Stability regulation of Gcn4p in *Saccharomyces cerevisiae*

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SUMMARY

Selective rapid protein degradation is an important mechanism for distinct biological events, including cell cycle progression, signal-transduction, and differentiation. Thus, the stability of proteins involved in these cellular processes such as transcription factors, cyclin-dependent kinases, and cyclins depend on different environmental changes. The *JUN* homolog *GCN4* of *Saccharomyces cerevisiae* encodes a global key regulator of a genetic network known as the 'general amino acid control' and is therefore able to respond to starvation of amino acids. The amount of Gcn4p is mainly regulated via control of its protein synthesis in the cytoplasm and control of protein degradation in the nucleus. In sated cells, Gcn4p is weakly expressed and highly unstable, whereas its translation rate and protein stability increase upon amino acid limitation conditions.

The amino acid-dependent Gcn4p degradation pathway is regulated via the phosphorylation by the nuclear cyclin-dependent kinase Pho85p in complex with the cyclin Pcl5p. As initial step of Gcn4p stabilization upon amino acid starvation the disassembly of the Pho85p/Pcl5p complex was identified. Furthermore, the proteins Pho81p, a cyclin-dependent kinase inhibitor, and Pcl7p, another Pho85p cyclin, were shown to be required for amino acid-dependent Gcn4p stabilization. Both proteins are nuclear and constantly present and not required for dissociation of Pho85p/Pcl5p in response to amino acid starvation conditions. Whereas Pho81p interacts with Pcl5p only when Gcn4p is degraded, binding to Pcl7p is a constitutive process.

The Pho85p substrate specificity for Gcn4p is mediated by the unstable cyclin Pcl5p. The control of Pcl5p sub-cellular localization was analyzed as a possible mechanism required for functional specificity of the kinase/cyclin complex. Nuclear localization of Pcl5p is independent of the availability of amino acids, Pho85p, Gcn4p, and Pho81p. In contrast, the β-importin Kap95p has been identified to be required for nuclear import of this cyclin. Deletion and transfer experiments of Pcl5p and Pcl5p/Pho80p chimera were performed to analyse important domains of cyclin Pcl5p. These investigations identified a central substrate specificity domain of Pcl5p followed by a C-terminal domain providing nuclear targeting.

Amino acid starvation-induced adherence depends on *GCN4* expression and the cell-surface flocculin Flo11p. To analyse the importance of Gcn4p turnover for the transcriptional activity of this protein, a stabilized Gcn4p version was created by the expression of $GCN4^{LEU267SER}$ or deletion of *PCL5*. In both cases, the amino acid starvation-induced Gcn4p activity is affected, and furthermore, adhesion and *FLO11* expression are not inducible in response to amino acid limitation. This suggests a linkage between transcriptional activation and protein degradation.

ZUSAMMENFASSUNG

Die selektive schnelle Proteindegradation ist ein wichtiger Mechanismus für verschiedene biologische Ereignisse wie Zell-Zyklus-Abläufe, Signal-Transduktion und Differenzierung. Folglich hängt die Stabilität von Proteinen wie Transkriptionsfaktoren, Zyklin-abhängigen Kinasen und Zyklinen, die in diese zellulären Prozesse involviert sind, von verschiedenen Umweltveränderungen ab. Das dem *JUN* homologe *GCN4* aus *Saccharomyces cerevisiae* kodiert für den globalen Schlüsselregulator eines genetischen Systems, das als die "Allgemeine Kontrolle der Aminosäurebiosynthese" bezeichnet wird und ist dementsprechend in der Lage, auf Aminosäuremangelbedingungen zu reagieren. Die Menge von Gcn4p wird hauptsächlich über die Kontrolle der Proteinsynthese im Zytoplasma und die Kontrolle der Proteindegradation im Kern reguliert. In gesättigten Zellen ist Gcn4p ein schwach exprimiertes und sehr instabiles Protein, wohingegen dessen Translationsrate und Stabilität als Antwort auf Aminosäuremangel ansteigt.

Die Aminosäure-abhängige Gcn4p Degradation wird über dessen Phosphorylierung durch die nukleäre Zyklin-abhängige Kinase Pho85p mit dem Zyklin Pcl5p reguliert. Als initiierender Schritt der Gcn4p Stabilisierung wurde die Dissoziation dieses Kinase/Zyklin Komplexes identifiziert. Des Weiteren konnten der Zyklin-abhängige Kinase Inhibitor Pho81p und ein weiteres Pho85p-Zyklin, Pcl7p, identifiziert werden, die für die Aminosäure-abhängige Stabilisierung von Gcn4p notwendig sind. Beides sind kernlokalisierte und konstitutiv exprimierte Proteine, die nicht für die Dissoziation von Pho85p/Pcl5p unter Aminosäuremangel benötigt werden. Pho81p interagiert mit Pcl5p nur unter Gcn4p-degradierenden Bedingungen, wohingegen die Interaktion zu Pcl7p einen konstitutiven Prozess darstellt.

