G1/S transition of mitotic cell cycle
YNL030W	2.15452	0.00534383	*HHF2*	Chromatin assembly/disassembly
YAR010C	2.15567	1.46E-005		
YCLX10C	2.19033	0.000323545		
YJR026W	2.1916	0.00120061		
YNR022C	2.19683	0.0023424	*MRPL5 0*	Protein biosynthesis
YKL153W	2.19708	9.79E-006		Biological_process unknown
YAL044C	2.20571	2.01E-007	*GCV3*	One-carbon compound metabolism
YMR046C	2.23124	0.000180343		
YBL101W-A	2.23564	3.41E-007		
YCL008C	2.26208	0.000172662	*STP22*	Protein-vacuolar targeting
YBR009C	2.277	0.0030872	*HHF1*	Chromatin assembly/disassembly
YPL031C	2.28497	0.00114161	*PHO85*	Protein amino acid phosphorylation
YLR109W	2.29352	1.15E-006	*AHP1*	Regulation of redox homeostasis
YBR012W-A	2.31756	0.00152655		
YLR194C	2.33043	0.000158075		Biological_process unknown
YDL055C	2.37734	0.000383171	*PSA1*	Protein amino acid glycosylation
YPR130C	2.40332	0.00421118		Biological_process unknown
YNL058C	2.4129	0.00276898		Biological_process unknown
YJL066C	2.42848	0.00112734	*MPM1*	Biological_process unknown
YBR173C	2.45641	1.82E-005	*UMP1*	Protein catabolism
YCR004C	2.45924	0.000107159	*YCP4*	Biological_process unknown
YPR126C	2.46424	0.00972435		Biological_process unknown
YCL020W	2.47199	0.00341342		
YER177W	2.47447	0.000593076	*BMH1*	Pseudohyphal growth
YPL252C	2.50048	7.73E-005	*YAH1*	Heme a biosynthesis
YMR199W	2.5009	2.35E-006	*CLN1*	G1/S transition of mitotic cell cycle
YJL158C	2.50222	0.0010844	*CIS3*	Cell wall organization and biogenesis
YLR237W	2.51013	0.00140223	*THI7*	Thiamin transport
YBL064C	2.51237	0.000285283		Regulation of redox homeostasis
YDR158W	2.51826	9.93E-005	*HOM2*	Methionine metabolism
YKR058W	2.52546	0.00147385	*GLG1*	Glycogen biosynthesis
YMR051C	2.54606	0.00386494		
YDL198C	2.56962	0.000387988	*YHM1*	Transport
YHR183W	2.60927	7.93E-005	*GND1*	Glucose metabolism

