Localization and Stability of the Transcriptional Activator Gcn4p of *Saccharomyces cerevisiae*

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Ralph Pries
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Contents

Summary

The ´general amino acid control´ (GAAC) of the yeast *Saccharomyces cerevisiae* is a well characterized regulatory network which secures a sufficient amino acid supply of the organism. This system can be induced by different environmental stimuli like e. g. amino acid starvation, purine limitation, UV radiation, glucose limitation or high salinity. Induction of the general amino acid control results in the transcriptional activation of more than 500 target genes from many different biosynthetic pathways. In the last decades numerous investigations have broadened the understanding of this network and primarily of its central trancriptional activator protein Gcn4p.

Gcn4p represents a classical bZIP transcription factor, which recognizes as a homodimer conserved consensus sites within the promoter regions of its target genes. Gcn4p is known as a weakly expressed and highly unstable protein in sated cells. The Gcn4p translation rate and protein stability significantly increase in response to amino acid limitation.

One aim of this work was to localize *S. cerevisiae* Gcn4p and the homologous protein CPCA of the mold *Aspergillus nidulans* within the cell. Fluorescence microscopy demonstrated that Gcn4p/CPCA are predominantly nuclear proteins, independently of the presence or absence of sufficient amounts of amino acids. Deletion and heterologous transfer experiments revealed that nuclear import of *S. cerevisiae* Gcn4p is secured by two functional nuclear localization sequences (NLS), from which only one is conserved in *A. nidulans* CPCA. Analyses of a set of *S. cerevisiae* importin mutant strains gave evidence that Gcn4p translocation requires importin α Srp1p and importin β Kap95p.

A second aim was to study protein stability regulation of Gcn4p. Regulation of stability of Gcn4p was dependent on its subcellular localization, demonstrating a compartment specific degradation of Gcn4p within the nucleus. The cyclin dependent kinase (CDK) Pho85p, which initiates Gcn4p decay by phosphorylation at Thr165, is as well as Gcn4p a predominantly nuclear protein. Pho85p activity is affected by the CDK inhibitor Pho81p in cells starved for amino acids. Preventing Gcn4p to enter the yeast nucleus either by deleting its NLSs or because of an affected nuclear import cycle in a *yrb1* mutant strain resulted in stabilized Gcn4p, indicating that efficient amino acid dependent Gcn4p stability regulation occurs exclusively in the yeast nucleus.

Zusammenfassung

Die `Allgemeine Kontrolle der Aminosäurebiosynthese` der Hefe *Saccharomyces cerevisiae* ist ein gut charakterisiertes regulatorisches Netzwerk, welches die ausreichende Aminosäureversorgung des Organismus gewährleistet. Dieses System wird durch Stimuli wie beispielsweise Aminosäuremangel, Purinmangel, UV Strahlung, Glukosemangel oder hohe Salzkonzentrationen aktiviert. Die Aktivierung der `Allgemeinen Kontrolle der Aminosäurebiosynthese` resultiert in einer gesteigerten Transkription von mehr als 500 Zielgenen aus vielen unterschiedlichen Biosynthesewegen. In den vergangenen Jahrzehnten haben zahlreiche Untersuchungen die Kenntnisse über dieses Netzwerk und dessen zentralen Transkriptionsaktivator Gcn4p enorm erweitert.

Gcn4p ist ein klassischer bZIP Transkriptionsaktivator, der als Homodimer an konservierte Konsensussequenzen in den Promotorregionen seiner Zielgene bindet. Bei ausreichender Aminosäureversorgung ist Gcn4p ein schwach exprimiertes und sehr instabiles Protein. Aminosäuremangel führt zu einer gesteigerten Expression und Stabilität von Gcn4p.

Ein Ziel dieser Arbeit war die Lokalisierung von Gcn4p aus *S. cerevisiae* und des homologen Proteins CPCA aus *Aspergillus nidulans*. Mittels Fluoreszenzmikroskopie wurde gezeigt, dass Gcn4p und CPCA unabhängig von der Aminosäureversorgung im Zellkern lokalisiert sind. Deletions- und heterologe Transportexperimente haben gezeigt, dass der Kerntransport von Gcn4p durch zwei funktionelle Kernlokalisierungssequenzen (NLS) vermittelt wird, von denen jedoch nur eine in CPCA von *A. nidulans* konserviert vorliegt. Untersuchungen verschiedener *S. cerevisiae* Importin-Mutantenstämme haben verdeutlicht, dass Importin α Srp1p und Importin β Kap95p für den Kerntransport von Gcn4p erforderlich sind. Des weiteren war es ein Anliegen dieser Arbeit, die Regulation der Proteinstabilität von Gcn4p zu untersuchen. Die Stabilität von Gcn4p ist abhängig von der intrazellulären Lokalisierung, wobei die spezifische Degradation von Gcn4p auf den Zellkern beschränkt ist. Die zyklinabhängige Kinase (CDK) Pho85p bewirkt durch Phosphorylierung von Thr165 die Degradation von Gcn4p und ist ebenfalls ein überwiegend im Zellkern lokalisiertes Protein. Die Aktivität von Pho85p wird unter Aminosäuremangelbedingungen durch den CDK Inhibitor Pho81p beeinflusst. Ein gestörter Gcn4p-Kerntransport durch Deletion der NLS Motive oder durch einen defekten Transportmechanismus in einer *yrb1* Mutante resultiert in stabilisiertem Gcn4p, was belegt, dass die Regulation der Gcn4p-Stabilität in Abhängigkeit von der Aminosäureversorgung im Zellkern stattfindet.

Chapter 1

Introduction

1. Regulation of protein stability in *S. cerevisiae*

Selective and programmed protein degradation represents an important regulatory tool for distinct biological processes in *Saccharomyces cerevisiae*. Numerous regulatory proteins such as cyclins, cyclin dependent kinases and transcription factors have an altering stability with respect to different environmental cues. These differences are essential in order to control cellular events like cell cycle progression or various biosynthetic pathways. *S. cerevisiae* is a eukaryotic model organism which is able to adapt quickly to changing metabolic conditions by stabilizing or destabilizing proteins. Distinct endomembrane organelles allow *S. cerevisiae* to carry out competing processes by separating them into different compartments like e. g. biosynthesis and degradation with respect to the particular requirements. Protein degradation in yeast occurs either in the vacuole or via the proteasome (and the mitochondrium, which will not be discussed) (for a review see Hochstrasser, 1995).

1. 1. Vacuolar protein degradation

The vacuole of *S. cerevisiae* shares features with lysosomes and comprises about 25 % of the cellular volume, whereas its morphology is correlated with the cell cycle state (Wiemken and Durr, 1974; Jones *et al*., 1993). Exponentially growing yeast cells often harbour a multilobed vacuole with interconnections, whereas cells of the stationary phase typically have a single large vacuole (Jones *et al.*, 1993). The vacuole functions as reservoir for phospate, amino acids, allantoin, and serveral divalent cations, whereas polyphosphates are used to secure a neutral cytoplasmic pH. In addition, the vacuole is known to play an important role for turnover of long-lived proteins (Serrano, 1991). Therefore it contains various endo- and exoproteases in its acidic matrix, which is maintained at pH 6.2 (Preston *et al*., 1989). Delivery of proteins, which are designated for vacuolar degradation occurs either directly from the Golgi cisternae by a normal delivery pathway, selective endocytosis or via

autophagy (Jones and Murdock, 1994). In the process of autophagy, whole organelles and cytosol are taken up for degradation (Khalfan and Klionsky, 2002). An example is the methylotrophic yeast *Hansenula polymorpha*, which synthesizes large peroxisomes when grown on methanol. When methanol is replaced by glucose as carbon source, the peroxisomes and the enzymes within are rapidly degraded by vacuolar hydrolases (van der Klei and Veenhuis, 2002). About 40 % of protein degradation in yeast can be attributed to vacuolar proteolysis, whereas short-lived proteins regulating transcription and cell cycle are degraded presumably by the proteasome, as well as defective proteins (Teichert *et al*., 1989).

1. 2. Protein degradation by the 26S proteasome

The ubiquitin system is involved in endocytosis and downregulation of transporters and triggers the selective proteasomal degradation of many short-lived proteins in eukaryotic cells. In addition, there are strong indications that the ubiquitin system is involved in development and apoptosis. Proteins are targeted for degradation by covalent ligation of ubiquitin, which is a highly conserved 76-amino-acid-residue protein (Hochstrasser, 1996).
The ubiquitin pathway can be subdivided in three steps: (I) ATP dependent activation of the C-terminal Gly residue of ubiquitin by a specific activating enzyme E1; (II) Transfer of activated ubiquitin to an active site Cys residue of a ubiquitin-carrier protein, E2; (III) catalyzed by a ubiquitin-protein ligase E3, ubiquitin is linked by its C-terminus in an amide isopeptide linkage to an ε-amino group of the substrate protein´s Lys residues (Figure 1). Ubiquitin itself is thought to dissociate from its substrate protein before proteasomal degradation and therefore can be directly used for the next round of ubiquitination (Hochstrasser, 2002). Usually there are various species of E2 and E3 multiprotein complexes, but only a single E1 (for a review see Hershko and Ciechanover, 1998). In *S. cerevisiae* 13 genes have been identified that encode E2-like proteins, which are also called Ubiquitin-conjugating enzymes or Ubc (Hochstrasser, 1996). Though E3 ubiquitin-protein ligases play an important role in determining protein degradation, they are difficult to identify, since they are associated with large multisubunit complexes, and it is often unclear which subunits are responsible for the ubiquitin-protein ligase activities. Ligation of polyubiquitin chains results in ATP dependent protein degradation by the 26S proteasome complex, which is assembled of a 20S proteasome, that contains the protease catalytic sites, with a 19S cap (Rechsteiner,

1997) (Figure 1). It was recently shown that the 20S proteasomes are imported as precursor complexes into the nucleus of *S. cerevisiae*, suggesting that nuclear 20S proteasomes are finally matured inside the nucleus (Lehmann *et al.*, 2002).

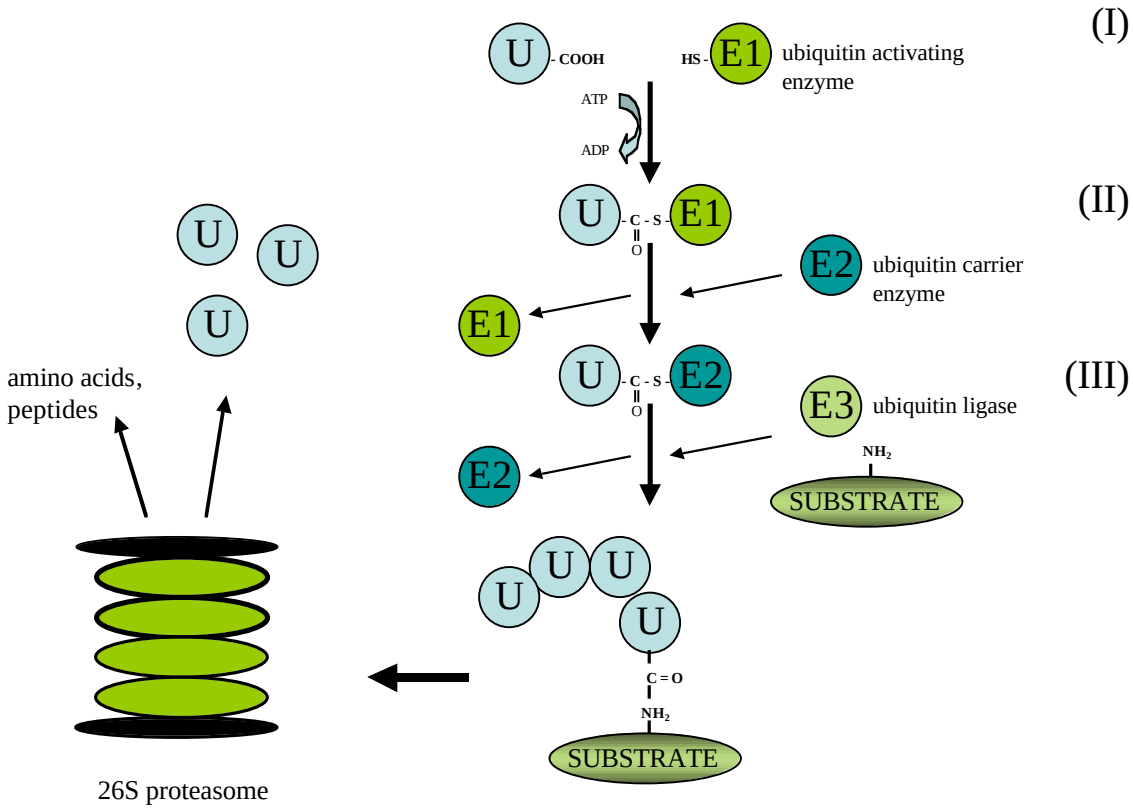

Figure 1: Model of the ubiquitin system. Illustrated are the three steps of the ubiquitin pathway that result in protein degradation at the 26S proteasome: (I) ATP dependent activation of the C-terminal Gly residue of ubiquitin (U) by a specific activating enzyme E1; (II) activated ubiquitin is transferred to an active site Cys residue of a ubiquitin-carrier protein, E2; (III) the ubiquitin-protein ligase E3 catalyzes ubiquitin linkage to the substrate protein´s Lys residues. Protein degradation at the 26S proteasome results in amino acids, peptides and ubiquitin molecules, whereas ubiquitin dissociates from its substrate protein and therefore it is not degraded.

Whereas the ubiquitination and degradation of mitotic cyclins and other cell-cycle regulators is determined by an N-terminal destruction box (Sudakin *et al.*, 1995), it was previously shown that many rapidly degraded proteins are targeted to the proteasome by phosphorylation. Phosphorylation of specific residues or within PEST regions, which are enriched in Pro, Glu, Ser, and Thr residues, is required for example for the ubiquitination and degradation of the yeast G1 cyclins Cln2 and Cln3 (Lanker *et al.*, 1996) or the transcription

factor Gcn4p, which controls amino acid biosynthesis in *S. cerevisiae* (Meimoun *et al*., 2000, see also the following chapters).

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

Every organism has to secure a sufficient amino acid supply, either by amino acid uptake from the environment or via *de novo* amino acid biosynthesis. Fungi are enabled to synthesize all 20 amino acids. In some fungi a tremendous regulatory network has been characterized, which upregulates the transcription of a huge number of genes from many different biosynthetic pathways in response to starvation for a single amino acid.

Regulatory networks of amino acid biosynthesis were first identified in the filamentous ascomycetes *Neurospora crassa* (Carsiotis *et al*., 1974) and *Aspergillus nidulans* (Piotrowska, 1980), where it was recognized that proline starvation resulted in an increased activity of enzymes belonging to the arginine biosynthetic pathway. Furthermore, it could be shown for *Neurospora crassa* that starvation for a single amino acid activates the majority of the 20 amino acid biosynthetic pathways (Barthelmess and Kolanus 1990; Kolanus *et al*., 1990).

In the bakers´ yeast *Saccharomyces cerevisiae* this network is well characterized and known as the `general amino acid control` (GAAC) (Hinnebusch and Natarajan, 2002), whereas the homologous system in the filamentous fungus *Aspergillus nidulans* is called `cross pathway control`. The central transcriptional activator proteins of these regulatory networks are *S. cerevisiae* Gcn4p and *A. nidulans* CPCA, respectively. CPCA of *A. nidulans* and as well the homologous proteins cpcA of the related fungus *Aspergillus niger* or CPC1 of *Neurospora crassa* have got strong similarities with *S. cerevisiae* Gcn4p and are functionally exchangeable (Hoffmann *et al.*, 2001; Wanke *et al.*, 1997). They all belong as also CpCPC1 of *Cryphonectria parasitica* (Wang *et al.*, 1998) or even human c-Jun to the AP1 family of transcription factors, which are known to be involved in cell proliferation in mammalian cells.

2. 1. Recognition and regulation of the amino acid availability

Sensing of the extra- as well as intracellular concentrations of available amino acids represents an essential part of the functioning of the amino acid supply of any cell. In the past

decades, research on fungi has broadened the understanding of how eukaryotic cells take inventories of the nutrient conditions and react accordingly. Whereas intracellular fungal sensors for monitoring the internal amino acid pool have been investigated for several years, the detection of sensors for amino acids in the environment is more recent and yet restricted to the budding yeast.

The intracellular ribosome-attached kinase Gcn2p of the yeast *S. cerevisiae* (Wek *et al*., 1995) functions as sensor of amino acids. A homologous protein (CPC3) was as well identified in the mold *N. crassa* (Sattlegger *et al*., 1998). Gcn2p monitors amino acids indirectly by recognition of uncharged tRNA molecules. This leads to an increased Gcn4p translation, while the overall translation rate is diminished. Gcn2p like kinases seem to be typical for eukaryotic cells, although they react to different stimuli in other organisms.

Amino acid limitation leads to a stabilization of the normally short half-lived transcription factor Gcn4p. Part of this control are distinct cyclin dependent kinases (CDK). Cyclins are highly unstable proteins which are involved e. g. in various steps of the cell division cycle by activating the eukaryotic protein kinase Cdc2p/Cdc28p (Mendenhall and Hodge, 1998). The cyclin Pcl5p is part of an additional system controlling Gcn4p levels in the fungal cell. Whereas Gcn2p controls the synthesis of Gcn4p at the level of translation initiation, Pcl5p is involved in the regulated degradation of Gcn4p by activating Pho85p (Shemer *et al*., 2002).

There are further protein kinases, which are important for efficiently monitoring translation and inducing an appropriate cellular response. One highly conserved type of kinases is part of the target of rapamycin (TOR) pathway, an essential control network which acts to facilitate efficient translation. TOR seems to be important for cellular growth in response to nutrient availability in any fungal as well as any eukaryotic cell (Schmelzle and Hall, 2000). Fungal *TOR* genes are primarily analysed in yeast where two partially redundant kinases are present, Tor1p and Tor2p. Dominant mutations in *TOR1* and *TOR2* were isolated in a selection for yeast cells resistant to the immunosuppressant rapamycin. The TOR mediated starvation response cannot only be induced by the addition of rapamycin but also by a shift to a poor nitrogen source. The TOR network causes the ubiquitination and subsequent degradation of high affinity amino acid transporters like Tat2p (Beck *et al*., 1999; Schmidt *et al*., 1998). In contrast to Tat2p, the amino acid permease Gap1p is stabilized under low nitrogen conditions. Npr1p, a serine/threonine kinase, acts as key player in the inverse regulation of both Gap1p and Tat2p stability. The *NPR1* gene is in addition regulated by Gcn4p (Natarajan *et al*., 2001). Gap1p is the predominant transporter of a yeast cell growing in the presence of poor nitrogen sources in the absence of amino acids. Gap1p expression is also controlled by the Gcn4p

system. The addition of ammonium or glutamine to cells growing on poor nitrogen sources results in endocytic internalization combined with ubiquitination and subsequent degradation of Gap1p in the vacuole (Stanbrough and Magasanik, 1995).

When amino acids are present in the environment and transported into the fungal cell by the action of the various amino acid permeases, they become compartimentalized resulting in discrete amino acid pools. A large pool of basic amino acids like arginine and lysine and in addition histidine is primarily located in the vacuole. Arginine is high in nitrogen content and can be mobilized from the vacuolar storage room upon nitrogen starvation by the action of arginase activity. Other vacuolar amino acids as e. g. histidine cannot be catabolized by yeast and are only used for protein synthesis. Acidic amino acids are primarily located in the cytoplasm, whereas other amino acids are located in both compartments (Klionsky *et al*., 1990).

2. 2. General amino acid control of *S. cerevisiae*

Abundant investigations have been carried out concerning the general amino acid control (GAAC) of *S. cerevisiae*. A huge number of genes involved in the regulation of this network have been identified and subdivided into two groups. Mutations resulting in a loss of transcription activation function in response to amino acid limitation are called *GCN* (general control non-derepressable), whereas *GCD* (general control derepressed) summerizes mutations with a constitutively active GAAC (Harashima and Hinnebusch, 1986).

The general amino acid control is not only induced by amino acid limitation, but also in response to various environmental stimuli. Gcn4p is activated by limitation for purines (Mösch *et al.*, 1991) and tRNA synthetases (Meussdoerffer and Fink, 1983), UV radiation (Engelberg *et al.*, 1994), glucose limitation (Yang *et al.*, 2000), rapamycin treatment (Valenzuela *et al.*, 2001), and high salinity (Goossens *et al.*, 2001) (Figure 2). Because of the high basal expression level of many amino acid biosynthetic enzymes, shifting the cells from rich to minimal medium is not sufficient to induce the general amino acid control. To create amino acid limitation conditions, amino acid analoga like 3-amino-triazole (3AT) (Klopotowsky and Wiater, 1965), 5-methyl-tryptophan (5MT) (Schürch *et al.*, 1974) or 8-aza-adenine (8azA) (Rolfes and Hinnebusch, 1993) are added to the medium or alternatively auxotrophic or bradytrophic (leaky auxotrophic) mutant strains can be used (Kornitzer *et al.*, 1994).

Figure 2 illustrates the numerous stimuli, different regulatory mechanisms, and targets of yeast Gcn4p. In response to Gcn4p induction, more than 500 target genes from various different biosynthetic pathways are transcriptionally upregulated and likewise the same number of genes is downregulated (Natarajan *et al.*, 2001). Regulation of the transcriptional activator Gcn4p occurs at the levels of translation initiation, protein stability, and activity (Figure 2), which will be described in more detail in the following sections.

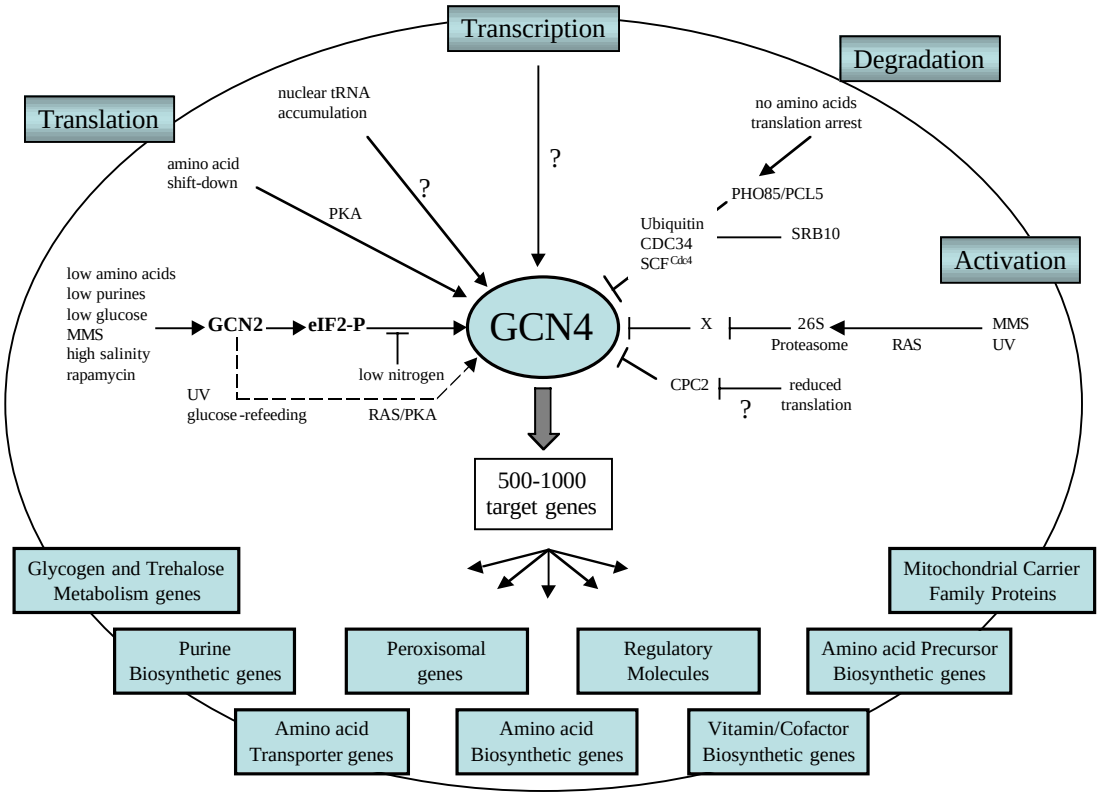

Figure 2: Model of the Gcn4p regulatory network. Gcn4p regulation occurs at different levels in response to various environmental stimuli and finally results in transcription activation of a huge number of target genes from many different biosynthetic pathways.

The Gcn4p bipartite DNA-binding motif consists of a leucine zipper dimerization domain and a highly charged basic region, that directly contacts DNA. In most cases transcriptional activation of Gcn4p target genes is triggered via consensus sequences (5`-ATGA (C/G) TCAT-3`) in their promoter regions (Arndt and Fink, 1986), called `general control responsive elements` (GCRE), whereas *Aspergillus niger* cpcA recognizes specifically 5`-ATGACTAAT-3` (Hinnebusch, 1984; Hope and Struhl, 1986). Furthermore it was recently

shown that Gcn4p is also able to bind with high affinity to DNA sites containing only a single Gcn4p consensus half-site (Hollenberg and Oakley, 2000).

The yeast Gcn4p dependent transcriptional activation is mediated by the coactivator multiprotein bridging factor (Mbf1p), which is highly conserved from *S. cerevisiae* to man and is required for bridging the DNA-binding region of Gcn4p and TATA-binding protein (Takemaru *et al.*, 1998).

2. 3. Gcn4p translational control

In *S. cerevisiae,* most of the environmental stimuli activate Gcn4p translation via the kinase Gcn2p, which consists of a C-terminal histidyl-tRNA synthetase (HisRS) related domain and an N-terminal protein kinase domain (Harashima and Hinnebusch, 1986; Zhu *et al*., 1996; Wek *et al.*, 1995). *GCN4* mRNA is synthesized even under nonstarvation conditions, whereas starvation for amino acids leads to an increased mRNA translation (Abastado *et al.*, 1991). When cells are objected to amino acid limitation, uncharged tRNA molecules are recognized by Gcn2p, which thereupon phosphorylates the α subunit of the eukaryotic elongation factor eIF2 on serine 51. Therefore the guanine nucleotide exchange factor eIF2B is inhibited to exchange bound GDP for GTP. This results in a downregulation of the cellular translation efficiency caused by lower amounts of ternary complexes containing eIF2, Met-tRNA$_i^{Met}$ and GTP. The entire yeast protein Gcn2p has a deduced molecular weight of approximately 180 kDa including 60 kDa for the HisRS segment.

Gcn2p function *in vivo* requires the Gcn1p/Gcn20p complex which binds to the N-terminal domain of Gcn2p (Garcia-Barrio *et al.*, 2000; Kubota, *et al*., 2001; Kubota, *et al.*, 2000). *GCN1* and *GCN20* share sequence similarities with translation elongation factor EF3 (Marton *et al*., 1993; Vazquez de Aldana *et al.*, 1995). Gcn1p seems to bind close to the ribosomal acceptor site and is assumed to promote Gcn2p activation by uncharged tRNA molecules.

While the translation efficiency in general is negatively affected, translation of the normally weakly translated *GCN4* mRNA increases because of four short upstream open reading frames (uORFs) in its 5´ leader sequence, which prevent efficient *GCN4* mRNA translation under non starvation conditions. Under these conditions ribosomes start translation at uORF1 and about 50% of the ribosomes continue scanning as 40S ribosomal subunits, reinitiate at uORF4 and dissociate from the mRNA before reaching the *GCN4* coding sequence. The

lower amount of ternary complexes under starvation conditions leads to an affected ribosome reinitiation, which therefore occurs at the *GCN4* start codon instead of uORF4 (Figure 3).

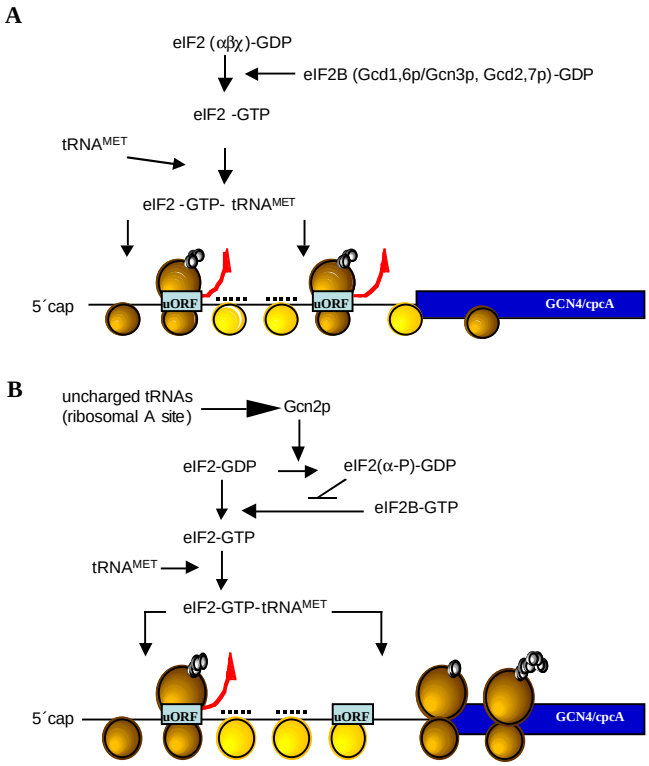

Figure 3: Translational control of *S. cerevisiae* Gcn4p under nonstarvation (A) and starvation (B) conditions. When cells are objected to amino acid limitation, uncharged tRNA molecules are recognized by Gcn2p, which thereupon phosphorylates the α subunit of the eukaryotic elongation factor eIF2 on serine 51. Therefore the guanine nucleotide exchange factor eIF2B is inhibited to exchange bound GDP for GTP, which results in a downregulation of the cellular translation efficiency caused by lower amounts of ternary complexes containing eIF2, Met-tRNA$_i^{Met}$, and GTP. The lower amount of ternary complexes under starvation conditions leads to an affected ribosome reinitiation, which therefore occurs at the *GCN4* start codon instead of uORF4.

Gcn4p activation was shown to be a biphasic process. Whereas amino acid limitation results in an increased translation of *GCN4* mRNA within about 20 min, an increased *GCN4* transcript level can be observed after 3-4 hours of starvation (Albrecht *et al.*, 1998).

In contrast, a repression of *GCN4* mRNA translation can be observed in response to nitrogen starvation, which occurs independently of eukaryotic initiation factor 2α (eIF2α) phosphorylation by Gcn2p (Grundmann *et al.*, 2001).

2. 4. Regulation of Gcn4p stability

In addition to the regulation of Gcn4p at the level of translation initiation, amino acid limitation leads to an increased stability of the highly unstable protein from a half-life of about 5 minutes or less to up to 20 minutes. Gcn4p stabilization does not require Gcn2p (Kornitzer *et al.*, 1994). Rapid degradation of Gcn4p is triggered by phosphorylation by the cyclin dependent kinases (CDKs) Pho85p and Srb10p (Meimoun *et al.*, 2000; Chi *et al.*, 2001), which marks the protein for ubiquitination by the SCF^{Cdc4} ubiquitin ligase complex and results in its degradation at the 26S proteasome (Figure 4). SCF (Skp1-Cdc53p/Cullin-F-box) complexes are the largest family of E3 ligases, which link ubiquitin to various substrates and were described in more detail in section 1 (Zheng *et al*., 2002).

The Srb10p-dependent Gcn4p degradation seems to occur constitutively. The fact that Gcn4p can interact with the polymerase II mediator complex (Drysdale *et al.*, 1995; Han *et al.*, 1999) and that Srb10p is part of the mediator (Myer *et al.*, 1998), suggests that the recruitment of the mediator by Gcn4p to the promoter leads to phosphorylation and subsequent degradation of Gcn4p (Hinnebusch and Natarajan, 2002).

Correspondingly, *pho85Δ* and *srb10Δ* mutations stabilize Gcn4p even under non starvation conditions. Accordingly, mutations concerning the specific ubiquitin conjugating enzymes Cdc34p (Ubc3p) and Rad6p (Ubc2p) give rise to Gcn4p stabilization (Kornitzer *et al.*, 1994). It was recently shown that the cyclin dependent kinase Pho85p requires the cyclin Pcl5p for Gcn4p phosphorylation at Thr165 and that a *pcl5Δ* mutation results in stabilized Gcn4 protein (Shemer *et al.*, 2002).

When translation is efficiently working in sated cells, Pcl5p is constantly produced as essential part of the Gcn4p destruction machinery. Under starvation conditions Pcl5p disappears presumably due to its own constitutively rapid turnover (Shemer *et al*., 2002), which cannot be counteracted by Gcn4p activating *PCL5* transcription (Jia *et al.*, 2000). The consequence is an increased stability of Gcn4p and therefore an increased transcriptional activation of Gcn4p regulated target genes. Pcl5p has therefore been proposed as a sensor of cellular protein biosynthetic capacity, which permanently requires efficient translation for function (Shemer *et al*., 2002).

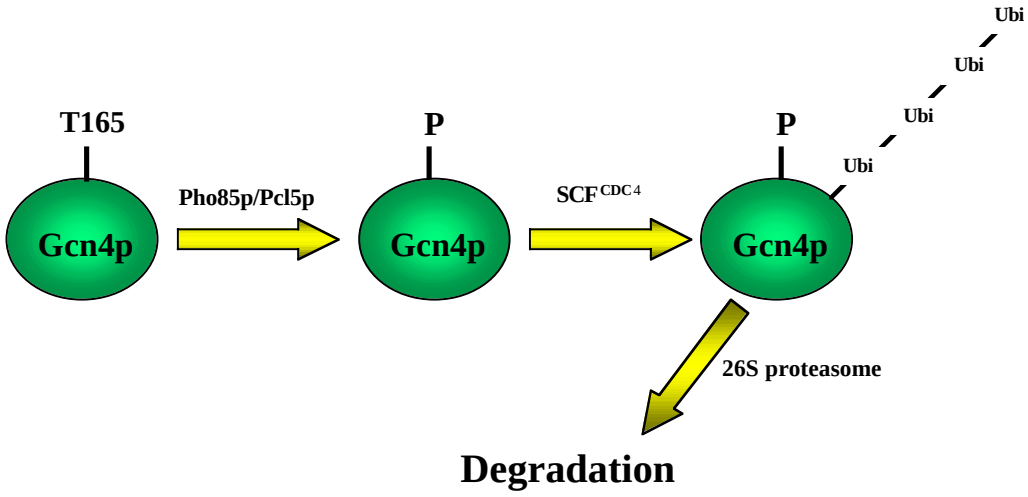

Figure 4: Proposed model of the Gcn4p degradation pathway (Meimoun *et al.*, 2000). Upon phosphorylation at Thr165 by the Pho85p/Pcl5p cyclin dependent kinase complex Gcn4p becomes ubiquitinated by the SCF^{Cdc4} ubiquitin ligase complex and is subsequently degraded at the 26S proteasome.

While *GCN4* expression has been studied primarily by the use of bradytrophs or the addition of amino acid analoga, Gcn4p stabilization analyzes have been carried out under conditions of severe amino acid starvation induced by shifting amino acid auxotrophic cells on minimal medium. It is possible that stabilization of Gcn4p only occurs under more severe starvation conditions, where the overall translation is fully arrested (Hinnebusch and Natarajan, 2002). Furthermore, there appears to be a regulation of Gcn4p function in addition to the regulation of translation and stability. The Gβ-like WD repeat protein Cpc2p seems to be involved in this regulation. A *cpc2* mutation increases the transcription of Gcn4p target genes even in the absence of amino acid starvation, but without increasing the Gcn4 protein level (Hoffmann *et al.*, 1999). A corresponding phenotype was observed concerning the homologous gene *cpc2* of *Neurospora crassa* (Paluh *et al.*, 1988) and *cpcB* of *Aspergillus nidulans* (Hoffmann *et al.*, 2001).

2. 5. Functional dissection of Gcn4p and homologous proteins

The *GCN4* open reading frame encodes for a protein of 281 amino acids with a molecular weight of about 31 kDa (Thireos *et al.*, 1984). Whereas the mammalian homologous proteins c-Jun and c-Fos are able to form homo- and as well heterodimers (Turner and Tjian, 1989), *S. cerevisiae* Gcn4p and *N. crassa* CPC1 are only known to form homodimers (Paluh and Yanofsky, 1991).

Figure 5 illustrates the functional domains of Gcn4p and its homologous proteins of *Neurospora crassa*, *Aspergillus nidulans*, and *Aspergillus niger* (Hoffmann *et al.*, 2001; Wanke *et al.*, 1997).

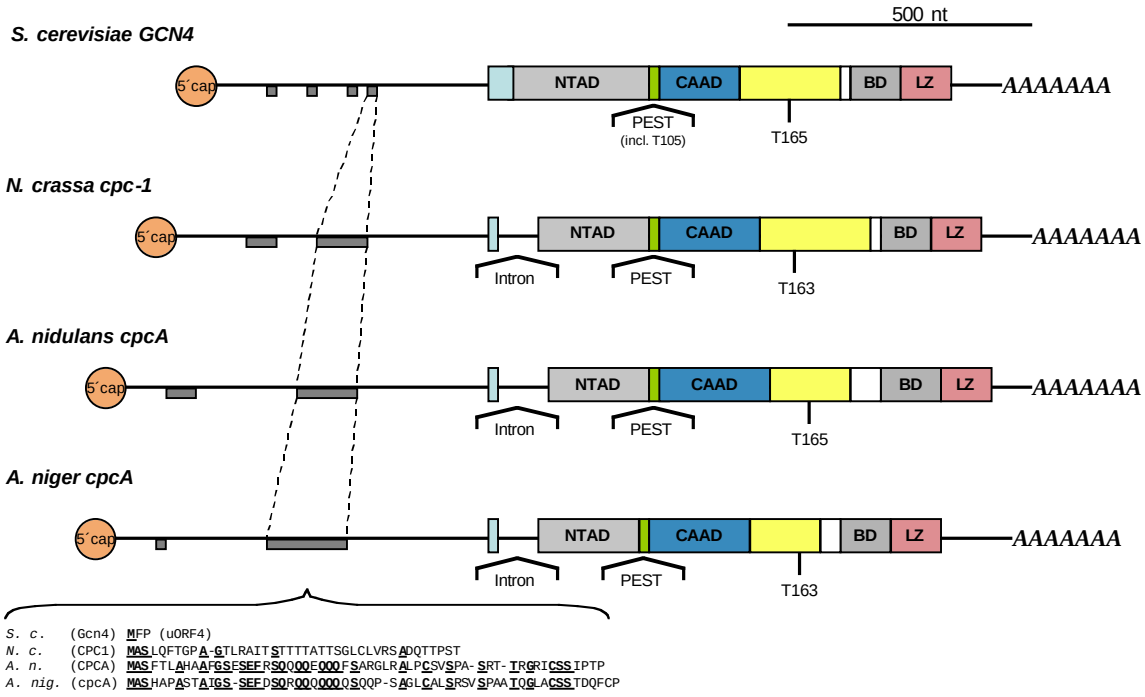

Figure 5: Functional domains of *S. cerevisiae* Gcn4p and its homologous proteins *cpc-1* of *Neurospora crassa*, *cpcA* of *Aspergillus nidulans*, and *cpcA* of *Aspergillus niger*.

The activation domain consists of an N-terminal activation domain (NTAD) and a central acidic activation domain (CAAD) and harbours the protein instability region (PEST). The basic leucine zipper mediates DNA binding (DB) and protein dimerization (LZ). Gcn4p degradation is initiated by Pho85p mediated phosphorylation at Thr165, which can also be found within the homologous proteins at position 163 or 165, respectively. Furthermore Thr105 is known to be involved in the degradation of Gcn4p, whereas it is not conserved in the illustrated homologous proteins of *N. crassa*, *A. nidulans*, and *A. niger*. All presented mRNAs harbour four or two upstream open reading frames (uORFs) in their 5´ untranslated region, respectively.

S. cerevisiae Gcn4p can be divided into an activation domain, a DNA binding domain and a leucine zipper necessary for protein dimerization. The Gcn4p activation domain consists of an N-terminal activation domain (NTAD), a central acidic activation domain (CAAD) and also harbours the instability region (PEST) of the protein (Figure 5).

Both activation domains were shown to have nearly the same activation potential (Drysdale *et al.*, 1995). In general leucine zippers are characterized by four repeats of leucine residues separated by seven amino acids (Landschulz *et al.*, 1988). Surprisingly, the characteristic leucine residues are missing within the *A. niger* cpcA leucine zipper (Hinnebusch, 1984; Hope and Struhl, 1986).

GCN4 mRNA harbours four upstream open reading frames (uORF) in its 5´ untranslated region for its translational regulation, whereas there are only two uORFs present in the homologous proteins of *N. crassa*, *A. nidulans*, and *A. niger*. Gcn4p degradation is initiated by Pho85p mediated phosphorylation at Thr165, which marks the protein for ubiquitination and subsequent decay at the 26S proteasome. Figure 5 shows the conserved character of the threonine residue at position 163 or 165 within the aligned proteins, respectively. Thr105 is known to be required for proper Gcn4p degradation but is not conserved within the other proteins. Among the four illustrated mRNAs, *GCN4* mRNA is the only one without an intron.

Whereas the stabilization of *S. cerevisiae* Gcn4p in response to amino acid starvation has clearly been demonstrated (Kornitzer *et al.*, 1994), it still has to be investigated whether the homologous proteins of *N. crassa*, *A. nidulans*, and *A. niger* are regulated similarly.

Transcription activation function of transcriptional activator proteins requires their import into the nucleus. Therefore the principles of protein import into the nucleus of eukaryotic cells will be represented in the following section.

3. Nuclear protein import

Nuclear import and export mechanisms are known to be involved in the regulation of different biosynthetic pathways. Pho4p is the central transcription factor of the phosphate metabolism and as Gcn4p a substrate of the CDK Pho85p. Whereas Pho4p is nuclear localized under low phosphate conditions, phosphorylation by Pho85p triggers its export into the cytoplasm under high phosphate conditions (Kaffman and O´Shea, 1999).

Gcn4p is the central transcriptional activator of the `general amino acid control` and has to enter the nucleus to fulfill its function in activating the transcription of its target genes.

The nucleus of eukaryotic organisms is characterized by an endoplasmatic reticulum associated nuclear membran which excludes it from the cytoplasm. This subcellular compartimentation results in a spatial separation of transcription and translation and therefore requires bidirectional intracellular trafficking of proteins and RNAs. This exchange of macromolecules is regulated via the nuclear pores, which are present in the nuclear envelope (Kaffman and O´Shea, 1999). So all nuclear proteins have to be transported into the nucleus after being synthesized in the cytoplasm (Görlich and Mattaj, 1996). Smaller molecules are able to shuttle between these subcellular compartments by passive diffusion, whereas molecules with a weight higher than 40 kDa need to be transported in a regulated manner mediated by specific transport proteins. In addition, also molecules which are objected to passive diffusion are often transported in an active way, since this allows a more efficient transport which can be regulated due to the environmental conditions.

The classical mechanism of nuclear protein import depends on the formation of a heterodimeric importin α/β complex in the cytosol followed by recognition of the cargo NLS (nuclear localization sequence) and its translocation into the nucleus via the nuclear pore complex. During this process importin β is thought to remain at the nuclear pore (Görlich *et al.*, 1995), whereas the importin α/cargo complex enters the nucleoplasm. After the cargo protein is released inside the nucleus importin α and importin β have to return into the cytoplasm for the next round of translocation (Figure 6). *S. cerevisiae* harbors one importin α homologue and eight importin β family member proteins, which will be described in detail in chapter 3.

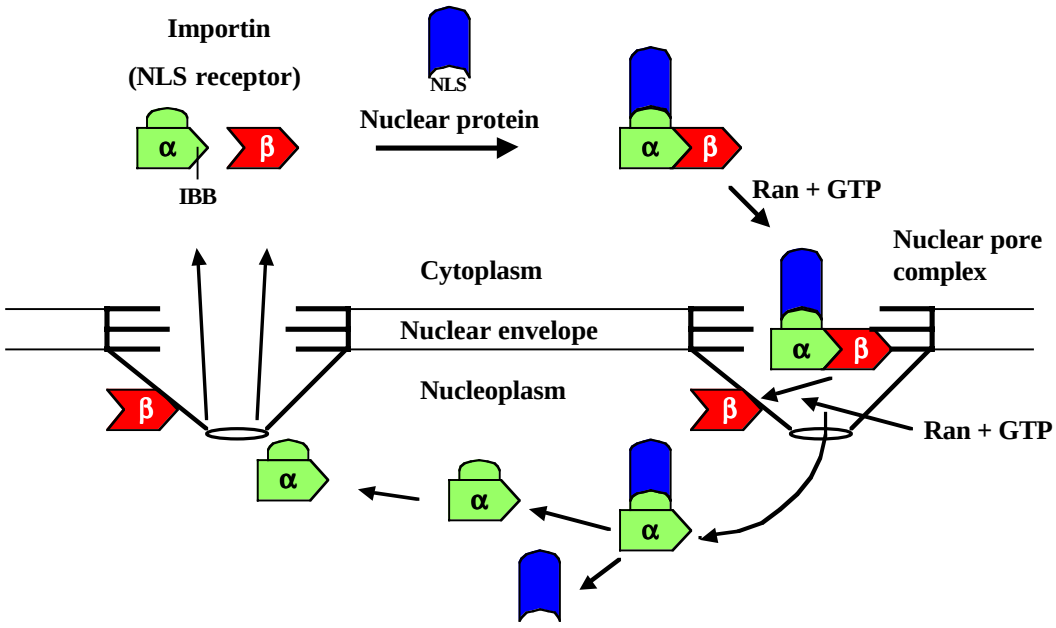

Figure 6: Model of nuclear protein import. The classical way of nuclear import of proteins requires an importin α/β complex and a nuclear localization signal (NLS) harbouring cargo protein. First importin α binds the NLS of the cargo protein, which leads to an importin α/β complex formation via the importin α `importin β binding domain` (IBB). In a GTP hydrolysis by Ran dependent process this heterotrimeric complex enters the nuclear pore complex by importin β mediated docking. Dissociation of importin β occurs and importin α enters the nucleoplasm with its substrate. For the next round of nuclear protein import the importins have to return into the cytoplasm.