Supplementary Table 4: Continued

ORF	Ratio	p-value	*Gene*	Function[A]
YCR046C	2.61735	0.000352394	*IMG1*	Protein biosynthesis
YER052C	2.61985	0.000131508	*HOM3*	Methionine metabolism
YCL030C	2.63761	0.000111603	*HIS4*	Histidine biosynthesis
YAR068W	2.66286	4.89E-005		Biological_process unknown
YKL097W-A	2.70799	4.78E-007		
YHR214W-A	2.71177	0.000206786		Biological_process unknown
YMR119W-A	2.73828	0.00161108		Biological_process unknown
YBR012W-B	2.74307	1.14E-005		
YDL131W	2.84261	4.94E-006	*LYS21*	Lysine biosynthesis
YFL021W	2.87832	1.28E-005	*GAT1*	Transcription initiation from Pol II promoter
YDL179W	2.8947	0.00360139	*PCL9*	Cell cycle
YGL037C	2.92871	5.49E-006	*PNC1*	Chromatin silencing at telomere
YMR173W	2.94607	0.00124729	*DDR48*	DNA repair
YNL078W	2.97141	0.000154484	*NIS1*	Regulation of mitosis
YBR214W	2.97653	1.60E-005	*SDS24*	Meiosis
YLR286C	2.97756	0.00120566	*CTS1*	Cytokinesis
YDR380W	2.98955	0.000165938	*ARO10*	Leucine catabolism
YGL121C	3.03448	0.00417774	*GPG1*	Signal transduction
YDR019C	3.07179	0.00181711	*GCV1*	One-carbon compound metabolism
YNL300W	3.15738	0.000302431		Biological_process unknown
YCLX11W	3.22222	1.32E-006		
YIL119C	3.27068	0.000151216	*RPI1*	Thiamin biosynthesis
YMR097C	3.29959	0.000394149	*MTG1*	Biological_process unknown
YNR037C	3.31739	0.000374499	*RSM19*	Protein biosynthesis
YJL088W	3.32675	0.00150877	*ARG3*	Arginine biosynthesis
YPL014W	3.36592	0.00269871		Biological_process unknown
YDR007W	3.3912	1.96E-006	*TRP1*	Tryptophan biosynthesis
YKL043W	3.51451	0.000713758	*PHD1*	Pseudohyphal growth
YJL067W	3.5361	0.00714499		Biological_process unknown
YMR276W	3.5427	1.64E-005	*DSK2*	Spindle pole body duplication (sensu *Saccharomyces*)
YBR147W	3.58883	5.37E-005		Biological_process unknown
YOR192C	3.67894	0.00123757		Transport
YDL182W	3.7091	1.69E-006	*LYS20*	Lysine biosynthesis
YER175C	3.89457	0.000760784	*TMT1*	Biological_process unknown
YMR321C	3.90822	0.000295953		Biological_process unknown
YJR025C	3.92418	0.00057755	*BNA1*	Nicotinamide adenine dinucleotide biosynthesis
YDL048C	3.96486	1.06E-005	*STP4*	Biological_process unknown
YMR173W-A	4.13281	3.20E-005		Biological_process unknown
YKR075C	4.37146	1.15E-005		Biological_process unknown
YBL029W	4.52298	1.12E-005		Biological_process unknown
YDR008C	4.54938	2.09E-006		Biological_process unknown
YNL208W	4.88215	3.36E-005		Biological_process unknown
YOL055C	5.03223	1.49E-005	*THI20*	Thiamin biosynthesis
YHR071W	5.34532	0.00161831	*PCL5*	Cell cycle
YBL003C	6.17036	3.81E-005	*HTA2*	Chromatin assembly/disassembly
YLR004C	6.246	3.97E-005		Biological_process unknown
YDR277C	6.33555	4.83E-005	*MTH1*	Signal transduction
YIR019C	6.404	0.00112353	*MUC1*	Pseudohyphal growth
YGL117W	6.87523	1.06E-005		Biological_process unknown

Supplementary Table 4: Continued

ORF	Ratio	p-value	Gene	Function[A]
YGR286C	6.8805	0.000801267	*BIO2*	Biotin biosynthesis
YDR464W	7.06046	0.00320997	*SPP41*	Negative regulation of transcription from Pol II promoter
YMR094W	8.11124	0.00442289	*CTF13*	Mitosis
YNR058W	8.35143	0.000589213	*BIO3*	Biotin biosynthesis
YOL058W	8.356	3.07E-005	*ARG1*	Arginine biosynthesis
YJR073C	8.50526	0.000335702	*OPI3*	Phosphatidylcholine biosynthesis
YJR155W	9.84365	0.00185956	*AAD10*	Aldehyde metabolism
YLR327C	11.2823	2.77E-006		Biological_process unknown
YMR195W	17.364	0.000468006	*ICY1*	Biological_process unknown
YNR057C	18.2823	0.000265922	*BIO4*	Biotin biosynthesis
YEL009C	32.7697	0.00141982	*GCN4*	Regulation of transcription from Pol II promoter
YMR096W	35.1403	0.000385527	*SNZ1*	Thiamin biosynthesis

[A] according to the *Saccharomyces* Genome Database (http://www.yeastgenome.org)

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Curriculum vitae

Malte Kleinschmidt

Geburtsdatum:	14.08.1974
Geburtsort:	Hoya/Weser
1981 – 1983	Grundschule Scholen
1983 – 1985	Grundschule Asendorf
1985 – 1987	Orientierungsstufe Bruchhausen-Vilsen
1987 – 1991	Realschule Bruchhausen-Vilsen
1991 – 1994	Gymnasium Sulingen
1994 – 1995	Zivildienst am Landesbildungszentrum für Hörgeschädigte Osnabrück
1995 – 2001	Studium Diplom Biologie an der Georg-August-Universität zu Göttingen
Febr. 2000	Diplomprüfungen (Hauptfach: Mikrobiologie; Nebenfächer: Genetik, Organische Chemie)
2000 – 2001	Diplomarbeit am Institut für Mikrobiologie und Genetik bei Prof. Dr. Gerhard H. Braus Thema: „Charakterisierung des WD-Proteins Cpc2p in *Saccharomyces cerevisiae* “
April 2001 – September 2005	Doktorarbeit am Institut für Mikrobiologie und Genetik der Georg-August-Universität zu Göttingen bei Prof. Dr. Gerhard H. Braus Thema: „Regulation of gene expression and adhesion in *Saccharomyces cerevisiae*“