This signal mediated nuclear import requires GTP hydrolysis by Ran, NLS motifs and soluble factors (Görlich and Mattaj, 1996). In general, NLS motifs are characterized by one or two clusters of basic amino acids, called monopartite or bipartite NLS motifs. For example the `SV40 large T-antigen` NLS is a region of seven amino acids from which five are basic ones, whereas the ´nucleoplasmin´ NLS-signal represents a classical bipartite motif consisting of two basic clusters separated by a ten amino acid spacer region (Jans and Huber, 1996). Signals involved in nuclear protein export are less conserved and harder to define, they are often leucine rich protein regions (Kaffman and O´Shea, 1999).

4. Aim of this work

The aim of this work was to elucidate the mechanisms of subcellular protein localization as a regulatory tool of biosynthetic pathways in eukaryotic cells with respect to the regulation of *S. cerevisiae* Gcn4p. Therefore the localizations of *S. cerevisiae* Gcn4p and the homologous protein CPCA of the mold *Aspergillus nidulans* were investigated under different metabolic conditions. The green fluorescent protein (GFP) was used to analyse the localization of both transcription factors in living cells by fluorescence microscopy. Deletion and heterologous transfer experiments were carried out to identify *S. cerevisiae* Gcn4p NLS motifs, which were compared with homologous proteins of other fungi. Subsequently, the karyopherins responsible for Gcn4p nuclear import in yeast should be identified by characterizing the corresponding mutant strains.

To gain new insight into the regulation of Gcn4p degradation and stabilization, the subcellular localizations of various proteins involved in this process were analysed in starved and sated yeast cells. Our investigations focused on the mechanisms and place of Gcn4p stability regulation in *Saccharomyces cerevisiae* and aimed to get an extended survey about this field.

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Chapter 2

Amino acid dependent Gcn4p stability regulation occurs exclusively in the yeast nucleus

Abstract

The c-Jun-like transcriptional activator Gcn4p controls biosynthesis of translational precursors in the yeast *Saccharomyces cerevisiae*. Protein stability is dependent on amino acid limitation and *cis*-signals within Gcn4p which are recognized by cyclin-dependent protein kinases including Pho85p. The Gcn4p population within unstarved yeasts consists of a small relatively stable cytoplasmic fraction and a larger less stable nuclear fraction. Gcn4p contains two nuclear localization signals (NLS) which function independently of the presence or absence of amino acids. Expression of NLS-truncated Gcn4p results in an increased cytoplasmic fraction and an overall stabilization of the protein. The same effect is achieved for the entire Gcn4p in a *yrb1* yeast mutant strain impaired in the nuclear import machinery. In the presence of amino acids, controlled destabilization of Gcn4p is triggered by the phosphorylation activity of Pho85p. A *pho85Δ* mutation stabilizes Gcn4p without affecting nuclear import. Pho85p is localized within the nucleus in the presence or absence of amino acids. Therefore there is a strict spatial separation of protein synthesis and degradation of Gcn4p in yeast. Control of protein stabilization which antagonizes with Gcn4p function is restricted to the nucleus.

Introduction

In the yeast *Saccharomyces cerevisiae* a large number of genes encoding enzymes of different biosynthetic pathways are co-regulated by a genetic network known as the general control system of amino acid biosynthesis (Natarajan *et al*., 2001). Starvation for a single amino acid results in an increased expression and stability of the transcriptional activator Gcn4p, whichsubsequently upregulates the transcription of multiple target genes in various biosynthetic pathways for translational precursors. Like mammalian c-Jun, Gcn4p belongs to the bZIP family of transcription factors. Gcn4p expression is regulated at the level of translation initiation and protein stability. Four short upstream open reading frames (uORFs) prevent an efficient translation of the *GCN4* mRNA under nonstarvation conditions (Hinnebusch, 1994). In addition, *GCN4* mRNA translation is repressed by nitrogen starvation (Grundmann *et al*., 2001). When cells are starved for amino acids, uncharged tRNA molecules bind to the tRNA synthetase domain of the kinase Gcn2p. As a consequence, the kinase becomes activated and phosphorylates the α-subunit of the initiation factor eIF-2 (Dever *et al*., 1992; Wek *et al*., 1995). Phosphorylation inhibits eIF-2B which normally exchanges eIF-2 bound GDP for GTP. This results in a reduced amount of active eIF-2 that is available for translation initiation (Merrik, 1992; Voorma *et al*., 1994). The diminished overall translation efficiency is counteracted by increased expression of Gcn4p, because the modified translational apparatus allows the utilization of the *GCN4* start codon. A strain lacking the kinase Gcn2p is not able to turn on the general control of amino acid biosynthesis in response to amino acid limitation.

Whereas translational regulation of the *GCN4* mRNA has been studied for many years, the regulation of Gcn4p stability is a more recent research field. Gcn4p is a highly unstable protein with a half-life of about 5 minutes. Starvation for specific amino acids increases the half-life of the protein (Kornitzer *et al*., 1994). Rapid degradation of Gcn4p depends on phosphorylation by cyclin-dependent protein kinases such as Pho85p. Amino acid residue Thr165 has been identified as one of the crucial phosphorylation sites. Another cyclin-dependent protein kinase which was recently identified to be involved in Gcn4p stability is Srb10p (Chi *et al*., 2001). Correspondingly, a *pho85Δ* or a *srb10Δ* mutation results in Gcn4p stabilization (Meimoun *et al*., 2000; Chi *et al*., 2001). Phosphorylated Gcn4p subsequently serves as substrate for ubiquitinylation by the SCF^{Cdc4} ubiquitin ligase complex.

S. cerevisiae cells are subdivided into the typical eukaryotic compartments which separate different cellular processes. The Gcn4 protein is synthesized in the cytoplasm and has to be

transported into the nucleus to fullfill its transcriptional activation function. To analyze whether Gcn4p stability is regulated at the level of its subcellular localization, we investigated the localization of Gcn4p and Pho85p in living yeast cells under various conditions. Gcn4p is predominantly localized in the nucleus in the presence or absence of amino acid limitation due to two nuclear localization signals (NLS). Nuclear localization of Gcn4p does not require a functional general control system. Pho85p which triggers Gcn4p degradation by initial phosphorylation is as well predominantly localized within the nucleus, independently of the availability of amino acids. Neither functional Pho85p nor a functional Srb10p are required for Gcn4p transportation into the nucleus. Gcn4p stability is regulated within the nucleus in response to the amount of available amino acids. Correspondingly, Gcn4p can be stabilized by preventing its entering into the nucleus. Our results show that the regulation of Gcn4p synthesis and the regulation of Gcn4p stability are two independent compartment-specific processes. Amino acid limitation as initial stimulus increases the synthesis of Gcn4p in the cytoplasm and increases the stability of the protein within the nucleus, respectively.

Materials and Methods

S. cerevisiae strains and growth conditions

Yeast strains used in this study are either congenic to the *S. cerevisiae S288c* (RH1347, RH1376, RH1408) or W303 genetic background. Details of the yeast strains used in this study are given in Table I. Standard methods for genetic crosses and transformation were used and standard yeast culture YPD and YNB media were prepared as described (Guthrie and Fink, 1991).

Plasmids

Plasmids pME2126, pME2127, pME2128, pME2129, pME2134 and pME2135 expressing different GFP-Gcn4p derivatives from the *MET25* promoter, were obtained by amplifying the different *GCN4* fragments with *Pfu*-polymerase and introducing them via *Sma*I/*Hin*dIII into p426MET25 (Mumberg *et al*., 1994) or low copy GFP-N-Fus vector (Niedenthal *et al*., 1996). A *Bgl*II site was introduced in front of the coding region for insertion of a 750 bp *Bgl*II fragment encoding the GFPuv variant of GFP that was amplified from plasmid pBAD-GFPuv (Clontech, Heidelberg, Germany).

Plasmids pME2130, pME2131, pME2132, pME2133, pME2136, pME2137 and pME2138 expressing GFP-Aro7p and GFP-Aro7p fused with different Gcn4p fragments driven from the *MET25* promoter, were constructed similar to the GFP-Gcn4p plasmids. *GCN4* fragments were fused via *Eco*RI/*Cla*I to the 3´-end of *ARO7*.

PHO85 was introduced as a *Sma*I/*Cla*I fragment into p426MET25 and GFPuv was inserted as a *Bgl*II-fragment at the *PHO85* 5´-end.

Plasmids KB294 and pME2140, expressing a triple myc epitope-tagged version of either wt Gcn4 or $Gcn4_{aa1\text{-}169}$ under the control of the *GAL1* promoter, were obtained by insertion of a 120 bp *Bam*HI fragment carrying the triple myc epitope (myc^3) into a *Bgl*II restriction site after the fourth amino acid.

Plasmids pME2316 and pME2317 express a GFP tagged $GCN4_{aa1\text{-}169}$ *Sma*I/*Hin*dIII fragment in a high copy vector from a *GAL1* promoter without and with the inserted $NLS2_{aa215\text{-}249}$ between GFP and $GCN4_{aa1\text{-}169}$, respectively.

GFP fluorescence microscopy

Yeast strains harbouring plasmids encoding GFP-fusion proteins were grown to exponential phase in selective minimal medium. Cells from 1 ml 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). 4´,6-Diamidino-2-phenylindole (DAPI) staining was used for visualization of nuclei using standard DAPI filter sets. Cells were photographed using a Xillix Microimager digital camera and the Improvision Openlab software (Improvision, Coventry, UK).

Protein analysis

Preparation of whole yeast cell extracts were performed as described (Surana *et al*., 1993). Routinely, 10 µg of crude protein extracts were separated. After separation on SDS gels, proteins were transfered to nitrocellulose membranes. Proteins were visualized using ECL technology (Amersham) after incubation of membranes with polyclonal mouse anti-Myc or mouse anti-GFP antibodies and a peroxidase-coupled goat anti-mouse secondary antibody.

Table I. Strains used in this study

Strain	Genotype	Source
RH1347	*MAT**a**, aro7, ura3-52*	Our collection
RH1376	*MAT**a**, ura3-52*	Our collection
RH1408	*MAT**a**, gcn4-103, ura3-52, gal2*	Our collection
RH1479	*MAT**α**, gcn2, ura3-52*	Our collection
W303	*MAT**a**, ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11,15, ura3*	Naysmith *et al*., 1990
EY0140	W303 *pho85Δ*::*LEU2*	O´Neill *et al*., 1996
RH2663	W303 *yrb1-51*	Bäumer *et al*., 2000
RH2713	W303 *srb10Δ::KAN^R*	Our collection

Table II. Plasmids used in this study

Plasmid	Description	Reference
p426MET25	pRS426 containing *MET25* promoter and *CYC1* terminator	Mumberg *et al.*, 1994
pME2232	pGFP-N-Fus	Niedenthal *et al.*, 1996
pME2231	*MET25prom-GFP-GCN4* fusion in pGFP-N-Fus	this study
pME2126	*MET25prom-GFP-GCN4* fusion in p426MET25	this study
pME2127	*MET25prom-GFP-GCN4*$_{aa1-249}$ fusion in p426MET25	this study
pME2128	*MET25prom-GFP-GCN4*$_{aa1-221}$ fusion in p426MET25	this study
pME2129	*MET25prom-GFP-GCN4*$_{aa1-169}$ fusion in p426MET25	this study
pME2130	*MET25prom-GFP-ARO7* fusion in p426MET25	this study
pME2131	*MET25prom-GFP-ARO7-GCN4*$_{aa167-200}$ fusion in p426MET25	this study
pME2132	*MET25prom-GFP-ARO7-GCN4*$_{aa221-281}$ fusion in p426MET25	this study
pME2133	*MET25prom-GFP-ARO7-GCN4*$_{aa231-249}$ fusion in p426MET25	this study
pME2134	*MET25prom-GFP-GCN4* $^{S214A, S218A, S224A}$ fusion in p426MET25	this study
pME2135	*MET25prom-GFP-GCN4* $^{S214D, S218D, S224D}$ fusion in p426MET25	this study
pME2136	*MET25prom-GFP-ARO7-GCN4*$_{aa167-249}$ fusion in p426MET25	this study
pME2137	like pME2136 but using *GCN4* $^{S214A, S218A, S224A}$	this study
pME2138	like pME2136 but using *GCN4* $^{S214D, S218D, S224D}$	this study
pME2139	*MET25prom-GFP-PHO85* fusion in p426MET25	this study
KB294	*GAL1prom-myc*^{3}*-GCN4* fusion in *URA3*-marked 2µm vector	Kornitzer, D.
pME2140	*GAL1prom-myc*^{3}*-GCN4*$_{aa1-169}$ fusion in *URA3*-marked 2µm vector	this study
pME2316	*GAL1prom*-GFP-*GCN4*$_{aa1-169}$ fusion in *URA3*-marked 2µm vector	this study
pME2317	*GAL1prom*-GFP-NLS$_{aa215-249}$-*GCN4*$_{aa1-169}$ fusion in *URA3*-marked 2µm vector	this study

Results

The transcription factor Gcn4p is targeted to the yeast nucleus in the presence or absence of amino acids

The transcriptional activator Gcn4p has to be imported into the nucleus to fulfill its function. Gcn4p is an unstable protein which can be stabilized in response to amino acid limitation (Kornitzer *et al*., 1994). In the presence of sufficient amounts of amino acids, only small amounts of the Gcn4 protein are synthesized. Amino acid limitation results in increased translation of *GCN4* mRNA. We asked whether Gcn4p stability correlates with its subcellular localization. The localization of Gcn4p was monitored *in vivo* by expressing the coding region of the green fluorescent protein (GFP) variant GFP-uv (Crameri *et al*., 1996), which was fused to the 5´-end of the *GCN4*-ORF. The chimeric gene was analyzed in yeast strain RH1408 (*ura3*, *gcn4Δ*) by fluorescence microscopy. The N-terminal half of Gcn4p carries the transcriptional activation domain, whereas the C-terminal part includes the bZIP region for DNA-binding and dimerization. The *GFP-GCN4* hybrid open reading frame on a low and as well on a high copy vector was driven from the *MET25* promoter which allows for downregulation by adding methionine to the medium.

Expression of GFP-Gcn4p was verified by Western analysis of *S. cerevisiae* cell extracts using polyclonal anti-GFP antibodies. GFP signals of the expected size could be visualized in cells expressing GFP-Gcn4p (Figure 1) and were compared to the expression levels of the unregulated house-keeping gene *ARO7* (Schmidheini *et al*., 1990) which was used as control. The *GCN4* deficient yeast strain RH1408 is unable to grow under amino acid starvation conditions because the amino acid biosynthetic genetic network cannot be induced by the transcriptional activator Gcn4p. The GFP-Gcn4p fusion protein was able to complement the *gcn4Δ* phenotype. Transcription factor function was tested *in vivo* by inducing amino acid starvation conditions using the analogue 3-amino-triazole (3AT), which acts as competitive inhibitor of the *HIS3* gene product (Klopotowski and Wiater, 1965) and therefore prevents growth without functional Gcn4p induced gene expression.

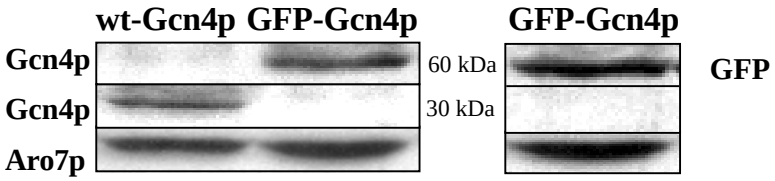


Figure 1: Expression of the GFP-Gcn4p fusion protein in *S. cerevisiae*.
For Western hybridization analysis polyclonal anti-Gcn4p and anti-GFP antibodies were used. As loading control, expression levels of Aro7p were measured in the same yeast extracts using a polyclonal anti-Aro7p antibody. Crude protein extracts were prepared from yeast strain RH1376 (*GCN4* wildtype) and RH1408 (*gcn4-103*) expressing GFP-Gcn4p from a *MET25* promoter on a 2µm plasmid (GFP-Gcn4). Used antibodies are indicated, illustrating the expression of GFP-Gcn4p

Localization of Gcn4p was examined by fluorescence microscopy in cells grown in the absence of amino acid limitation which were compared to cells starved for histidine by adding the analogue 3AT. Gcn4p was clearly concentrated in the yeast nucleus which was visualized by DAPI staining (Russel *et al.*, 1975). Low amounts of GFP-Gcn4p derived from a centromere plasmid as well as higher amounts derived from a 2µm vector were efficiently transported into the nucleus independently of the absence or presence of 3AT (Figure 2). The nuclear localization of Gcn4p in 3AT starved cells was corroborated by testing yeasts starved for tryptophan or leucine. Starvation for tryptophan was induced by adding the analogue 5-methyl-tryptophan (5MT) to the medium (Schürch *et al.*, 1974). Leucine starvation was analyzed without the use of amino acid analogues in a leucine auxotrophic mutant strain (W303, Table 1) which was transferred from leucine containing medium to minimal medium lacking leucine. The fluorescence of the GFP-Gcn4p fusion protein of tryptophan or leucine starved yeasts was similar to histidine starved cells and strictly correlated with the DAPI staining of the nucleus. These data imply that most Gcn4p is immediatly transported into the nucleus subsequent to translation and that therefore the majority of Gcn4p is localized within the nucleus in both the unstable and stable state.

Translation of *GCN4* mRNA is regulated by the general control regulatory network. We next asked whether an intact general control system is required for nuclear import of Gcn4p. The kinase Gcn2p senses amino acid starvation and is activated by an increasing concentration of uncharged tRNA molecules in response to limiting amounts of amino acids. Phosphorylation of eIF2 by Gcn2p leads to an increased translation of the *GCN4* mRNA (Hinnebusch, 1994). Gcn4p localization was analyzed in a *gcn2Δ* mutant strain, which is unable to induce increased *GCN4* mRNA translation. In this strain Gcn4p is also predominantly localized in the nucleus suggesting that the kinase Gcn2p is not required for its trafficking to the nucleus (Figure 2).

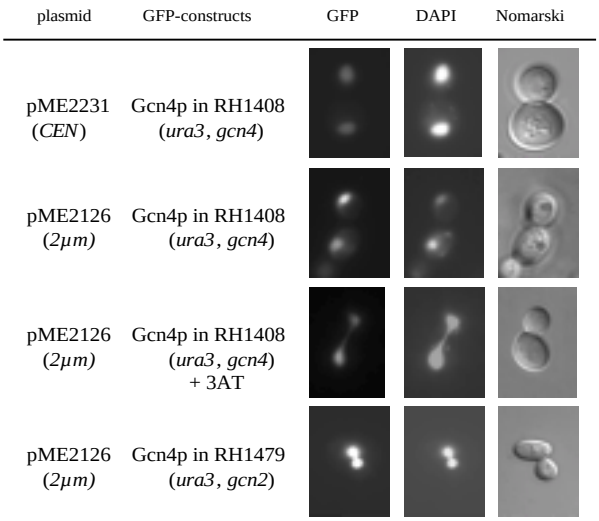


Figure 2: Localization of GFP-Gcn4p in yeast.
Yeast cells (RH1408) expressing wild-type GFP-Gcn4p fusion protein from the *MET25* promoter on a low copy plasmid (pME2231) and on a 2µm plasmid (pME2126) were grown to early log phase in selective medium at 30 °C, and analyzed by differential interference contrast microscopy (right column) and fluorescence microscopy (left column GFP, middle column DAPI). Nuclear localization of wild-type GFP-Gcn4p (pME2126) was also determined in *gcn2Δ* (RH1479) mutant cells.

In summary, these results indicate that Gcn4p transport from the cytoplasm into the nucleus does neither depend on the presence or absence of amino acids nor on an intact general control system. These data suggest that the stabilization of Gcn4p in response to amino acid limitation has to be regulated within the nucleus or during a very narrow time window between translation in the cytoplasm and immediate targetting to the nucleus.

Gcn4p nuclear import is triggered by two functional NLS-motifs

We constructed yeast strains mislocalizing the protein to distinguish the Gcn4p population synthesized in the cytoplasm from the bulk of Gcn4p localized in the nucleus. We initially performed deletion and heterologous transfer experiments to identify sequences required for nuclear localization of Gcn4p. Different C-terminal truncations of the GFP-Gcn4p fusion protein were constructed by deleting the corresponding parts of the gene. Mutant proteins were analyzed by fluorescence microscopy. In addition, we verified putative NLS-signals by testing whether they could mislocalize a cytoplasmic protein into the nucleus.

Our results showed that Gcn4p transport into the nucleus does not require the leucine zipper which mediates the dimerization (pME2127, Figure 3). Even truncated Gcn4p lacking the DNA binding domain and leucine zipper was still predominantly localized in the nucleus (pME2128, Figure 3), whereas the 169 N-terminal amino acids of Gcn4p were not sufficient to enter the nucleus. The corresponding GFP fusion protein significantly accumulated in the cytoplasm (pME2129, Figure 3), suggesting the existence of a NLS-motif between Gcn4p amino acids 170 and 221. It is known that the activity of the human bZIP proteins c-Jun and c-Fos depends on phosphorylation and dephosphorylation of serine and threonine residues N-terminal of the basic region. While phosphorylation of these residues leads to a deactivation of the protein, their dephosphorylation results in an increased DNA binding activity (Boyle *et al.*, 1991). These serine and threonine residues of the c-Jun protein are conserved in *S. cerevisiae* Gcn4p.

The putative phosphorylation sites serine 214, 218 and 224 are not involved in Gcn4p nuclear import. This was shown by comparing the localization of wild-type Gcn4p and two Gcn4p mutant derivatives where the three serine residues were either mutated to alanine in order to mimic a constitutive dephosphorylation or to aspartatic acid in order to mimic a constitutive phosphorylation at these positions. Nuclear import of Gcn4p was never compromised (data not shown).

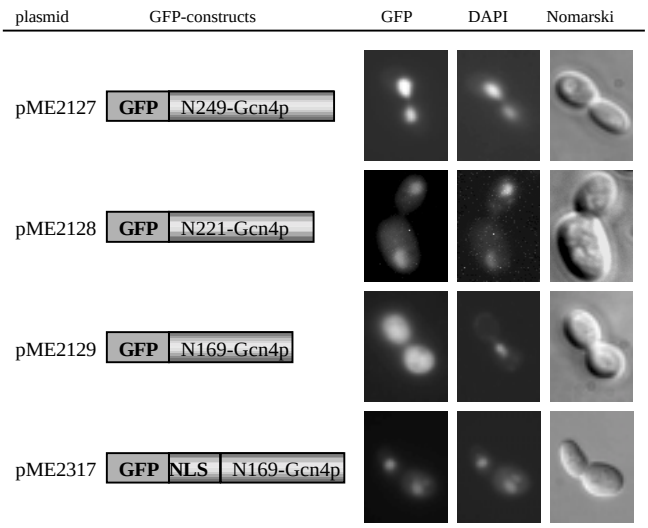


Figure 3: Localization of C-terminal truncated Gcn4p fused to GFP in yeast.
S. cerevisiae cells (RH1408, *gcn4-103*) expressing different GFP-Gcn4p derivatives from the induced *MET25* promoter were grown to early log phase in selective medium at 30 °C, and analyzed by differential interference contrast microscopy (right column) and fluorescence microscopy (left column GFP, middle column DAPI). Localization of GFP-Gcn4p derivatives lacking either the leucine-zipper amino acid residues 250 to 281 (pME2127), the leucine-zipper and the DNA-binding-domain from position 221 to 281 (pME2128) or the 112 C-terminal amino acids of Gcn4p (pME2129) are shown. Fusing back NLS2 from position 215 to 249 to the N-terminus of truncated Gcn4p (pME2129) enabled the protein to enter the nucleus again (pME2317). The N-terminal amino acids of Gcn4p fused to GFP are indicated.

Yeast chorismate mutase [E.C 5.4.99.5] was used as reporter protein to verify putative Gcn4p NLS-motifs by fusing different *GCN4* fragments to its C-terminus. This protein, involved in the biosynthesis of the aromatic amino acids tyrosine and phenylalanine, is encoded by the *ARO7* gene in *S. cerevisiae* and its expression is independent of Gcn4p (Schmidheini *et al*., 1990). The GFP open reading frame was fused to the 5´-end of *ARO7*. The resulting fusion protein was localized exclusively in the cytoplasm when analyzed by fluorescence microscopy (pME2130, Figure 4). The protein was also functional which was shown by its potential to complement an *aro7Δ* phenotype. Heterologous transfer experiments were carried out to verify the Gcn4p deletion experiments. A Gcn4p stretch of the 34 amino acid residues from position 167 to 200 fused to the C-terminus of Aro7p was able to mislocalize yeast chorismate mutase to the nucleus (pME2131, Figure 4) and therefore functions as NLS-motif (NLS1). The amino acids 221-281 consisting of the DNA binding domain and the leucine

zipper of Gcn4p were also able to cause nuclear import of the cytoplasmic chorismate mutase (pME2132, Figure 4), indicating the existence of a second NLS-motif within the 60 C-terminal amino acids of Gcn4p. The second NLS-motif (NLS2) was narrowed down to 19 amino acids (aa 231 to 249) which are sufficient to mistarget a chimeric chorismate mutase to the nucleus instead of the cytoplasm (pME2133, Figure 4). NLS2 was further verified by its capability to target the truncated cytoplasmic Gcn4p1-169 back into the nucleus when fused to its N-terminus (pME2317, Figure 3).

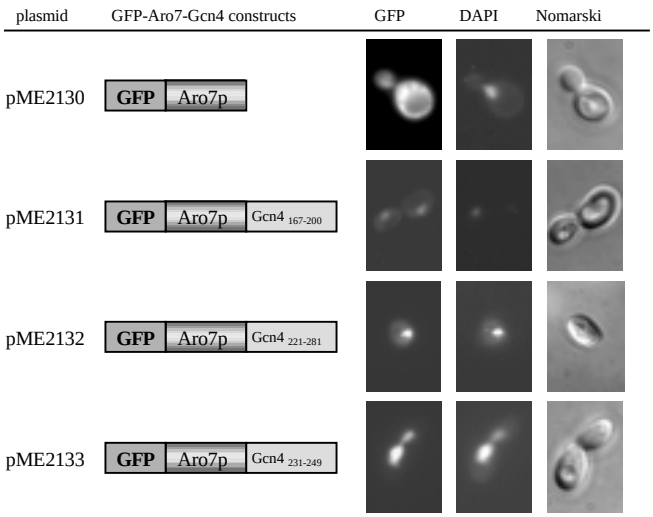


Figure 4: NLS-motifs of Gcn4p target the cytoplasmic Aro7 protein to the nucleus.
Yeast cells (RH1347) expressing different GFP-Aro7p constructs from the induced *MET25* promoter were grown to early log phase in selective medium at 30 °C, and analyzed by differential interference contrast microscopy (right column) and fluorescence microscopy (left column GFP, middle column DAPI). Localization of GFP-Aro7p (pME2130) was compared with GFP-Aro7p fused either with Gcn4p amino acids 167-200 (pME2131), 221-281 (pME2132) and Gcn4p amino acids 231-249 (pME2133), respectively. Bars illustrate the different GFP constructs and the fused Gcn4p amino acids are indicated.

NLS2 of Gcn4p resembles a classical bipartite NLS-motif, consisting of two basic clusters separated by a 10 amino acid spacer region. The first cluster is formed of two basic amino acids, whereas the second cluster consists of seven amino acids including four basic residues. The entire C-terminal amino acid residues 167 to 249 of Gcn4p, containing NLS1 and NLS2 interrupted by a stretch including the three serine residues, were also fused to the C-terminus of chorismate mutase. The nuclear localization of this GFP-Aro7p-Gcn4p chimera was

indistinguishable to the GFP-Gcn4 protein when analyzed by fluorescence microscopy (data not shown).

These results suggested two functional NLS-motifs located within Gcn4p. There is no hint that phosphorylation of the conserved serine residues 214, 218 and 224 which are located between NLS1 and NLS2 is required for Gcn4p translocation (Figure 5). The identification of Gcn4p NLS-motifs allowed the construction of mislocalized mutant proteins to compare the stability of cytoplasmic and nuclear Gcn4p.

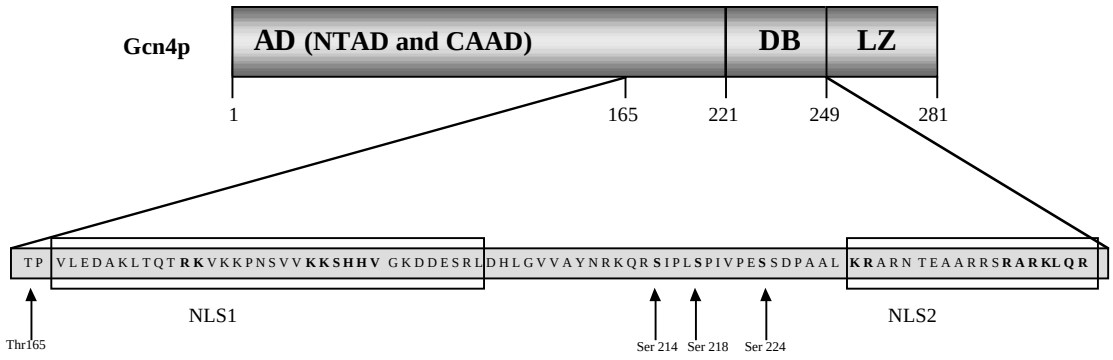


Figure 5: Scheme of the two Gcn4p NLS-motifs.

The positions of the two identified NLS-motifs within the amino acid sequence of the transcription factor Gcn4p are shown schematically. The two Gcn4p NLS-motifs NLS1 and NLS2 consist of the amino acids 167 to 200 and 231 to 249, respectively. Thr165 is the phosphorylation site of Pho85p (Meimoun *et al*., 2000). The activation domain (AD) consists of a N-terminal activation domain (NTAD) and a central acidic activation domain (CAAD). DB and LZ are the DNA binding domain and the leucine zipper of Gcn4p. The conserved serine residues 214, 218 and 224 regulate the DNA binding activity in the mammalian c-Jun (Boyle *et al.*, 1991).

Mislocalization of Gcn4p in the cytoplasm stabilizes the protein

The Gcn4 protein of unstarved yeast cells is an unstable protein (Kornitzer *et al*., 1994). We wondered whether the stability of Gcn4p varies depending on the cellular compartment where it is localized. Therefore we analyzed whether mislocalization of Gcn4p affects protein stability. The myc-tagged Gcn4p lacking the identified NLS-motifs was shown to be unable to enter the nucleus (pME2129, Figure 3). A promoter shut-off experiment of the fusion gene

revealed a stabilization of the resulting mutant protein when compared to Gcn4p carrying wildtype NLS1 and NLS2 expressed from the same promoter (Figure 6A). Therefore efficient proteolysis of Gcn4p correlates with nuclear localization. No additional protein stabilization of the truncated cytoplasmic Gcn4p could be observed in response to amino acid limitation (Figure 6A). We also tested protein stability of the truncated Gcn4p (amino acids 1-169) where nuclear import was restored by fusing Gcn4p amino acids 215 to 249 to its N-terminus (pME2317, Figure 4). In accordance to the previous data, a more efficient degradation of the again nuclear protein could be observed (Figure 6B).

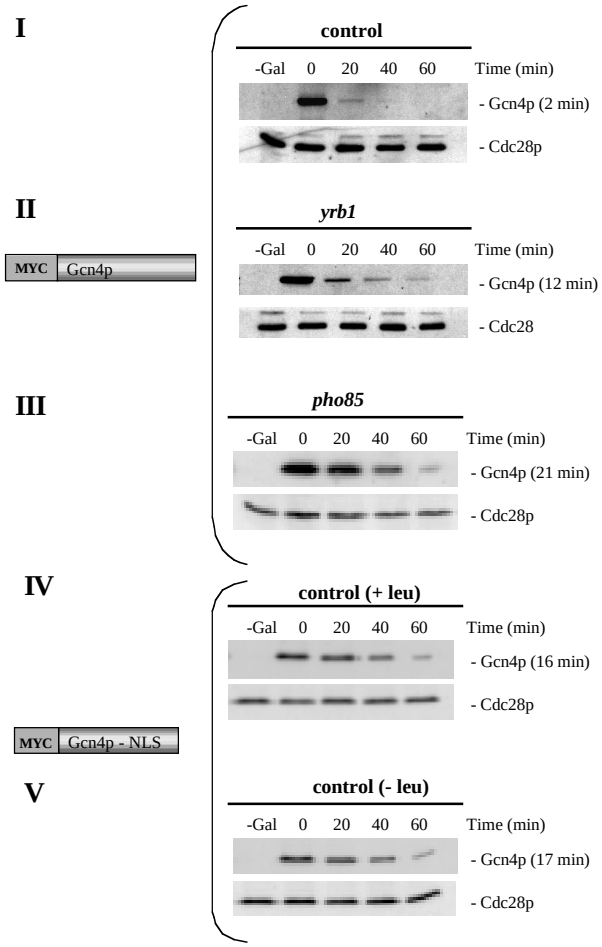
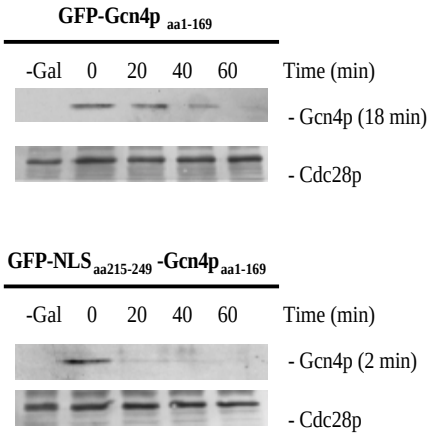


Figure 6:

Stability analysis of *S. cerevisiae* Gcn4p.

A: Cytoplasmic Gcn4p and nuclear Gcn4p are stabilized in the absence of Pho85p compared to wildtype Gcn4p. The isogenic yeast strains W303 (control: I), RH2663 (*yrb1-51*: II), and EY0140 (*pho85Δ*: III) were transformed to express non-truncated *GAL-myc-GCN4* on the high-copy number plasmid (KB294). In addition, the *leu2*-deficient control strain W303 expressed *GAL- myc-GCN4* lacking the two Gcn4p NLS-motifs (amino acids 169 to 281) on the high copy number plasmid (pME2140) in the presence (IV) and absence (V) of leucine were pre-

grown in selective minimal medium containing raffinose. 2% galactose was added to these cycling cultures to express *MYC-GCN4*. Cells were collected by filtration and incubated in minimal medium containing glucose. Samples were collected at the indicated time points after the shift to glucose medium (0 min time-point). Levels of Myc-tagged Gcn4p were determined by immunoblotting using Myc-antibodies. The kinase Cdc28p was used as loading control. The analyzed Myc-Gcn4p fusion proteins with (Myc-Gcn4p) and without (Myc-Gcn4p–NLS) the NLS motifs are illustrated. Protein half-lives were calculated based on the band intensities of at least three independent experiments and put in brackets.



B: Nuclear localization correlates with efficient Gcn4p degradation in non-starved yeast cells.
A control strain (W303) containing *GAL-GFP-GCN4*$_{aa1-169}$ lacking the two NLS motifs and *GAL-GFP-NLS-GCN4*$_{aa1-169}$ on the high copy number plasmids pME2316 and pME2317, respectively, were pre-grown in selective minimal medium containing raffinose. 2% galactose were added to these cycling cultures to express *GFP-GCN4*. Cells were collected by filtration and incubated in minimal medium containing glucose. Samples were analyzed at the indicated time points after the shift to glucose medium (0 min time-point). Levels of GFP-tagged Gcn4p were determined by immunoblotting using GFP-antibodies. Cdc28p was used as loading control. The analyzed GFP-Gcn4p fusion proteins without (GFP-Gcn4$_{aa1-169}$) or with (GFP-NLS-Gcn4$_{aa1-169}$) the NLS motif are illustrated. Protein half-lives were calculated based on the band intensities of at least three independent experiments and put in brackets.

The compartment specific stability of Gcn4p was verified by mislocalizing an intact protein in a yeast strain impaired in nuclear transport. *YRB1* (yeast Ran BP1) is the yeast homologue of the mammalian Ran BP1 (Butler and Wolfe, 1994). Temperature sensitive mutants of *YRB1* show defects in nuclear import at their restrictive temperature (Schlenstedt *et al.*, 1995). The nuclear import of Gcn4p in *yrb1-51* mutant cells was inhibited in response to the *yrb1* defect after cells have been shifted to their restrictive temperature. Simultaneously an accumulation of GFP-Gcn4p in the cytoplasm could be observed by fluorescence microscopy (Figure 7). As

additional control for nuclear import in the cell, the localization of the chimeric GFP-Aro7-$Gcn4_{aa231-249}$ (pME2133) fusion protein was analyzed in the *yrb1-51* mutant strain, confirming the defective nuclear transport machinery (Figure 7).

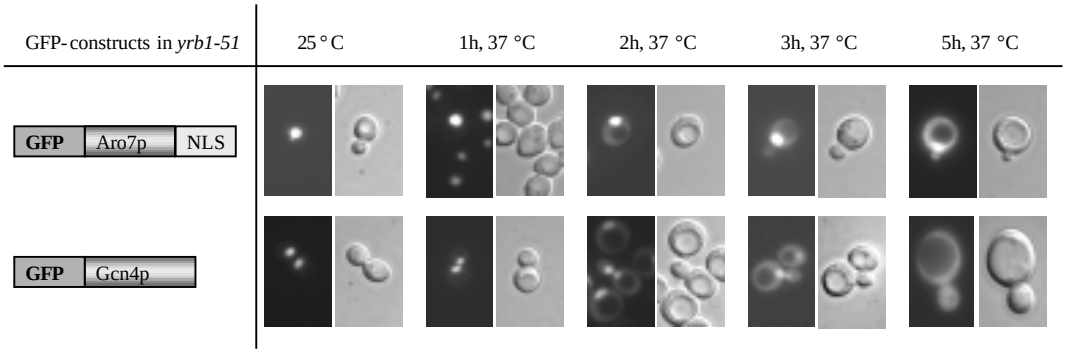


Figure 7: A y*rb1-51* mutation impairs Gcn4p import into the yeast nucleus.
Temperature sensitive *yrb1-51* mutant cells (RH2663) expressing either Aro7p-Gcn4p/NLS-GFP (pME2133) or non truncated Gcn4p-GFP (pME2126) were grown to early log phase in selective medium at the permissive temperature of 23 °C and then shifted to the restrictive temperature of 37 °C. Samples were collected at the indicated time points after shifting and analyzed by differential interference contrast microscopy and fluorescence microscopy.

The stability of intact cytoplasmic Gcn4p in the *yrb1-51* mutant was analyzed by a promoter shut-off experiment and Western hybridization. Wild-type and temperature sensitive *yrb1-51* cells were transformed with a plasmid containing a *GAL1*-promoter controlled gene construct expressing a myc-tagged Gcn4 protein. The *GCN4* containing gene fusion was transiently expressed in exponentially growing wild-type and *yrb1-51* mutant cells. This experiment showed that cytoplasmic Gcn4p in *yrb1-51* cells is stabilized compared to the nuclear Gcn4p of the control (Figure 6A).

Taken together, these data suggest that the stability of Gcn4p depends on the compartment of the cell where it is localized. Whereas the cytoplasmic Gcn4p is relatively stable, the nuclear Gcn4p is highly unstable in unstarved yeast cells where sufficient amounts of amino acids are present.

Destabilization of Gcn4p is secured by the nuclear localization of Pho85p protein kinase

Efficient degradation of the transcription factor Gcn4p requires its phosphorylation by cyclin-dependent kinase Pho85p. A *pho85Δ* mutation results in Gcn4p stabilization (Meimoun *et al.*, 2000). Phosphorylated Gcn4p is a substrate for the SCF^{Cdc4} ubiquitin ligase complex which labels the protein for degradation by the proteasome. Pho85p phosphorylates Gcn4p at Thr165 which targets it to the SCF^{Cdc4} complex. The F-box protein Cdc4p is exclusively nuclear (Blondel *et al.*, 2000). We asked whether phosphorylation by Pho85p kinase as the initial step leading to Gcn4p instability is a compartment-specific process. Therefore we examined the localization of Pho85p in *S. cerevisiae* by using a GFP-Pho85p fusion protein. The kinase Pho85p was detected predominantly inside the nucleus in the presence or absence of amino acid limitation. Therefore Pho85p colocalized with the large unstable fraction of the Gcn4 protein within the nucleus of unstarved yeast cells which corresponds to preceding investigations (Hinnebusch, 1997) (Figure 8). Promoter shut-off experiments confirmed the previously described Gcn4p stabilization in response to the *pho85Δ* mutation (Figure 6A). A *pho85Δ* mutation leads to Gcn4p stabilization without affecting its localization in the nucleus. This was shown by a GFP-Gcn4p in a *pho85Δ* mutant strain which was not affected in Gcn4p nuclear import (Figure 8). In addition, we tested Gcn4p localization in a *srb10* mutant strain since the kinase Srb10p was also shown to be involved in Gcn4p degradation. Gcn4p nuclear import is not impaired in a *srb10* mutant strain (Figure 8).

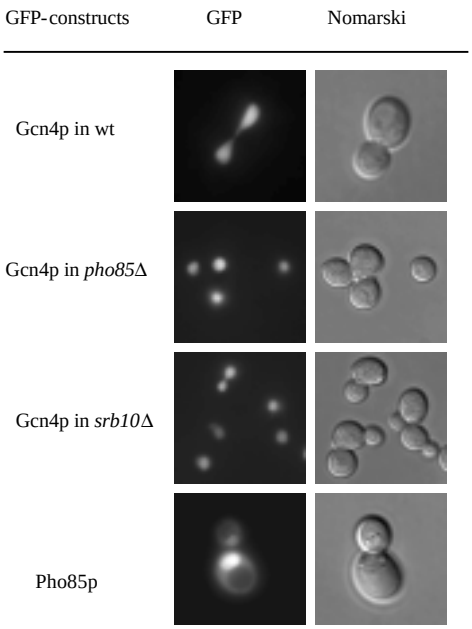


Figure 8: Nuclear localization of GFP-Pho85p in wildtype cells (RH1376) and GFP-Gcn4p in *pho85* (EY0140) and *srb10* (RH2663) mutant strains.
GFP-Pho85p localization was identical in the presence or absence of amino acid limitation. Yeast cells were grown to early log phase in selective medium at 30 °C, and analyzed by differential interference contrast microscopy and fluorescence microscopy.

We conclude that the cytoplasmic Gcn4p is relatively stable whereas destabilization of Gcn4p is spatially separated and restricted to the nucleus. The localization of the Pho85p kinase in the nucleus suggests that the degradation of Gcn4p is initiated within this compartment. The nuclear localization of Cdc4p suggests that also the second step in the degradation pathway is performed in the nucleus. Therefore the yeast cell strictly separates regulation of Gcn4p synthesis and regulation of Gcn4p stability by restricting them to two different cellular compartments.

Discussion

Separation of various processes of gene expression into different subcellular organelles is a typical feature of eukaryotic cells. Whereas transcription occurs inside the nucleus, the built mRNA has to be exported into the cytoplasm in order to be translated. Numerous proteins required for transcription have to be transported into the nucleus after being synthesized in the cytoplasm. We showed here that regulation of synthesis and degradation of the yeast transcriptional activator Gcn4p are spatially separated. Regulation of yeast Gcn4p synthesis is regulated on translational level in the cytoplasm, whereas Gcn4p degradation is regulated inside the nucleus to further modulate its function as transcription factor (Figure 9).

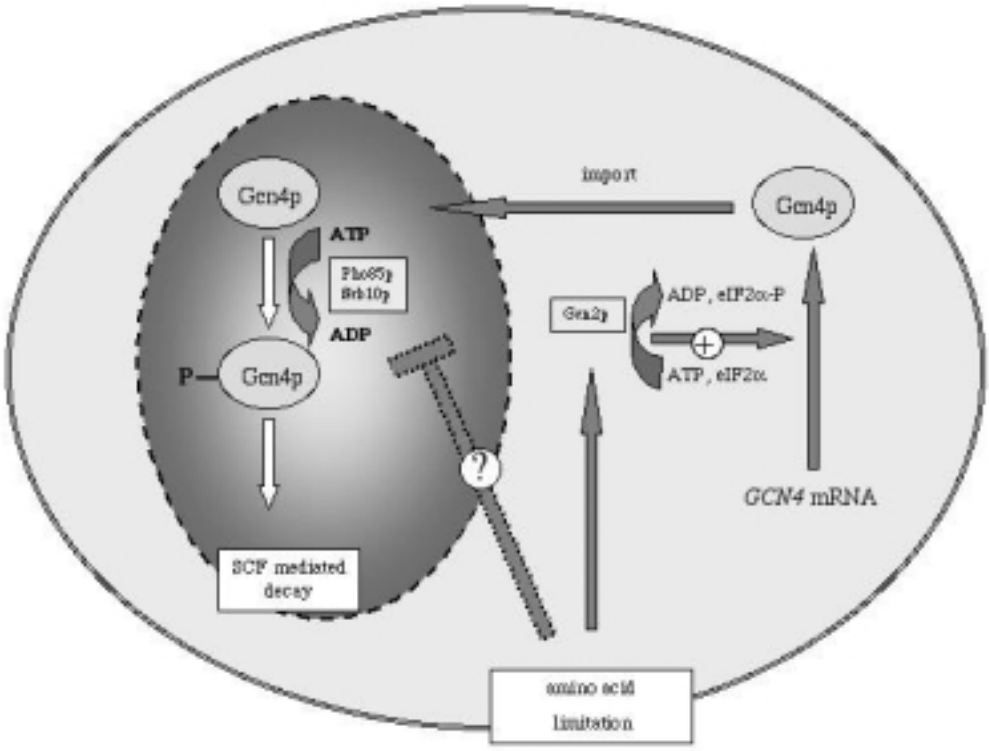


Figure 9: Gcn4p translational and stability control are spatially separated processes in yeast.
In the presence of amino acids *GCN4* mRNA is weakly translated and efficiently transported into the nucleus. The nuclear Gcn4p in unstarved yeast cells is highly unstable. The labeling for the degradation pathway occurs primarily by phosphorylation by the kinases Pho85p or Srb10p and subsequent SCF^{Cdc4} mediated ubiquitinylation. Amino acid limitation results in increased *GCN4* mRNA translation in the cytoplasm and in a more stable Gcn4p within the nucleus. A simple hypothesis for increased stability postulates an inhibition mechanism for the nuclear kinase activities.

Gcn4p in the presence or absence of amino acid limitation

Expression and stability regulation of the yeast transcriptional activator Gcn4p depend on the amount of available amino acids (Kornitzer *et al*., 1994) and on the amount of available nitrogen source (Grundmann *et al*., 2001). In the presence of sufficient amounts of amino acids, Gcn4p is a weakly expressed and unstable protein. *GCN4* mRNA translation in the cytoplasm is repressed and rapid protein degradation is initiated in the nucleus. Gcn4p degradation starts by phosphorylation at Thr165 by the protein kinase Pho85p and is followed by ubiquitinylation by the SCF^{Cdc4} ubiquitin ligase complex (Meimoun *et al*., 2000) which targets it for degradation by the 26S proteasome (Hershko and Ciechanover, 1998).

When cells are starved for amino acids, increased Gcn4p expression in the cytoplasm is accompanied by protein stabilization in the nucleus. Uncharged tRNA molecules are recognized by the cytoplasmic sensor kinase Gcn2p which is localized at the ribosome and phosphorylates the general translation initiation factor eIF-2α. Increased eIF2α-P levels are required to overcome the translational repression of the *GCN4* mRNA resulting in increased amounts of Gcn4 protein (Dever *et al*., 1992). The sensor kinase Gcn2p is only involved in regulating Gcn4p synthesis but does not affect its stability (Kornitzer *et al*., 1994). We showed here that the cytoplasmic fraction of the Gcn4 protein seems to be relatively small. Therefore Gcn4p is predominantly a nuclear protein independently of the presence or absence of amino acid limitation. Nuclear import has to occur immediately after the protein has been synthesized in the cytoplasm. Low amounts of Gcn4p are required for the basal level transcription of genes including *ADE4*, *ARO3* and *LEU2* under nonstarvation conditions (Mösch *et al*., 1991; Gedvilaite and Sasnauskas, 1994). Therefore even under these conditions the transport of Gcn4p expressed under repressed translational conditions into the nucleus seems to be highly efficient.

We found no indication that nuclear transport of Gcn4p is regulated as it is common for other yeast transcription factors. The metabolic transcription factor Pho4p (O´Neill *et al*., 1996) is required for induction of the *PHO5* gene in response to phosphate starvation. Pho4p was shown to be nuclear localized when yeast cells were starved for phosphate and predominantly cytoplasmic in phosphate-rich medium. Amino acid starvation did not affect the predominant nucleus localization of the metabolic transcription activator Gcn4p. Pho4p as well as Gcn4p activities depend on the kinase Pho85p. The regulation of Pho4p localization is triggered by phosphorylation by the Pho80p-Pho85p cyclin-CDK (cyclin-dependent kinase) complex resulting in nuclear export of Pho4p to the cytoplasm (O´Neill *et al*., 1996). In case of Gcn4p,

the same protein kinase presumably in combination with a yet undetermined cyclin initiates a different cellular process by initiating the Gcn4p protein degradation pathway within the nucleus. The nucleus does not necessarily regulate protein stability only for proteins which fullfill their function there. Far1p is required in the cytoplasm to polarize the actin cytoskeleton along a morphogenic gradient (Valtz *et al*., 1995). Far1p stability is regulated in the nucleus and depends as Gcn4p on SCF^{Cdc4} but has to be exported to the cytoplasm to fulfill its function (Blondel *et al*., 2000).

Specific protein degradation of Gcn4p within the nucleus

The amount of Gcn4p is carefully regulated within the cell. The signal transduction pathway resulting in a higher synthesis rate of the protein in the cytoplasm depends on intracellular sensing of the presence or absence of uncharged tRNAs reflecting the availability of protein precursors. It is yet unclear whether uncharged tRNAs are also the signal which is perceived by the nucleus and results in protein stabilization of Gcn4p. In the presence of amino acids, protein destabilization is triggered by at least two different protein kinases. One of these protein kinases is Pho85p which phosphorylates Gcn4p at residue Thr165 and subsequently targets the protein to the SCF^{Cdc4} ubiquitin ligase complex. Accordingly, a deletion of *PHO85* leads to a stabilization of Gcn4p (Meimoun *et al.*, 2000). In contrast to the situation of the transcription factor Pho4p, Pho85p phosphorylation of Gcn4p does not change the nuclear localization of the protein. It is unclear how amino acid availability regulates Pho85p activity. We show here that Pho85p is predominantly localized in the nucleus in the presence or absence of amino acid limitation and that a *pho85Δ* mutation does not affect Gcn4p nuclear import. Furthermore recent studies show evidence that Cdc4p is likewise exclusively nuclear (Blondel *et al.*, 2000). Therefore a signal monitoring the availability of protein precursors seems to be transduced the nucleus to initiate or inhibit Gcn4p phosphorylation as initial step in the protein degradation pathway. Regulation of Gcn4p stability is a highly complicated process, because Pho85p is not the only protein kinase which phosphorylates Gcn4p resulting in protein destabilization. The protein kinase Srb10p also phosphorylates Gcn4p and thereby marks it for recognition by SCF^{Cdc4} ubiquitin ligase (Chi *et al*., 2001). Similar as shown for Pho85p, Srb10p is predicted to be a nuclear and DNA-associated protein (Cooper *et al*., 1999). It remains to be elucidated whether and how the kinase activities of both proteins Pho85p and Srb10p are coordinated and whether they respond to similar or different signals. Since Gcn4p stabilization is exclusively regulated inside the nucleus the next task will be to

identify the factors involved in Pho85p and Srb10p regulation. In addition, there has to be a sensor for amino acids. Gcn2p does not seem to be this sensor for the nuclear degradation machinery. Additional factors might be involved in the crosstalk between cytoplasm and nucleus.

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Chapter 3

Nuclear import of yeast Gcn4p requires karyopherins Srp1p and Kap95p

Abstract

Yeast transcription factor Gcn4p contains two stretches of amino acid residues, NLS1 and NLS2, which are able to relocate the cytoplasmic chorismate mutase into the nucleus. Both NLSs have to be impaired to prevent the nuclear entry. Only NLS2 is conserved among fungi. A truncated version of CPCA, the counterpart of Gcn4p in *Aspergillus nidulans*, lacking the conserved NLS results in an accumulation of CPCA in the cytoplasm instead of the nucleus. Yeast Gcn4p NLS1 mediated nuclear transport is impaired by various defects in genes for karyopherins without any specificity, whereas NLS2 specifically requires the α-importin Srp1p and the β-importin Kap95p for nuclear transportation. Defects in either of the two karyopherins result in yeast strains unable to respond to amino acid starvation. We identified yeast Gcn4p as a substrate for the Srp1p/Kap95p karyopherin complex. Our data suggest that NLS2 is the essential and specific nuclear transport signal, whereas NLS1 might have an additional auxiliary function.

Introduction

An endoplasmatic reticulum associated nuclear membrane excludes the nucleus of eukaryotic organisms from the cytoplasm. This subcellular compartimentation results in a spatial separation of transcription and translation and therefore requires bidirectional intracellular trafficking of proteins and RNAs. This exchange of macromolecules is regulated via the nuclear pores, which are present in the nuclear envelope (Kaffman and O´Shea, 1999). Nuclear proteins are synthesized in the cytoplasm and subsequently transported into the nucleus (Görlich and Mattaj, 1996). Whereas smaller molecules are able to shuttle between these subcellular compartments by passive diffusion, molecules with a weight higher than 40 kDa need to be transported in a regulated manner mediated by specific transport proteins. In addition, the passive diffusion of molecules, which are able to enter the nucleus is often enhanced by active transport to allow a more efficient and regulated nuclear import.

The classical mechanism of nuclear protein import requires the formation of a heterodimeric importin α/β complex in the cytosol followed by the recognition of the cargo NLS (nuclear localization signal) and its translocation into the nucleus via the nuclear pore complex. After the cargo protein is released inside the nucleus, importin α and importin β have to return to the cytoplasm for the next round of translocation. This signal mediated nuclear import requires GTP hydrolysis by Ran, NLS motifs, and soluble factors (Görlich and Mattaj, 1996; Moore and Blobel, 1993). Most NLS motifs are characterized by one or two clusters of basic amino acids, called monopartite or bipartite NLS motifs (Dingwall and Laskey, 1991). For example, the monopartite `SV40 large T-antigen´ NLS includes five basic amino acids within a seven amino acid region. The ´nucleoplasmin´ NLS-signal is another well studied example representing a classical bipartite motif consisting of two basic clusters separated by a ten amino acid spacer region (Jans and Huber, 1996). Another type of nuclear trafficking signals are nuclear export sequences (NES). NES are less conserved and often harbour leucine rich regions (Kaffmann and O´Shea, 1999).

In *S. cerevisiae*, Srp1 is the only known importin α subunit, while higher eukaryotes express different importin α family member proteins (Yano *et al.*, 1992). A group of thirteen proteins in *S. cerevisiae* show similarities to importin β, from which at least seven were characterized as import and four as export receptors, respectively (Kaffman and O´Shea, 1999) (Table I).

Nuclear import and export mechanisms are known to be involved in the regulation of different biosynthetic pathways in yeast. Pho4p is the central transcription factor of the phosphate

metabolism of *S. cerevisiae*. When yeast is cultivated in the presence of low phosphate, Pho4p is localized in the nucleus, whereas the protein is exported into the cytoplasm under high phosphate conditions. Pho4p therefore possesses NLS as well as NES regions (Kaffmann and O´Shea, 1999).

Table I. *S. cerevisiae* importins

Importin (family)	Cargo example	Reference
Srp1 (α)	20S proteasome	Yano *et al.*, 1992 Lehmann *et al.*, 2002
Kap95 (β)	20S proteasome	Shulga *et al.*, 1996 Lehmann *et al.*, 2002
Mtr10 (β)	Npl3	Pemberton *et al.*, 1997
Kap104 (β)	Nab2, Nab4	Aitchison *et al.*, 1996
Pse1 (β)	Pho4, L25	Kaffman *et al.*, 1998 Rout *et al.*, 1997
Kap123 (β)	L25	Schlenstedt *et al.*, 1997 Rout *et al.*, 1997
Nmd5 (β)	Hog1	Ferrigno *et al.*, 1998
Sxm1 (β)	Lhp1	Rosenblum *et al.*, 1997
Pdr6	unknown function	

Gcn4p is the central transcriptional activator of the `general amino acid control`, which regulates the transcription of more than 500 target genes from many different biosynthetic pathways in response to starvation for a single amino acid as well as other environmental stimuli (Natarajan *et al.*, 2001; Hinnebusch and Natarajan, 2002). This regulatory network is also activated in response to purine starvation, glucose limitation or UV radiation (Mösch *et al.*, 1991; Yang *et al.*, 2000; Engelberg *et al.*, 1994). Starvation for amino acids leads to two effects: (1) an increased translation and (2) an increased stability of Gcn4p. The corresponding gene is weakly expressed and the protein is highly unstable under non starvation conditions (Kornitzer *et al.*, 1994). After Gcn4p has been synthesized in the cytoplasm, it has to enter the nucleus to fulfill its function as transcriptional activator of its target genes (Pries *et al.*, 2002).

Nuclear import of yeast Gcn4p is presumably a constitutive process, which does not depend on the availability of amino acids. Gcn4p harbours two nuclear localization signals, NLS1 and NLS2, which are located within the C-terminal part of Gcn4p. Each Gcn4p NLS has the potential to mislocalize a cytoplasmic protein into the nucleus. Correspondingly, only a deletion of both NLSs prevents Gcn4p to enter the nucleus (Pries *et al.*, 2002). The small cytoplasmic population of Gcn4p is more stable than the major nuclear fraction of the protein. Efficient degradation of Gcn4p seems to be restricted to the nucleus (Pries *et al*., 2002) and is triggered by phosphorylation followed by ubiquitination and degradation at the 26S proteasome (Meimoun *et al.*, 2000). The initial phosphorylation process requires the nuclear cyclin dependent kinases Pho85p and Srb10p (Meimoun *et al.*, 2000; Chi *et al.*, 2001). Furthermore it has recently been shown that Gcn4p specific Pho85p kinase activity requires its association with the cyclin Pcl5p (Shemer *et al.*, 2002).

Amino acid alignments revealed that only Gcn4p NLS2 is highly conserved among homologous proteins of other fungi. This prompted us to ask whether there is only one NLS in *Aspergillus nidulans* CPCA, the Gcn4p counterpart. We studied the localization of truncated versions of CPCA in *A. nidulans* and yeast. In contrast to the wildtype protein which was nuclear, truncated CPCA lacking the single conserved NLS accumulated in the cytoplasm. Therefore we investigated importin specificity of the two Gcn4p NLS motifs. Our results reveal that Gcn4p NLS2 specifically triggers Gcn4p nuclear import, whereas the non conserved NLS1 seems to have an additional auxiliary function. Gcn4p is a cargo for the yeast importin α Srp1p/ importin β Kap95p heterologous complex. The corresponding mutant strains are impaired to the appropriate response to amino acid starvation.

Materials and Methods

S. cerevisiae strains and growth conditions

Yeast strains used in this study are congenic to *S. cerevisiae* W303 genetic background. Details of the yeast strains used in this study are given in Table I. Standard methods for genetic crosses and transformation were used and standard yeast culture YNB minimal media were prepared essentially as described (Guthrie *et al.*, 1991). The *S. cerevisiae* strain RH2904 was obtained by insertion of a *HIS3* cassette into RH2705.

Transformation and cultivation of *Aspergillus nidulans*

For CPCA localization analysis we used the *Aspergillus nidulans* strain AGB10 (*pyrG*89, *pyroA*4) (Hoffmann *et al.*, 2001). Cultivation of *A. nidulans* strains was performed at 37°C on minimal medium (Bennett and Lasure, 1991). Transformation was carried out as described (Punt and van den Hondel, 1992) and transformants were selected on medium without uridine to select for the presence of the prototrophic marker *pyrG*. Expression of the *alcA* promoter was induced with 2 % ethanol and 2 % glycerol as sole carbon sources.

Fluorescence microscopy

Yeast strains harbouring plasmids encoding proteins fused to GFPuv (Crameri *et al.*, 1996) were grown to exponential phase in selective minimal medium. Cells 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). 4,6-diamidino-2-phenylindole (DAPI) staining was used for visualization of nuclei using standard DAPI filter sets. Cells were photographed using a Hamamatsu-Orca ER digital camera and the Improvision Openlab software (Improvision, Coventry, UK). In case of *A. nidulans* fluorescence microscopy a thin layer of solid medium without uridine to select for the presence of the prototrophic marker *pyrG* was spread on a glass slide which then was placed obliquely with one end in liquid medium which served as reservoir. The solid layer was inocculated with spores of the transformed *A. nidulans* strain and analysed by fluorescence microscopy after hyphae formation.

Table II. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
RH2701	*MATα, mtr10::HIS3, ade2, leu2, trp1, his3, ura3*	Senger *et al.*, 1998
RH2702	*MAT**a**, kap104::HIS3, ura3, his3, trp1, leu2, lys2*	Aitchison *et al*., 1996
RH2703	*MAT**a**, pse1-1, ura3, trp1, leu2*	Seedorf and Silver, 1997
RH2704	*MATα, rsl 1-4 (kap95ts), ura3, trp1, leu2, ade2*	Koepp *et al.*, 1996
RH2705	*MATα, srp1-31, ura3, his3, trp1, leu2*	Lehmann *et al*., 2002
RH2904	*MATα, srp1-31, ura3, trp1, leu2*	this work
RH2706	*MAT**a**, pse1-1, kap123::HIS3, ura3, trp1, leu2*	Seedorf and Silver, 1997
RH2707	*MATα, kap123::HIS3, ura3, his3, leu2*	Seedorf and Silver, 1997
RH2708	*MATα, nmd5::HIS3, ura3, his3, leu2, ade2, ade8*	Ferrigno *et al*., 1998
RH2709	*MAT**a**, pdr6::HIS3, ura3, his3, trp1, leu2*	Lau *et al*., 2000
RH2710	*MAT**a**, sxm1::HIS3, ura3, his3, trp1, leu2*	Seedorf and Silver, 1997
W303	*MAT**a**, ade2, trp1, leu2, his3, ura3*	Naysmith *et al.*, 1990

Table III. Plasmids used in this study

Plasmid	Description	Reference
p426MET25	pRS426 containing *MET25* promoter and *CYC1* terminator	Mumberg *et al.*, 1994
pME2126	*MET25prom-GFP-GCN4* fusion in p426MET25	Pries *et al.*, 2002
pME2128	*MET25prom-GFP-GCN4*$_{aa1-221}$ fusion in p426MET25	Pries *et al.*, 2002
pME2129	*MET25prom-GFP-GCN4*$_{aa1-169}$ fusion in p426MET25	Pries *et al.*, 2002
pME2130	*MET25prom-GFP-ARO7* fusion in p426MET25	Pries *et al.*, 2002
pME2131	*MET25prom-GFP-ARO7-GCN4*$_{aa167-200}$ fusion in p426MET25	Pries *et al.*, 2002
pME2133	*MET25prom-GFP-ARO7-GCN4*$_{aa231-249}$ fusion in p426MET25	Pries *et al.*, 2002
pME2446	*alcA* promoter, GFPS65T, *His2B* terminator, amp^{R}, *pyr4*	Fernandez *et al.*, 1998
pME2447	*A. nidulans* CPCA as *Kpn*I fragment in pME2446	this study
pME2448	*A. nidulans* CPCA$_{aa1-190}$ as *Kpn*I fragment in pME2446	this study
pME2494	*A. nidulans* CPCA-GFP in p426MET25	this study
pME2495	*A. nidulans* CPCA$_{aa1-190}$-GFP in p426MET25	this study

Results

Deletion of a conserved NLS of the Gcn4p counterpart CPCA impairs its nuclear transport in the filamentous fungus *A. nidulans*

S. cerevisiae Gcn4 protein harbous two regions, which are able to mediate nuclear localization of the cytoplasmic protein chorismate mutase (Pries *et al.*, 2002). Amino acid alignments revealed that the second Gcn4p nuclear localization sequence (NLS2) is highly conserved among homologous proteins of other fungi like e. g. *Aspergillus nidulans* CPCA, *Aspergillus niger* CpcA, and *Neurospora crassa* CPC1. Even the human AP-1 transcriptional activator c-Jun harbours a putative NLS motif with nearly 58 % identity to Gcn4p NLS2 (Figure 1 A), whereas NLS1 of *S. cerevisiae* Gcn4p is not conserved within these proteins.

CPCA of the filamentous fungus *Aspergillus nidulans* is the central transcription factor of the cross-pathway-control (*cpc*) of amino acid biosynthesis, which is the counterpart of the general amino acid control of *S. cerevisiae*. CPCA reveals strong similarities with *S. cerevisiae* Gcn4p (40 % identity) and is functionally exchangeable (Hoffmann *et al.*, 2001). We investigated the subcellullar localization of GFP marked CPCA in *A. nidulans* AGB10 (*pyrG*89, *pyroA*4) by fluorescence microscopy. The chimeric protein was driven from an ethanol inducable *alcA* promoter. *A. nidulans* expressing CPCA-GFP (pME2447) was grown on a thin layer of solid selective-minimal medium on glass slides and could thus directly be analysed after hyphae formation. CPCA was identified as a predominantly nuclear protein independently of the availability of amino acids, which was confirmed by DAPI staining of the nuclei (Figure 1 B). Nuclear localization of CPCA corresponds to the localization recently described for *S. cerevisiae* Gcn4p (Pries *et al.*, 2002). Localization of truncated *A. nidulans* $CPCA_{aa1\text{-}190}$-GFP (pME2448) lacking the conserved putative NLS motif resulted in cytoplasmic accumulation of the chimeric protein (Figure 1 B). This suggests that the conserved amino acid stretch is a functional and unique NLS in *A. nidulans* CPCA, whereas a Gcn4p NLS1 equivalent is missing.

Since *A. nidulans* CPCA and *S. cerevisiae* Gcn4p are functionally exchangeable, we also investigated the localization of entire and truncated *A. nidulans* CPCA versions in *S. cerevisiae*. Correspondingly to Gcn4p (Pries *et al.*, 2002), the entire *A. nidulans* CPCA is a nuclear protein in *S. cerevisiae* (Figure 2). Truncated $CPCA_{1\text{-}190}$-GFP lacking the conserved amino acid stretch accumulates in the yeast cytoplasm (Figure 2, pME2495), likewise as in *A.*

nidulans (Figure 1 B). Therefore the yeast import machinery is unable to recognize additional CPCA amino acid stretches as import signals.

A

	NLS2	% Identity to Gcn4p
S. cerevisiae (Gcn4p)	KRARNTEAARRSRARKLQR	100
A. nidulans (CPCA)	KRARNTEAARKSRARKLER	89,4
A. niger (CpcA)	KRARNTEAARKSRARKLER	89,4
N. crassa (CPC1)	KRARNTLAARKSRERKAQR	78,9
H. sapiens (c-Jun)	KRMRNRIAASKCRKRKLER	57,8

B

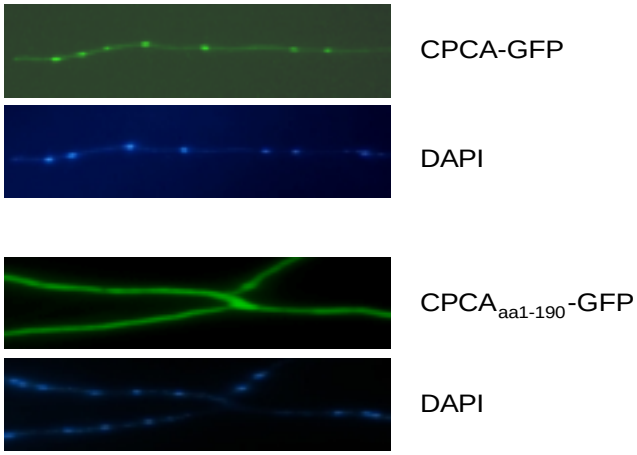

Figure 1: Nuclear import of *A. nidulans* CPCA is triggered by a single highly conserved NLS.

A: Amino acid alignments of *S. cerevisiae* Gcn4p with the homologous proteins CPCA of *Aspergillus nidulans,* CpcA of *Aspergillus niger*, CPC1 of *Neurospora crassa*, and human c-Jun show the highly conserved character of Gcn4p NLS 2.

B: The nuclear localization of CPCA of *A. nidulans* which was C-terminally conjugated with GFP was verified by DAPI staining of the nuclei. Truncated CPCA$_{aa1-190}$ lacking the conserved NLS motif which is accumulated in the cytoplasm is shown below. Both, CPCA-GFP and CPCA$_{aa1-190}$-GFP chimeric proteins were analysed in *A. nidulans* strain AGB10 and driven from an induced *alcA* promoter.

In contrast to *A. nidulans* CPCA, deletion of both NLS motifs is required to block Gcn4p import into the yeast nucleus (Figure 2, pME2129), whereas Gcn4p lacking only the conserved nuclear localization signal (NLS2) is still able to enter the nucleus (Figure 2,

pME2128) (Pries *et al.*, 2002). These data confirm that *A. nidulans* CPCA harbours only a single classical bipartite NLS motif, which is highly conserved amoung other fungi.

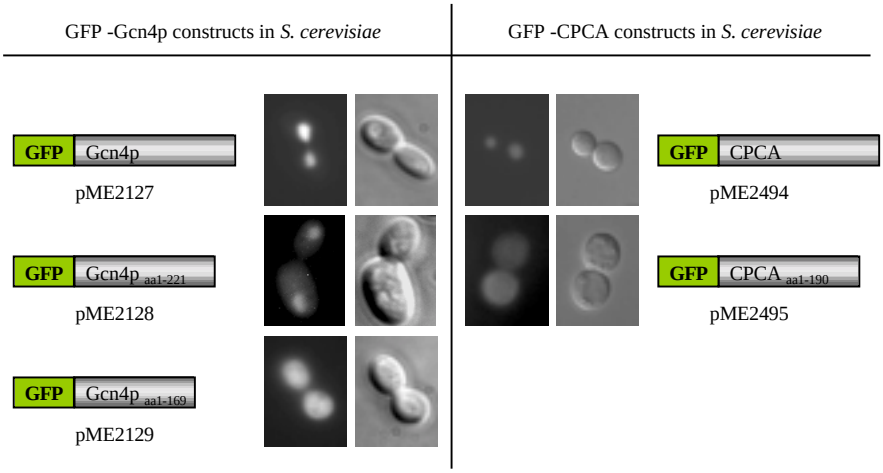

Figure 2: Truncated $CPCA_{aa1-190}$ of *A. nidulans* expressed in yeast accumulates in the cytoplasm.
Localizations of wildtype and truncated *A. nidulans* CPCA and S. cerevisiae Gcn4p expressed in yeast were compared. Both entire proteins, yeast Gcn4p (pME2126) and CPCA of *A. nidulans* (pME2494), were localized in the nucleus of *S. cerevisiae*. Correspondingly to the cytoplasmic localization in *A. nidulans* (Figure 1 B), truncated $CPCA_{aa1-190}$-GFP is impaired to enter the nucleus of *S. cerevisiae* (pME2495). In contrast, deletion of the conserved NLS2 of Gcn4p still results in nuclear protein (pME2128). Inactivation of both NLS is required for cytoplasmic localization of Gcn4p (pME2129).

Yeast Gcn4p NLS2 is a specific nuclear localization signal and requires the α-importin Srp1p and the β-importin Kap95p for nuclear import of a cytoplasmic protein

Nuclear import of *A. nidulans* CPCA is depending on a single NLS and yeast Gcn4p on two NLSs, respectively. We wondered, whether the two Gcn4p NLS motifs have different functions. Both NLS motifs were shown to confer nuclear localization of the cytoplasmic protein chorismate mutase, which is encoded by the *ARO7* gene (Pries *et al.*, 2002). To identify the NLS binding factors, subcellular localization of GFP-Aro7p-$Gcn4p_{aa167-200}$ (pME2131) and GFP-Aro7p-$Gcn4p_{aa231-249}$ (pME2133) were compared in a set of *S. cerevisiae* mutant strains, defect in various importin encoding genes. Yeast strains carrying the non-temperature sensitive importin mutations *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709) and *sxm1* (RH2710) were cultivated and analysed at a temperature of 30°C. Strains carrying

the temperature sensitive mutations *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH2703), *kap95* (RH2704), *pse1*/*kap123* (RH2706) and *srp1* (RH2904) were analysed at the permissive temperature of 25 °C and compared to the restrictive temperature of 37 °C.

The Gcn4 protein could clearly be visualized inside the nucleus in all analyzed mutant strains at the permissive temperature, whereas nuclear import in the *srp1* mutant strain was already partially affected at 25 °C. After transferring the cells to the restrictive temperature of 37 °C, localization analyses revealed that nuclear import of the chimeric protein GFP-Aro7p-NLS2$_{aa231-249}$ is only affected by the *srp1* (RH2904) and *kap95* (RH2704) mutations, respectively (Figure 3). This suggests that the classical bipartite NLS2$_{aa231-249}$ mediates specific protein translocation via an importin-α Srp1p/ importin-β Kap95p heterodimeric complex.

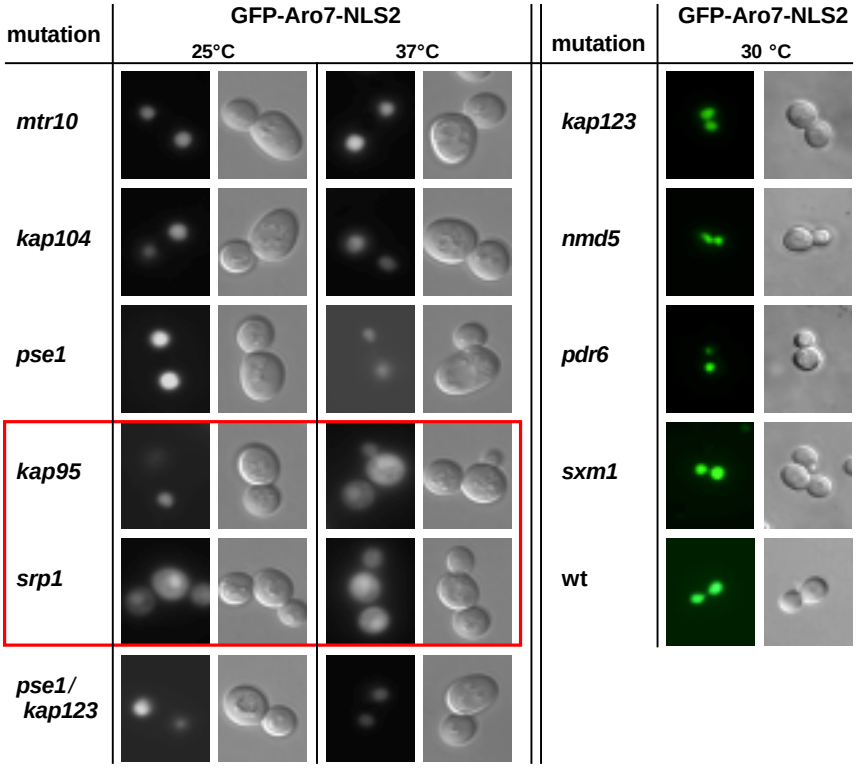

Figure 3: Nuclear import of GFP-Aro7p-NLS2 requires Srp1p and Kap95p in yeast.

Nuclear import of GFP-Aro7p-NLS2 (pME2133) was analysed in six temperature sensitive *S. cerevisiae* importin mutant strains. GFP-Aro7p-NLS2 translocation is not affected in the *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH2703), and *pse1*/*kap123* (RH2706) mutant strains, whereas the chimeric protein accumulated in the cytoplasm in *kap95* and *srp1* mutant strains at the restrictive temperature of 37 °C, which is marked by a frame. In addition, nuclear import of GFP-Aro7p-NLS2 (pME2133) was investigated in four strains carrying the non-temperature sensitive importin mutations *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709), and *sxm1* (RH2710). All four mutant strains were not affected in nuclear import of GFP-Aro7p-NLS2, correspondingly to the wild type control (W303).

Investigations concerning Gcn4p $NLS1_{aa167-200}$ obtained a substantial different result. Nuclear import of GFP-Aro7p-$NLS1_{aa167-200}$ was observed in the mutant strains *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709) and *sxm1* (RH2710) (Figure 4), but significantly impaired in the *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH2703), *kap95* (RH2704), *pse1*/*kap123* (RH2706) and *srp1* (RH2904) mutant strains at their restrictive temperature of 37 °C (Figure 4), suggesting that Gcn4p $NLS1_{aa167-200}$ might only have an auxiliary, unspecific function.

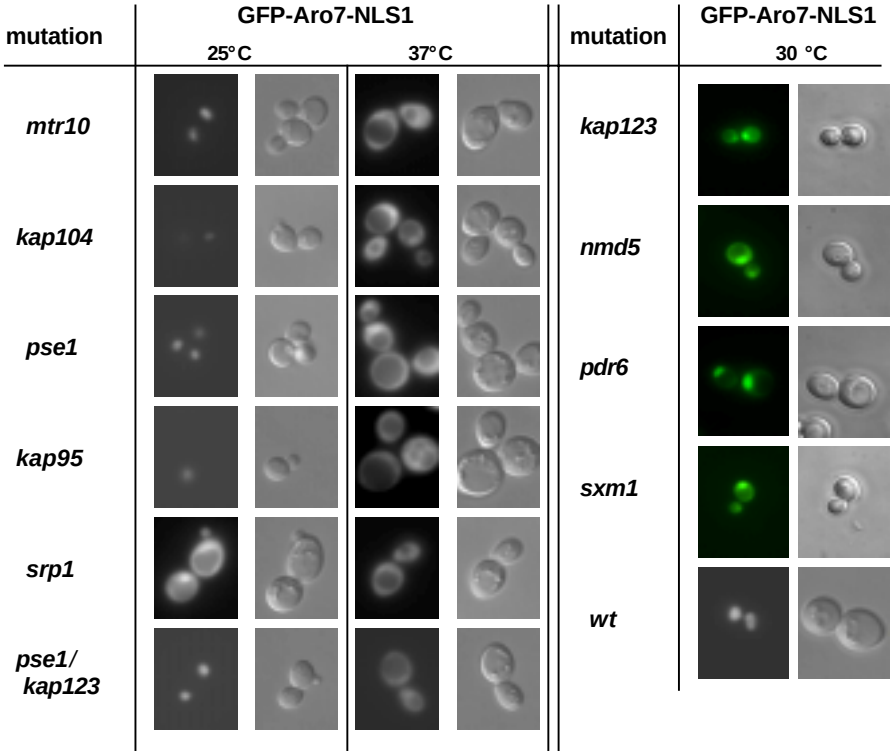

Figure 4: Nuclear import of GFP-Aro7p-NLS1 is affected by various importin mutations in yeast.

Nuclear import of GFP-Aro7-NLS1 (pME2133) was investigated in yeast strains carrying the temperature sensitive mutations *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH2703), *pse1*/*kap123* (RH2706), *kap95* (RH2704), and *srp1* (RH2904). Translocation of the chimeric protein is affected in all mutant strains at the restrictive temperature of 37 °C. Furthermore four mutant strains representing the non-temperature sensitive importin mutations *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709), and *sxm1* (RH2710) were analysed by fluorescence microscopy concerning nuclear import of GFP-Aro7p-NLS1. All four mutant strains were not affected in nuclear import of GFP-Aro7p-NLS1, correspondingly to the wild type control (W303).

Nuclear import of yeast Gcn4p requires Srp1p and Kap95p

Nuclear import is essential for Gcn4p to fulfill its function as transcription factor of numerous genes from different biosynthetic pathways in *S. cerevisiae*. To identify the karyopherins required for nuclear import of the entire Gcn4 protein, we analysed the localization of GFP-Gcn4p (pME2126) in various genetic backgrounds of *S. cerevisiae* carrying mutations in importin genes. GFP-Gcn4p was localized in the nucleus in the *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709) and *sxm1* (RH2710) mutant strains, similar to the wild type situation (Figure 5). Mutant strains carrying *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH2703), and *pse1*/*kap123* (RH2706) alleles also did not affect Gcn4p nuclear import. However, a cytoplasmic accumulation of GFP-Gcn4p was observed in *kap95* (RH2704) or *srp1* (RH2904) mutant strains. Nuclear import in the *srp1* mutant strain is already partially impaired at permissive 25 °C (Figure 5).

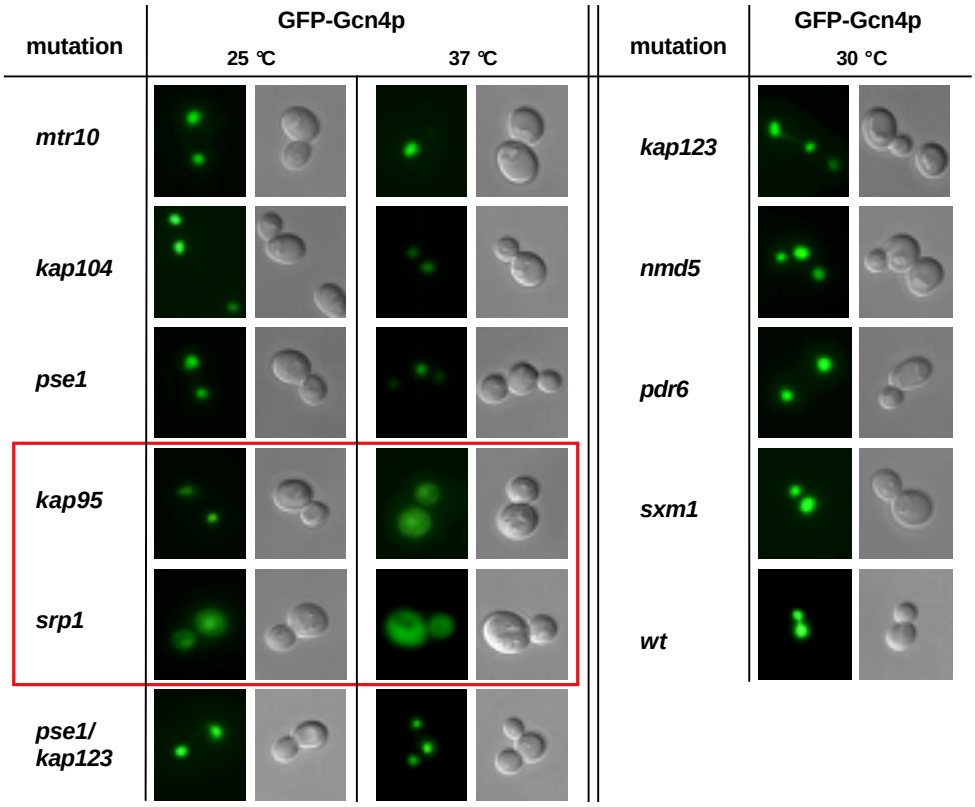

Figure 5: Yeast *kap95* and *srp1* mutant strains are affected in Gcn4p nuclear import.

A functional GFP-Gcn4p fusion protein (pME2126) was analysed in six temperature sensitive importin mutant strains by fluorescence microscopy at the permissive (25°C) and restrictive (37°C) temperature, respectively. Gcn4p translocation is not affected in the *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH2703) and *pse1*/*kap123*

(RH2706) mutant strains, whereas the *kap95* (RH2704) and *srp1* (RH2904) mutations clearly impair Gcn4p to enter the nucleus at the restrictive temperature of 37 °C, which is marked by a frame.
In addition, four mutant strains representing the non-temperature sensitive importin mutations *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709), *sxm1* (RH2710) and the wild type strain (W303) were analysed by fluorescence microscopy. All four mutant strains showed no defects concerning Gcn4p nuclear import compared to the wild type control (W303).

These data suggest that Gcn4p transport into the nucleus requires the α-importin Srp1p and the β-importin Kap95p. Together with the previous results, these data suggest that both karyopherins specifically interact with NLS2 which is presumably the major and conserved localization signal.
While the yeast transcriptional activator Pho4p was shown to be imported by the importin β family member Pse1p (Kaffman *et al.*, 1998), we present Gcn4p as a yeast substrate translocated *in vivo* by the classical importin α/β complex Srp1p/Kap95p.

Yeast strains with defects in the α-importin Srp1p or the β-importin Kap95p are unable to adapt to amino acid starvation

A functional `general amino acid control` network is essential for fungal growth in the presence of amino acid analogues causing amino acid limitation. Strains with an affected `general amino acid control` are called *gcn*, which stands for `general control nonderepressable` (Hinnebusch and Natarajan, 2002). Consequently, an affected nuclear import of Gcn4p should lead to a *gcn* phenotype. Therefore the sensitivity of the described importin mutant strains against amino acid limitation was analysed by use of the analogue 3-amino-triazole (3AT). This drug is a false competitive feedback-inhibitor of the *HIS3* gene product and therefore results in histidine starvation (Klopotowski and Wiater, 1965). Cell growth was compared on minimal medium (YNB) and YNB with 20 mM 3AT. As additional control growth on and YNB with 20 mM 3AT and histidine was monitored, in order to distinguish between any toxic effects of 3AT and histidine starvation. The yeast cells were incubated at 25 °C, 30 °C and 37 °C for 3 days to observe the effect of the conditional mutant strains (Figure 6).

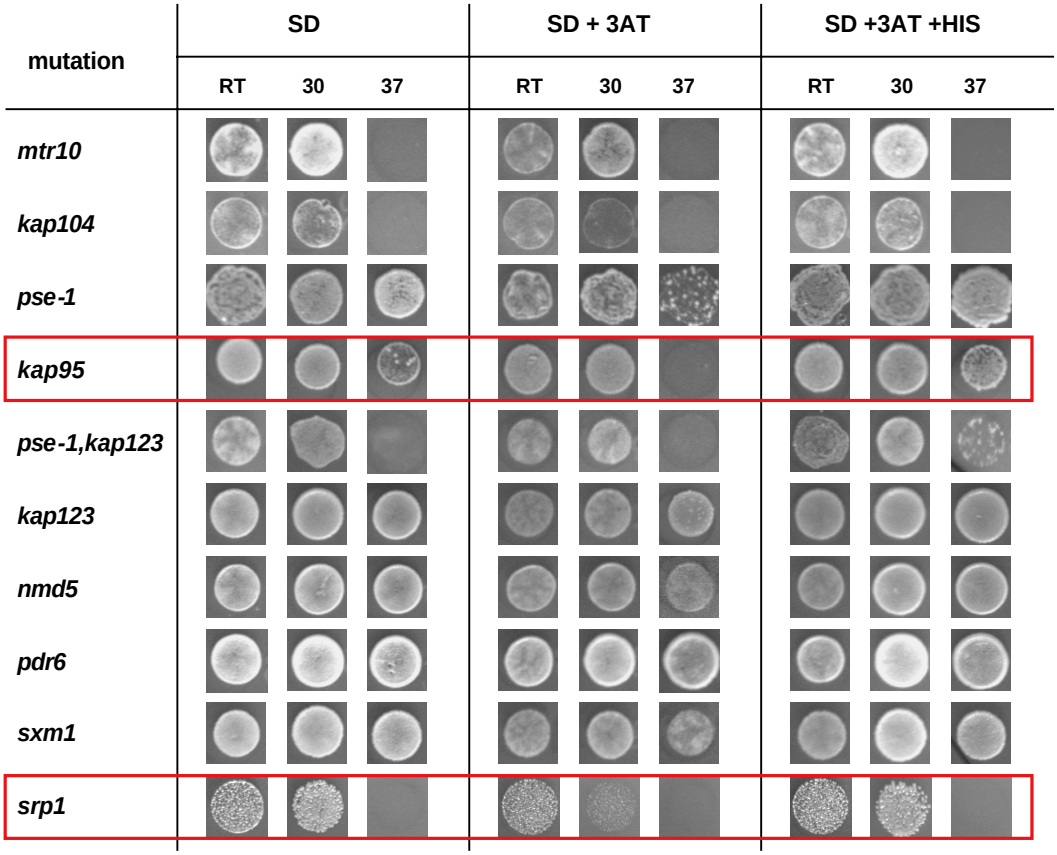

Figure 6: *kap95* and *srp1* mutant strains are unable to respond appropriately to amino limitation.
The importin mutant strains *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH27013), *pse1*/*kap123* (RH2706), *kap95* (RH2704), *srp1* (RH2904), *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709), *sxm1* (RH2710) were cultivated on plates containing minimal medium (YNB), YNB with 20mM 3AT, and YNB with 20mM 3AT and histidine. With respect to the conditional mutant strains, plates were incubated at 25°C, 30°C, and 37°C. Growth of the *srp1* and *kap95* mutant strains is affected on 3AT containing plates at 30°C and 37°C, respectively, whereas growth can be restored by the addition of histidine.

The data shown in Figure 6 demonstrate that yeast mutant strains impaired in *srp1* and *kap95* were unable to grow in the presence of the analogue 3AT at their restrictive temperature. The addition of histidine to the medium restored the resistance against the analogue. 3AT sensitivity of the *srp1* mutant strain was already obtained at 30 °C, whereas growth was completely blocked at 37 °C. Growth of the *mtr10*, *kap104* and *pse1*/*kap123* conditional mutant strains was clearly affected at 37 °C on all tested media, whereas no explicit 3AT sensitivity could be observed (Figure 6). These data corroborate our previous results and show

that the importin-α Srp1p and the importin-β Kap95p are required for a functional general control response of yeast to adapt to amino acid starvation. These data suggest that defects in these specific importins prevent the transcription factor to enter the nucleus and therefore Gcn4p is unable to fulfill its function.

Discussion

Relation of NLS and nuclear import

Regulated nuclear import of proteins in a eukaryotic cell requires energy, importins, and nuclear localization sequences (NLS). The yeast Gcn4 protein harbors two amino acid stretches, which are able to confer nuclear import of a cytoplasmic protein (Pries *et al.*, 2002). This seems to be unique amoung yeast nuclear proteins so far, whereas e.g. the human transcription factor NF-AT2 is known to harbour two NLS motifs. The inactivation of both NLS is required for its cytoplasmic localization (Beals *et al*., 1997). Our aim was to estimate the requirement of the two Gcn4p NLS motifs, since NLS2, but not NLS1 seems to be highly conserved amoung the homologous proteins of other fungi. Therefore both Gcn4p NLS motifs were analysed concerning their importin specificity and furthermore localization analyses of the Gcn4p counterpart CPCA of *A. nidulans* were carried out.

The data presented in this work indicate, that NLS2 mediates specific nuclear import of Gcn4p via the importins Srp1p and Kap95p, which was confirmed by fusing NLS2 to the chimeric GFP-Aro7 protein. NLS2 resembles a classical bipartite NLS motif, which is known from various nuclear proteins of *Saccharomyces cerevisiae* like e. g. Cdc16p, Cdc25p, Rad1p or Pho2p (Dingwall and Laskey, 1991). Nevertheless, there are also exceptions from this conclusion, like the targeting sequence of yeast Gal4p, which is supposed to consist of as many as 74 amino acids (Silver *et al.*, 1984).

A. nidulans CPCA lacking the conserved NLS2 motif is not able to enter the nucleus, neither in *A. nidulans* nor in *S. cerevisiae*, suggesting that CPCA harbous only a single NLS. Furthermore, Gcn4p-NLS1 could not be dedicated to specific *S. cerevisiae* karyopherins. Taken together, Gcn4p-NLS1 might only have an auxiliary or additive function, like e. g. it was shown that the insertion of an additional NLS has an additive effect on nuclear import of the yeast ribosomal protein L29 (Underwood and Fried, 1990). This does not mean that nuclear protein import is always related simply to the presence or absence of NLS. A functional NLS of a nuclear protein may not be able to target a non-nuclear protein to the nucleus, suggesting different strengths and potencies of nuclear targeting sequences (Dingwall and Laskey, 1991).

S. cerevisiae α-importin Srp1p and β-importin Kap95p

Importin α Srp1p is a 60 kDa protein and was originally identified as a genetic suppressor protein of RNA-polymerase I mutations (Enenkel *et al.*, 1995). It harbours a consensus sequence for a bipartite nuclear localization signal within the N-terminal part of the protein and could be localized inside the nucleus independently of the cell cycle stage (Kussel *et al.*, 1995).

Whereas Srp1p is the only known importin α family member in *S. cerevisiae*, there are different related importin α homologous proteins in higher eukaryotes. Human importin α *KPNA3* for example shows high similarities to human importin α *RCH1* and as well to yeast *SRP1* (Takeda *et al.*, 1997).

This study shows that *S. cerevisiae* Srp1p recognizes the classical bipartite NLS2 motif of the transcription factor Gcn4p and is required for its subsequent nuclear import. Former studies revealed that yeast Srp1p is even functional as a cytoplasmic/nuclear transporter protein in mammalian cells. Srp1p is able to bind the NLS motif of LEF-1 (lymphoid enhancer factor) and mediate its translocation into the nucleus (Prieve *et al.*, 1998).

S. cerevisiae Kap95p is a 95 kDa protein, which shows high homology to karyopherin β in vertebrates and was shown to mediate nuclear import of proteins in mammalian cells by formation of a heterodimeric complex together with Srp1p (Enenkel *et al.*, 1995).

GFP localization analyses identified *Saccharomyces cerevisiae* Gcn4p as an *in vivo* substrate for α-importin Srp1p and β-importin Kap95p. The corresponding mutant strains are unable to respond appropriately to amino acid starvation.

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Chapter 4

Stabilization of yeast Gcn4p in starved cells is secured by Pho81p and its stabilized cyclin Pcl7p

Abstract

Amino acid limitation results in the stabilization of the yeast transcriptional activator Gcn4p. Gcn4p regulates a genetic network which includes numerous amino acid biosynthetic genes. Rapid decay of Gcn4p is initiated by phosphorylation by the nuclear cyclin-dependent kinase (CDK) complex Pho85p/Pcl5p in the presence of amino acids. Gcn4p stabilization in response to amino acid starvation requires Pho81p. The protein is constitutively stable and predominantly nuclear within starved as well as sated cells. Pho81p is part of the Pho81p/Pcl7p CDK inhibitor complex which regulates Pho85p/Pcl5p. Amino acid limitation does not activate *PCL7* transcription. The cyclin Pcl7p was predominantly localized within the yeast nucleus, independently of the availability of amino acids. Amino acid limitation results in an increased stability of Pcl7p. We propose that Pcl7p plays a key role in stabilizing Gcn4p under starvation conditions.

Introduction

The *S. cerevisiae* bZIP transcriptional activator Gcn4p of the general amino acid control (GAAC) upregulates the transcription of more than 500 genes in response to environmental stimuli like e. g. amino acid starvation (Hinnebusch and Natarajan, 2002), purine limitation (Mösch *et al.*, 1991; Rolfes *et al.*, 1993), or UV radiation (Engelberg *et al.*, 1994). Besides an increased *GCN4* translation, amino acid starvation results in a stabilization of Gcn4p, which is highly unstable in sated cells with a half life of approximately five minutes or less (Kornitzer *et al*., 1994).

Rapid degradation of Gcn4p is initiated by phosphorylation by the two cyclin dependent kinases (CDKs) Pho85p and Srb10p. Deletions of either *PHO85* or *SRB10* result in a stabilization of Gcn4p (Chi *et al.*, 2001; Meimoun *et al*., 2000). Pho85p activity plays a more prominent role in Gcn4p stabilization than Srb10p activity (Shemer *et al*., 2002). Srb10p is a component of the RNA polymerase II holoenzyme complex and seems to be involved in degradation and in the transcriptional activation function of Gcn4p (Chi *et al*., 2001). Srb10p might be required to limit the number of transcripts for a given bound Gcn4p at a target promoter. A limited number of transcripts per promoter-bound Gcn4p allows the cell to rapidly turn down GAAC when the environmental conditions improve.

Phosphorylation of Gcn4p at Thr165 by the cyclin dependent kinase (CDK) Pho85p marks the protein for ubiquitination by the SCF^{Cdc4} ubiquitin ligase complex and subsequently results in its rapid decay by the 26S proteasome (Meimoun *et al.*, 2000). Both, Gcn4p and Pho85p were localized predominantly in the yeast nucleus independently of the availability of amino acids, whereas Gcn4p relocation into the cytoplasm stabilizes the protein (Kaffman *et al.*, 1998; Pries *et al.*, 2002).

Association of Pho85p with the cyclin Pcl5p was shown to be required for Gcn4p phosphorylation. Correspondingly, a *pcl5Δ* mutation leads to stabilized Gcn4p (Shemer *et al.*, 2002). Pcl5p itself is highly unstable and its continous synthesis is required for Pho85p/Pcl5p activity. Translation efficiency is affected in amino acid starved cells and therefore the amount of Pcl5p decreases in response to amino acid limitation (Shemer *et al.*, 2002).

Different cyclins are known to interact with Pho85p and thought to be responsible for distinct Pho85p functions. Association of Pho85p with the cyclin Pho80p is required for phosphorylation of Pho4p, the transcriptional activator of the yeast phosphate metabolism (Kaffman *et al.*, 1998). In contrast to the regulation of Gcn4p, Pho85p/Pho80p triggered

phosphorylation of Pho4p does not initiate its degradation but results in rapid nuclear export of the protein in phosphate rich medium (Kaffman *et al.*, 1998). Phosphate limitation leads to an inhibition of Pho85p activity by the cyclin dependent CDK inhibitor Pho81p, which allows Pho4p to fulfill its activation function inside the nucleus (Kaffman *et al.*, 1998; Schneider *et al.*, 1994).

We asked whether Pho85p kinase activity might also be inhibited by Pho81p in response to amino acid limitation. Therefore the role of Pho81p and its associated cyclin Pcl7p concerning the regulation of Gcn4p stability was investigated.

We show evidence that the CDK inhibitor Pho81p is required for proper Gcn4p stabilization in amino acid starved cells. Pho81p is predominantly nuclear and relatively stable in starved and sated cells, which excludes its regulation at the levels of subcellular localization or protein stability. We identified the Pho81p associated cyclin Pcl7p as a nuclear enriched protein with only a minor cytoplasmic population. Amino acid limitation does not activate *PCL7* transcription but leads to an increased stability of Pcl7 protein, suggesting Pcl7p as a sensor for amino acid starvation in yeast.

Materials and Methods

S. cerevisiae strains and growth conditions

Yeast strains used in this study are either congenic to the *S. cerevisiae S288c* (RH1168) or W303 (RH2711, RH2712) genetic background. Details of the yeast strains used in this study are given in Table I. Standard methods for yeast transformation were used and standard yeast culture YPD and YNB media were prepared as described (Guthrie and Fink, 1991).

Plasmids

The plasmids used in this study are described in Table II. Plasmid pME2228 expressing green fluorescent protein (GFP)-Pho81p from the *MET25* promoter was obtained by amplifying the *PHO81* open reading frame with *Pfu* polymerase and introducing it via *Cla*I into p426MET25 (Mumberg *et al*., 1994). Plasmid pME2230 expressing green fluorescent protein (GFP)-Pcl7p from the *MET25* promoter was constructed by amplifying the *PCL7* open reading frame with *Pfu* polymerase and introducing it via *Bam*HI/*Hin*dIII into p426MET25 (Mumberg *et al*., 1994). In case of pME2228 and pME2230 a *Bam*HI site was introduced in front of the coding region for insertion of a 750 bp *Bgl*II fragment encoding the GFP-uv variant of GFP that was amplified from plasmid pBAD-GFPuv (Clontech, Heidelberg, Germany). Plasmid KB294 expressing a triple myc epitope-tagged version of wild-type Gcn4p under the control of the *GAL1* promoter, was obtained by insertion of a 120 bp triple myc epitope (*myc*^{3}) into a *Not*I restriction site after the fourth amino acid (D. Kornitzer, Haiffa, Israel).

GFP fluorescence microscopy

Yeast strains harbouring plasmids encoding GFP-fusion proteins were grown to exponential phase in selective minimal medium. Cells 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). 4´,6-diamidino-2-phenylindole (DAPI) staining was used for visualization of nuclei using standard DAPI filter sets. Cells were photographed using a Hamamatsu Orca ER digital camera and the Improvision Openlab software (Improvision, Coventry, United Kingdom).

Protein analysis

Preparation of whole yeast cell extracts was performed as described (Surana *et al*., 1993). Routinely, 10 µg of crude protein extracts were separated. After separation on SDS gels, proteins were transfered to nitrocellulose membranes. Proteins were visualized using ECL enhanced technology (Amersham) after incubation of membranes with polyclonal mouse anti-Myc, mouse anti-GFP or rabbit anti-Cdc28 antibodies and a peroxidase-coupled goat anti-mouse or goat anti-rabbit secondary antibody.

Table I. Strains used in this study

Strain	Genotype	Source
RH1168	*MAT**a**, leu2-3, ura3-52, gal2*	Our collection
RH2711	*MAT**a**, ade2, trp1, leu, his3, ura3*	O´Neill *et al*., 1996
RH2712	*MAT**a**, ade2, trp1, leu2, his3,ura3, pho81Δ::TRP1*	O´Neill *et al*., 1996

Table II. Plasmids used in this study

Plasmid	Description	Reference
p426MET25	pRS426 containing *MET25* promoter and *CYC1* terminator	Mumberg *et al*.,1994
pME2228	*GFP-PHO81* fusion in p426MET25	this study
pME2230	*GFP-PCL7* fusion in p426MET25	this study
KB294	*GAL1prom-myc*^{3}*-GCN4* fusion in *URA3*-marked 2µm vector	Kornitzer, D.

Results

A *pho81Δ* mutation results in an affected Gcn4p stabilization

In sated yeast cells Gcn4p is phosphorylated at Thr165 by Pho85p/Pcl5p, which marks the protein for SCF^{Ccd4} mediated ubiquitination and guarantees subsequent Gcn4p decay at the 26S proteasome. Decreased amounts of the highly unstable cyclin Pcl5p are suggested to cause an increased Gcn4p stability in response to amino acid limitation (Shemer *et al.*, 2002). Besides Gcn4p, the cyclin dependent kinase Pho85p is known to phosphorylate the *S. cerevisiae* transcription factor Pho4p in phosphate rich medium, whereas Pho85p activity is inhibited by the cyclin dependent kinase inhibitor (CDKI) Pho81p in the absence of phosphate (Kaffman *et al.*, 1998).

Therefore we asked whether Pho85p mediated phosphorylation of Gcn4p might only be determined by the level of Pcl5 protein or if Pho85p activity might furthermore be regulated by Pho81p with respect to the availability of amino acids.

Gcn4p stability was compared in a *pho81Δ* mutant strain (RH2712) and the corresponding *PHO81* control strain (RH2711), which are both auxotrophic for leucine in the presence or absence of amino acid limitation. Therefore myc-tagged *GCN4* driven from an inducable *GAL1*-promoter on a 2*μm* plasmid (KB294) was expressed in exponentially growing cells with 2% galactose as sole carbon source. After 4 hours of induction, yeast cells were shifted to minimal medium with 2 % glucose to shut off the *GAL1*-promoter. One half of the culture was cultivated without, the other half in the presence of leucine to compare the effect of amino acid starvation. Subsequently, samples were collected 5, 15, and 30 minutes after promoter shut off and the amounts of myc-Gcn4p were detected by Western hybridization analyses with an anti-myc antibody. Our data revealed that the *pho81Δ* mutant strain is significantly impaired in stabilizing Gcn4p when compared to the *PHO81* wildtype control strain (Figure 1).

Therefore these data suggest that proper Gcn4p stabilization under amino acid starvation conditions requires the Pho85p inhibitor Pho81p.

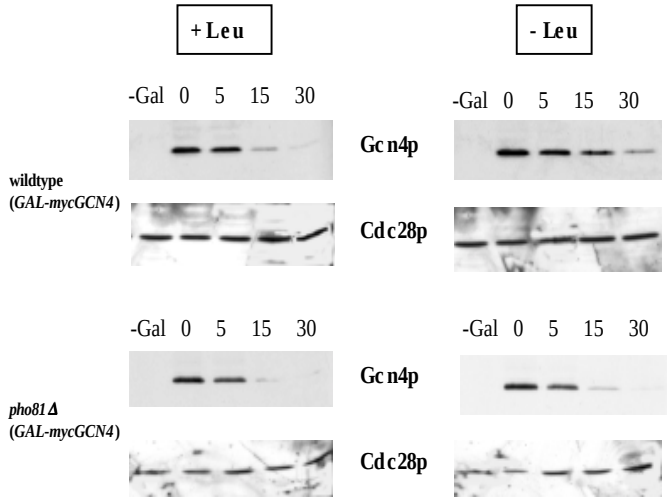

Figure 1: Pho81p is required for Gcn4p stabilization in starved yeast cells.
The *pho81Δ S. cerevisiae* mutant strain RH2712 and the *PHO81* control strain RH2711 were transformed to express *GAL-myc-GCN4* on a high copy plasmid (KB294). Cells were grown to early log phase in selective minimal medium at 30°C. A 2% galactose concentration was added to these cycling cultures to express *MYC-GCN4*. Cells were collected and incubated in minimal medium with or without leucine and glucose as a carbon source to shut off the *GAL1* promoter. Samples were collected at the indicated time points after the shift to glucose medium (0 min time point). Protein levels of Myc-tagged Gcn4p were determined by immunoblotting using Myc antibodies. The kinase Cdc28p was detected with an anti Cdc28 antibody and used as loading control.

Pho81p is a stable protein in starved and sated cells which is predominantly localized in the nucleus

Gcn4p stabilization in response to amino acid starvation is restricted to the yeast nucleus (Pries *et al*., 2002). Since Gcn4p could hardly be stabilized in *pho81Δ* mutant cells, we were interested in the subcellular localization of Pho81p in starved and sated cells. Localization of Pho81p was investigated in living yeast cells by use of chimera proteins with the green fluorescent protein (GFP). Chimeric GFP-Pho81p driven from a regulatable *MET25* promoter was analysed in *S. cerevisiae* by fluorescence microscopy. Pho81p-GFP was predominantly observed in the yeast nucleus in the presence and absence of amino acids, respectively (Figure 2A).

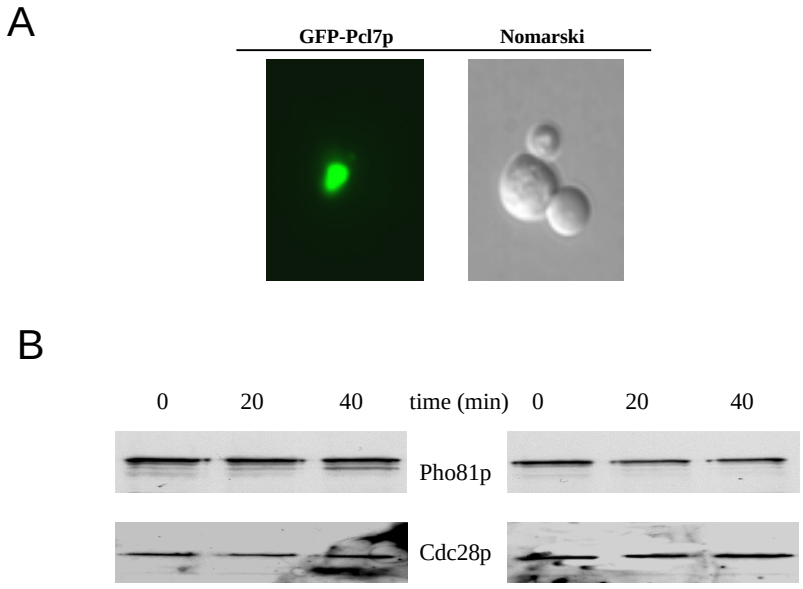

Figure 2:

Pho81p is a predominantly nuclear and relatively stable protein independently of the availability of amino acids.

A: Yeast cells (RH1168) expressing GFP-Pho81p fusion protein from the *MET25* promoter (pME2228) were grown to early log phase in selective minimal medium at 30°C and analysed by DIC microscopy (right column) and fluorescence microscopy (left column). GFP-Pho81p is predominantly nuclear localized in the presence and absence of leucine.

B: The yeast strain RH1168 was transformed to express *GFP-PHO81* from a *MET25* promoter (pME2228). Cells were pregrown to early log phase in selective minimal medium at 30°C. Subsequently, cells were collected and incubated in minimal medium with or without leucine and a 1mM methionine concentration to shut off the *MET25* promoter. Samples were collected at the indicated time points after the shift to methionine medium (0 min time point). Levels of GFP-Pho81p were determined by immunoblotting using GFP antibodies. The kinase Cdc28p was used as loading control. Stability of GFP-Pho81p is similar in the presence or absence of amino acid limitation.

Furthermore investigations were carried out concerning the stability of Pho81p in yeast cells starved and non starved for leucine. GFP-Pho81p was expressed in exponentially growing yeast cells and subsequently the *MET25* promoter was shut off by the addition of methionine to a final concentration of 1 mM. Gcn4p stabilization is thought to occur within a time

window of about 30 minutes (Kornitzer *et al.*, 1994). Hence, samples were taken 20 minutes and 40 minutes after promoter shut off and Pho81p stability was investigated in Western hybridization analyses with an anti-GFP antibody. Our results reveal a proper expression of the chimeric GFP-Pho81 protein. Equal amounts of protein were observed in starved and sated cells within the analysed time window (Figure 2B). In summary, our results exclude a regulation of Gcn4p stability at the levels of Pho81p subcellular localization or stability.

Equal amounts of *PCL7* transcripts are expressed in sated and starved yeast cells and result in predominantly nuclear localized protein

Our data show that Pho81p is required for the stabilization of Gcn4p in response to amino acid starvation. Pho81p activity was neither regulated at the level of subcellular localization nor protein stability. Since Pho81p activity is specified by its association with the cyclin Pcl7p, we analysed whether Pcl7p is affected by the amino acid availability (Kaffman *et al.*, 1998; Schneider *et al.*, 1994). Cyclins are known to underly rapid synthesis and turnover processes with respect to the cellular requirements. In order to elucidate whether Pho81p activity might be regulated via a varying availability of Pcl7p, *PCL7* transcription and Pcl7 protein stability were investigated in cells starved and non starved for amino acids.

Therefore localization of GFP-Pcl7p driven from a *MET25* promoter (pME2230) was investigated in living yeast cells under starvation and non starvation conditions for amino acids. Amino acid limitation was induced by shifting the transformed leucine auxotrophic yeast strain (RH1168) from rich to minimal medium lacking leucine. GFP-Pcl7p was localized mainly in the nucleus under both conditions, whereas in addition a weak cytoplasmic localization was observed (Figure 3A).

These data indicate that Gcn4p stabilization is not regulated at the level of nuclear/cytoplasmic trafficking of the CDK inhibitor Pho81p or its associated cyclin Pcl7p with respect to the availability of amino acids.

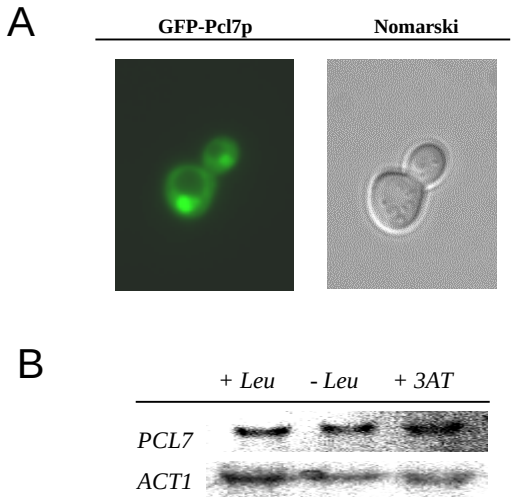

Figure 3: Pcl7p is not transcriptionally activated in starved cells and is enriched in the yeast nucleus

A: Yeast cells (RH1168) expressing GFP-Pcl7p fusion protein from the *MET25* promoter on a high copy plasmid (pME2230) were grown to early log phase in selective medium at 30°C and analysed by DIC microscopy (right column) and fluorescence microscopy (left column).

B. Total RNAs were isolated from exponentially growing yeast cells in the presence or absence of amino acids. Amino acid limitation was obtained by shifting the leucine auxotrophic cells to medium lacking leucine or by the addition of the amino acid analogue 3AT, which results in histidine starvation. Amino acid limitation did not affect *PCL7* transcription.

Total RNA was prepared from exponentielly growing yeast cells (RH1168), whereas amino acid limitation was induced by shifting the leucine auxotrophic cells from minimal medium to medium lacking leucine or by the addition of 20 mM 3AT to the medium, which is an inhibitor of the *HIS3* gene product and therefore results in histidine starvation (Klopotowski and Wiater, 1965).

PCL7 is equally transcribed in the presence and absence of amino acid limitation, which corresponds to the fact that no Gcn4p recognition elemets (GCRE) are present in the *PCL7* 5´region (Figure 3B).

Stabilization of Pcl7p in response to amino acid starvation

We asked whether the Pcl7 protein stability depends on the amino acid availability. Therefore promoter shut off experiments were carried out to analyse the relative turnover rates of Pcl7 protein in starved and sated yeast cells. A regulatable *MET25*-promoter was used to express GFP-tagged Pcl7 protein (pME2230) in exponential growing *S. cerevisiae* (RH1168) cells. The promoter was shut off by the addition of methionine to the medium. Subsequently, Pcl7p decay was investigated by Western hybridization analysis with an anti-GFP antibody.

Our results reveal the correct expression of the before localized GFP-Pcl7p chimeric protein and an increased stability of Pcl7p in response to leucine starvation (Figure 4). In the absence of methionine significant amounts of Pcl7p were detectable. One hour after methionine addition and promoter shut the protein was hardly detectable In contrast, GFP-Pcl7p was clearly stabilized when cells were shifted to minimal medium lacking leucine (Figure 4).

These data illustrate a correlation of Pcl7p and Gcn4p stabilization in response to amino acid starvation.

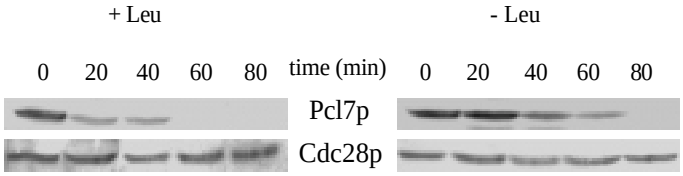

Figure 4: Yeast Pcl7p stability in response to leucine starvation.

Yeast strain RH1168 was transformed to express *MET25-GFP-PCL7* on the high copy plasmid pME2230. Cells were grown to early log phase in selective medium at 30°C. Cells were collected and incubated in minimal medium with or without leucine. 1mM Methionine were added at time point 0 to shut off the *MET25* promoter. Samples were collected at the indicated time points after the shift to methionine medium. Levels of GFP-Pcl7p were determined by immunoblotting using GFP antibodies. The kinase Cdc28p was used as loading control.

Discussion

Gcn4p phosphorylation by Pho85p/Pcl5p

The translational control mechanisms which are responsible for an increased Gcn4p synthesis in amino acid starved yeast cells are intensively studied. However, there are many questions concerning the regulation of Gcn4p stabilization within the nucleus (Pries *et al*., 2002). Gcn4p half life increases from hardly five minutes to about 20 min in response to amino acid limitation. The sensor kinase Gcn2p which is known to be required for the translational control of *GCN4*, is not required for Gcn4p stabilization (Kornitzer *et al*., 1994).

The amino acid dependent Gcn4p decay pathway in sated cells is rapidly initiated by phosphorylation by the nuclear Pho85p/Pcl5p CDK/cyclin complex, which subsequently leads to SCF^{Cdc4} mediated ubiquitination and Gcn4p degradation at the 26S proteasome (Shemer *et al.*, 2002). It was recently shown that the cyclin Pcl5p itself is a highly unstable protein with a half life of approximately two minutes (Shemer *et al.*, 2002), suggesting that constitutive Pcl5p synthesis is required to secure Gcn4p specific kinase activity of the CDK Pho85p. Overexpression of *PCL5* results in increased sensitivity to 3-amino-triazol (3AT) (Shemer *et al.*, 2002) and sulfometuron-methyl (SM) (Jia *et al.*, 2000), which are inhibitors of amino acid biosynthesis. Pcl5p is proposed to be a sensor for amino acid starvation, due to an affected overall translation in starved cells due to decreased amounts of ternary complexes (Shemer *et al.*, 2002). Surprisingly, the *PCL5* promoter harbors two potential Gcn4p binding sites in its 5´ region and therefore *PCL5* is transcriptionally activated under amino acid starvation conditions. At first sight this seems to be contradictory to a decreased *PCL5* translation in starved cells, but it might allow the cell to translate high levels of *PCL5* mRNA upon recovery of amino acid starvation and therefore to turn off the general amino acid control by rapid Gcn4p degradation.

Pho81p/Pcl7p is proposed to counteract Pho85p/Pcl5p activity in starved cells

We showed here that the cyclin dependent kinase inhibitor Pho81p is required for proper stabilization of Gcn4p in response to amino acid limitation. Neither Pho81p subcellular localization nor protein stability is affected in amino acid starved cells. In contrast to *PCL5*,

PCL7 is not transcriptionally activated by Gcn4p. The Pho81p associated cyclin Pcl7p is clearly stabilized in starved cells.

This suggests the cyclin Pcl7p to be an additional sensor of amino acid starvation, which is involved in Gcn4p stabilization besides Pcl5p via its varying stability. Since Gcn4p activity upregulates various different biosynthetic pathways, it is likely that Gcn4p activity is regulated in parallel at distinct levels.

The model in Figure 5 proposes a regulatory cascade in which Pho85p kinase activity is double checked with respect to the availability of amino acids.

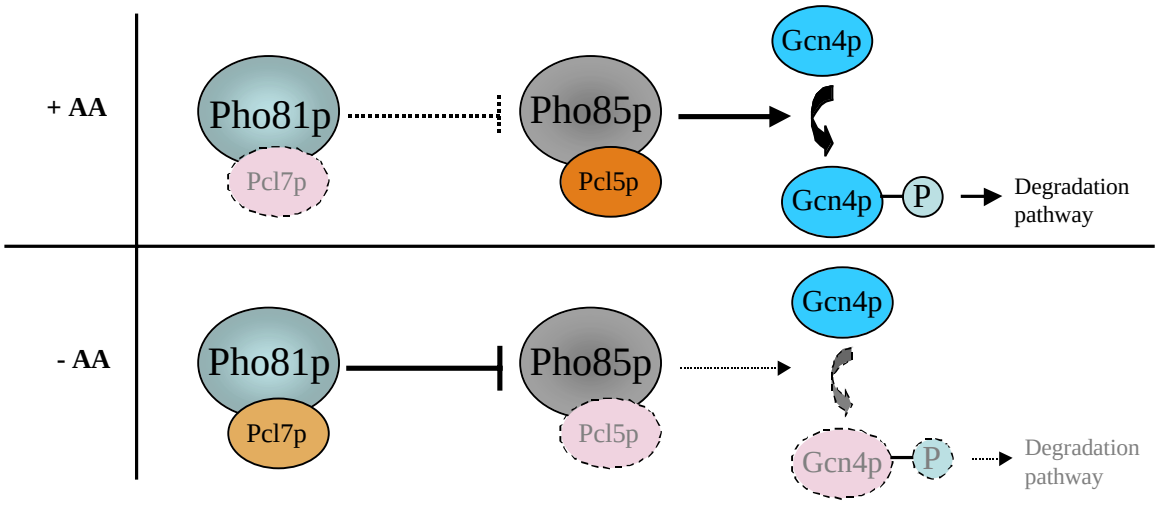

Figure 5: Model of Gcn4p stability regulation.

The model proposes an interplay of the Pho81p/Pcl7p and Pho85p/Pcl5p complexes concerning the regulation of Gcn4p stability. Rapid Gcn4p degradation in sated cells is known to be initiated by Pho85p/Pcl5p phosphorylation at Gcn4p threonine residue 165, whereas kinase activity is affected by low amounts of the Pho85p associated cyclin Pcl5p in amino acid starved cells. The cyclin dependent kinase inhibitor Pho81p and its associated cyclin Pcl7p have an inhibitory effect on Pho85p kinase activity, which is secured by high amounts of Pcl7p in amino acid starved cells due to its increased stability.

In sated cells constitutive Pcl5p synthesis secures Pho85p/Pcl5p activity and therefore rapid Gcn4p decay. When cells are starved for amino acids, Gcn4p phosphorylation by Pho85p is affected by lower amounts of available Pcl5 protein and Pho81p/Pcl7p triggered inhibition. Pho81p activity is guaranteed by an increased Pcl7p stability in response to amino acid starvation, which counteracts the overall decreased translation efficiency (Figure 5). Therefore investigations concerning the regulation of Pcl7p stability might identify a link between the state of limited amino acid availability and Gcn4p stabilization.

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Chapter 5

Conclusions and perspectives

Degradation and stabilization of Gcn4p

The transcriptional activator Gcn4p of *Saccharomyces cerevisiae* is a weakly expressed and highly unstable protein in sated cells. Amino acid starvation leads to an increased mRNA translation and protein stability and subsequently to more steady state amounts of Gcn4p within the given yeast cell.

Recent investigations have shown that the cyclin dependent kinase (CDK) Pho85p of *S. cerevisiae* initiates the rapid degradation of Gcn4p in sated cells by phosphorylation of the residue Thr165, which marks Gcn4p for ubiquitination by the SCF^{Cdc4} ubiquitin-ligase complex (Meimoun *et al.*, 2000). Pho85p is able to associate with ten different cyclins. Phosphorylation of Gcn4p specifically requires its association with the cyclin Pcl5p (Shemer *et al.*, 2002). Pcl5p itself is a highly unstable protein and consequently its efficient synthesis is required to secure Gcn4p phosphorylation by Pho85p/Pcl5p. Due to an overall reduced translation in starved cells, Pcl5p is suggested to be a sensor for amino acid starvation (Shemer *et al.*, 2002).

The data presented in this work show evidence that the cyclin dependent kinase inhibitor (CDKI) Pho81p, which is known to inhibit Pho85p kinase activity within the yeast phosphate metabolism (Kaffman *et al.*, 1998), might also be an inhibitor of Pho85p activity in starved cells. We observed an increased stability of the Pho81p associated cyclin Pcl7p in cells starved for leucine, which suggests a Pho81p activity regulation at the level of cyclin availability and Pcl7p as an additional sensor for amino acid starvation.

To support this model of a subtle double regulation of Gcn4p stability by Pho85p/Pcl5p and Pho81p/Pcl7p, it still has to be investigated in detail, which stimuli and sensor proteins are required and sufficient for the stabilization of Gcn4p in amino acid starved cells (Figure 1).

The kinase Gcn2p recognizes uncharged tRNA molecules in response to amino acid starvation and initiates an increased translation of *GCN4* mRNA, but is not required for the stabilization of Gcn4p in starved cells (Kornitzer *et al.*, 1994). It is yet unclear, whether stimuli besides amino acid starvation, such as UV radiation, which is known to cause an

increased *GCN4* translation in a Ras-dependent fashion (Engelberg *et al.*, 1994), also leads to an increased Gcn4p stability. Furthermore our data show evidence that the processes of Gcn4p degradation and stabilization occur inside the yeast nucleus (Pries *et al.*, 2002). Therefore the question arises where the cell recognizes the state of amino acid limitation and which signal transduction pathways regulate Gcn4p stability.

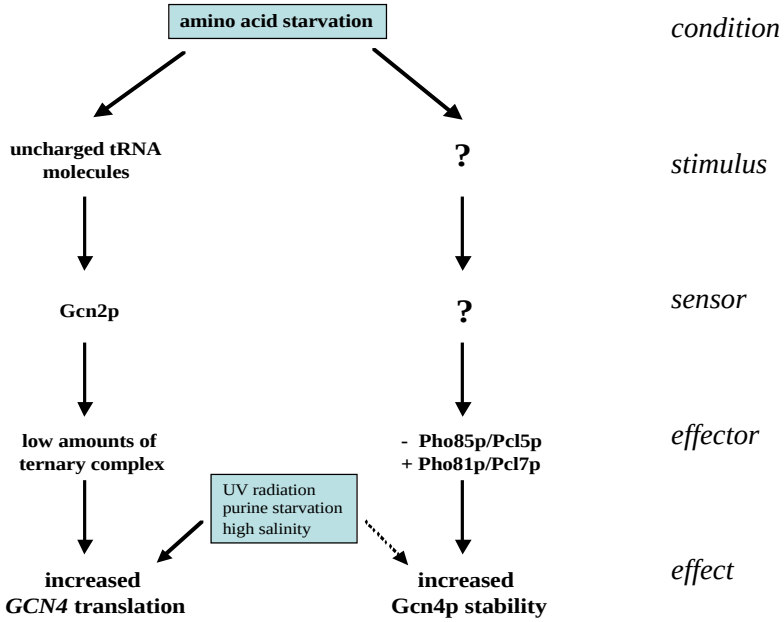

Figure 1: Model of *GCN4* translation and Gcn4p stability regulation.
Distinct pathways are supposed to regulate *GCN4* translation and Gcn4p stability in response to amino acid starvation. The kinase Gcn2p is known to recognize uncharged tRNA molecules in response to amino acid starvation and therefore initiates an increased *GCN4* translation. The stability of Gcn4p is proposed to be double checked by the CDK/cyclin complex Pho85p/Pcl5p and the CDK inhibitor/cyclin complex Pho81p/Pcl7p with respect to the amino acid availability, whereas the initiating stimuli and sensor proteins still have to be elucidated in detail. Furthermore it is not known yet, whether stimuli like e. g. UV radiation, purine starvation, and high salinity, which lead to an increased *GCN4* translation, also affect the Gcn4 protein stability.

Gcn4p Thr165 is conserved in its homologous proteins of *Neurospora crassa*, *Aspergillus nidulans*, and *Aspergillus niger*, whereas it has not been investigated so far whether these proteins are regulated at the level of their stability.

Furthermore various data suggest that different qualities of amino acid limitation might be recognized by different sensor proteins, which subsequently trigger distinct levels of Gcn4p regulation like translation and stability.

Strict and partial amino acid starvation

GCN4 expression studies have primarily been carried out by the use of bradytrophic mutant strains (leaky auxotroph) or amino acid analoga, whereas Gcn4p stabilization analyses were investigated by shifting amino acid auxotrophic cells on minimal medium lacking the required amino acid. Recent data suggest that stabilization of Gcn4p might only occur under more severe starvation conditions, where the overall translation is fully arrested (Hinnebusch and Natarajan, 2002). So it probably cannot simply be distinguished between sated and starved cells concerning the regulation of Gcn4p, but amino acid limitation has to be understood as an infinitely variable process instead (for a model see Figure 2).

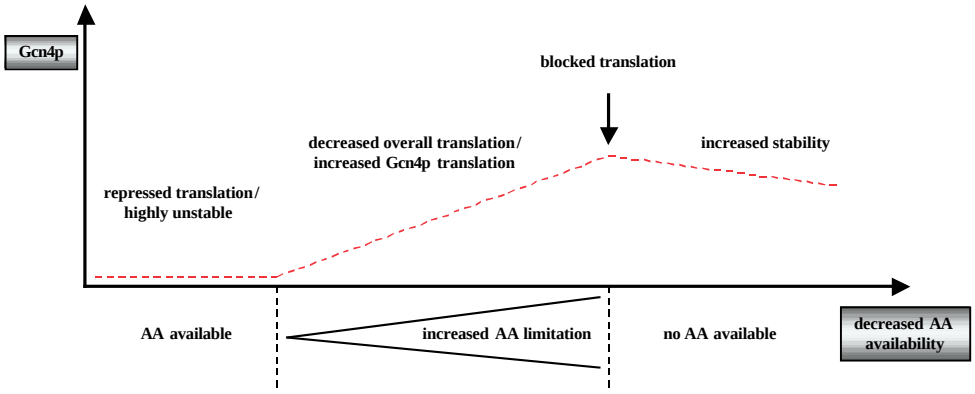

Figure 2: Model of the cellular response during a decreased amino acid availability.
A decreased amino acid availability leads to an increased translation of Gcn4p and subsequently to an increased protein stability. In sated cells, Gcn4p is weakly expressed and highly unstable. A limited or imbalanced amino acid pool results in a decreased overall translation and an increased translation of Gcn4p, which is induced by uncharged tRNA molecules that are recognized by Gcn2p. A total amino acid lack like e.g. in auxotrophic mutant cells, cannot be compensated by an increased translation and therefore Gcn4p stabilization is required to secure general amino acid control function.

It is known that amino acid imbalances or a limited availability of single amino acids, caused by amino acid analoga or bradytrophy, results in an increased translation of *GCN4* mRNA (Niederberger *et al.*, 1981). This reaction subsequently leads to a transcriptional activation of Gcn4p target genes, which allows the cell to restore a sufficient amino acid availability.

In contrast, when auxotrophic mutant strains are objected to minimal medium, the cells have to deal with an absolute lack of amino acids. Under this condition of severe amino acid starvation the kinase Gcn2p still recognizes uncharged tRNA molecules and therefore ribosome initiation occurs at the *GCN4* start codon, but efficient *GCN4* translation is affected because of missing amino acids. Therefore the stabilization of Gcn4p represents the option to preserve the current amount of Gcn4 protein and to secure an at least partially functional general amino acid control. Uncharged tRNA molecules were in addition proposed to lead to a stabilization of Gcn4p besides an increased *GCN4* translation. This was investigated by the use of a strain carrying a temperature-sensitive mutation in the $tRNA^{Ile}$ synthetase gene *ILS1*, which results in increased levels of uncharged $tRNA^{Ile}$ (Kornitzer *et al.*, 1994). In contrast to uncharged $tRNA^{His}$ molecules that arise in 3AT treated cells, the effect of tRNA synthetase mutant strains cannot be compensated by an increased *GCN4* translation and finally leads to a blocked translation which might be the reason for the observed Gcn4p stabilization (Figure 3).

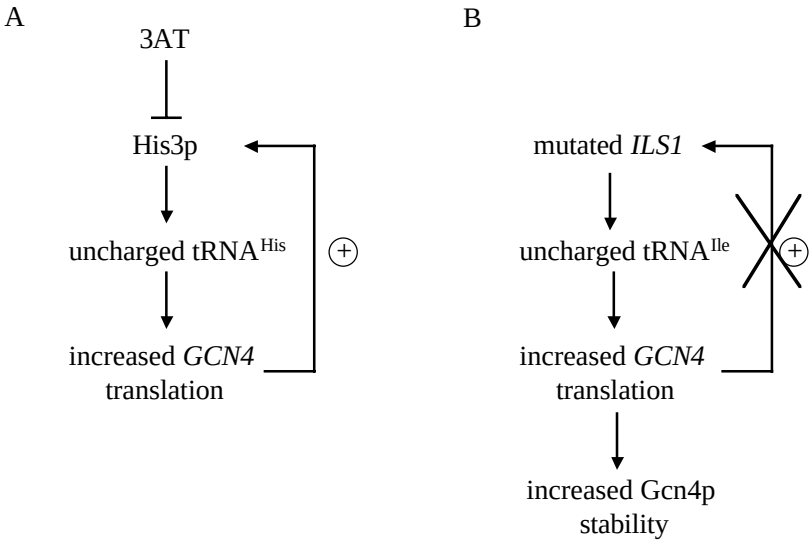

Figure 3: Mutated *ILS1* cannot be counteracted by an increased *GCN4* translation.

A: The amino acid analogue 3-amino triazole (3AT) is an inhibitor of the *HIS3* gene product and therefore leads to histidine starvation and consequently to uncharged $tRNA^{His}$ molecules (Klopotowski and Wiater, 1965), which induce an increased *GCN4* translation. Gcn4p activates the *HIS3* transcription and therefore subsequently overrules the inhibitory effect of 3AT.

B: Mutated *ILS1* cannot be counteracted by an increased *GCN4* translation and finally results in a blocked translation because of missing isoleucine. Stabilization of Gcn4p allows the cell to preserve the current amount of Gcn4 protein and therefore to rapidly turn on the general amino acid control when the environmental conditions improve.

The model of gradual Gcn4p regulation during a decreasing amino acid availability pays attention to the corresponding environmental conditions and furthermore excludes the toxical effect of excess amounts of Gcn4p, which can for example be observed when *GCN4* is overexpressed in *pcl5* mutant strains (Shemer *et al*., 2002). The cell does not require and even not endure an increased expression and stability of Gcn4p in parallel. Future projects should investigate the pathway of Gcn4p stabilization by analysing different kinds of amino acid limitation and other GAAC inducing stimuli concerning their effect on Gcn4 protein stability.

Role of the WD protein Cpc2p

The presented model of Gcn4p stability regulation proposes that a sensor of ribosomal function might be responsible for Gcn4p stabilization.

The WD-repeat protein Cpc2p of *S. cerevisiae* is proposed to be a ribosome associated protein (Link *et al*., 1999) and an inhibitor of the general amino acid control in sated cells. WD-repeat proteins are known from varying biosynthetic groups like e. g. signal transduction, RNA processing, transport of vesikels, or cell cycle (Neer *et al*., 1994; Smith *et al*., 1999), whereas they are suggested to be important concerning protein-protein interaction processes .

A *cpc2* mutation increases the transcription of Gcn4p target genes even in the absence of amino acid starvation (Hoffmann *et al.*, 1999). A corresponding phenotype was observed concerning the homologous gene *cpc2* of *Neurospora crassa* (Paluh *et al*., 1988).

A strain lacking a functional Gcn2p is impaired to induce an increased *GCN4* translation in response to amino acid limitation. Surprisingly, it was shown that a *gcn2* mutation can be abolished by an additional *cpc2* mutation. This suggests that Cpc2p might be involved in the stability regulation of Gcn4p, since the translational control is cut of in a *gcn2* mutant strain. With respect to this model of Cpc2p as an inhibitor of Gcn4p stabilization, it could be expected that correspondingly e. g. *pho85* or *pcl5* mutations would enable a *gcn2* mutant strain again to grow in the presence of amino acid limitation.

Due to its proposed ribosome association, Cpc2p might be a sensor for translation efficiency, which inhibits the general amino acid control by securing the highly unstable character of Gcn4p in sated cells and even under partial starvation for amino acids.

This work represents Gcn4p as a protein which is regulated by the given possibilities of compartimentation in eukaryotic cells. The yeast nucleus was shown as the place of Gcn4p stability regulation and furthermore several new players of this process were identified.
Therefore we did a good step forward in understanding the manifold regulation of *S. cerevisiae* Gcn4p and received various new questions to be answered.

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Curriculum Vitae

Ralph Pries

Geburtsdatum:	04.09.1972
Geburtsort:	Borghorst/Westf.
1979-1983	Marienschule Borghorst
1983-1992	Städtisches Gymnasium Borghorst
1992-1993	Zivildienst im Sonderkindergarten Morgenstern, Burgsteinfurt
1993-1998	Studium Diplom Biologie an der Georg-August-Universität zu Göttingen
1998-1999	Diplomarbeit am Institut für Mikrobiologie und Genetik, Georg-August-Universität zu Göttingen, bei Prof. G. H. Braus Thema: „Struktur des *HARO7*-Gens der Hefe *Hansenula polymorpha* (*Pichia angusta*)“
März 1999	Diplom in Biologie Hauptfach: Mikrobiologie, Nebenfächer: Immunologie und Organische Chemie
Juni 1999 - Dezember 2002	Doktorarbeit am Institut für Mikrobiologie und Genetik, Georg-August-Universität zu Göttingen, bei Prof. G. H. Braus Thema: „Localization and stability of the transcriptional activator Gcn4p in *Saccharomyces cerevisiae*“