Die Substrat-Spezifität von Pho85p für Gcn4p wird durch das instabile Zyklin Pcl5p vermittelt. Die Kontrolle der Pcl5p Lokalisierung in der Zelle wurde als potentieller, für die funktionelle Spezifität des Kinase/Zyklin Komplexes benötigter Mechanismus untersucht. Die Kernlokalisierung von Pcl5p verläuft unabhängig von Aminosäuremangelbedingungen und den Proteinen Pho85p, Gcn4p und Pho81p. Im Gegensatz dazu ist das β-Importin Kap95p als ein für den Kernimport dieses Zyklins benötigtes Protein identifiziert worden. Deletions- und Transferexperimente von Pcl5p und Pcl5p/Pho80p Chimären wurden durchgeführt, um wichtige Domänen des Zyklins zu ermitteln. Diese Untersuchungen identifizierten eine zentrale Substrat-Spezifitätsdomäne von Pcl5p gefolgt von einem C-terminalen, für die Kernlokalisierung ausreichenden Bereich dieses Zyklins.

Die Aminosäuremangel-induzierte Adhäsion ist abhängig von der *GCN4* Expression und dem Zelloberflächen-Flokkulin Flo11p. Um die Bedeutung der Degradation von Gcn4p für dessen transkriptionelle Aktivität zu untersuchen, wurde durch den Gcn4p Aminosäureaustausch Leu267Ser oder mittels einer *PCL5* Deletion eine stabilisierte Form von Gcn4p hergestellt. In beiden Fällen ist die Aminosäuremangel-induzierte Gcn4p Aktivität beeinträchtigt und die Adhäsion bzw. *FLO11* Expression durch Aminosäuremangel nicht mehr induzierbar. Das deutet darauf hin, dass Proteinabbau und transkriptionelle Aktivierung gekoppelte Prozesse in der Zelle sind.

CHAPTER I

Introduction

1 Cyclin-dependent kinases

The activities of cyclin-dependent kinases (CDKs) are required for eukaryotic cell cycle events, but furthermore for transcriptional and cellular processes such as sub-cellular localization or interaction with other proteins. They are defined as kinase catalytic subunits that require cyclin regulatory subunits for their activation and specificity. The definition of the cyclin subunit is extended from proteins whose levels fluctuate during the cell cycle (Evans et al., 1983) to a family of structurally related proteins that activate CDKs (Morgan, 1995). CDKs specifically phosphorylate their substrates and mark them, e.g. for degradation. Besides cyclin binding as a primary determinant of CDK function, the level of CDK activity, substrate recognition and sub-cellular localization are also modulated by various additional regulatory subunits and protein kinases.

1.1 Functions of cyclin-dependent kinases

1.1.1 Roles of CDKs in cell cycle control

Originally, CDKs were identified because of their role in the regulation of the events of the eukaryotic cell cycle (Levine and Cross, 1995). In the yeast *Saccharomyces cerevisiae* there are six different CDKs, of which Cdc28p and Pho85p are bound and activated by various cyclin subunits. Cdc28p regulates diverse cell-cycle transitions dependent on the association with multiple stage-specific cyclins. At initiation of the cell cycle, the kinase Cdc28p requires the Cln cyclins 1-3, in contrast to the B-type cyclins (Clb1-6) that activate Cdc28p for entry into S phase (Clb5p and Clb6p) or mitosis (Clb1-4) (Nasmyth, 1996). The CDK Pho85p, which can interact with ten different cyclins, only plays an auxiliary role in the cell cycle (Measday et al., 1997). In association with the cyclins Pcl1p and Pcl2p these kinase/cyclin complexes contribute to passage through G1. In the fission yeast *Schizzosaccharomyces pombe*, a single CDK, Cdc2p, functions in cell cycle control together with cyclins Cdc13p, Cig1p and Cig2p (Stern and Nurse, 1996). Of the three CDKs identified in the more complex filamentous fungus *Aspergillus nidulans*, $NIMX^{cdc2}$, the homolog of *S. cerevisiae* Cdc28p, is important for cell cycle progression (Osmani et al., 1994). In contrast, in vertebrates more than 10 Cdc2p related proteins were discovered, among which only Cdk1 and Cdk2 are

functionally homologous to yeast Cdc28p/Cdc2p and required for cell cycle functions. Cdk2 interacts with the cyclins E, A and B throughout the cell cycle (Edgar and Lehner, 1996; Nigg, 1995).

1.1.2 Functions of *S. cerevisiae* kinase Pho85p

Besides cell cycle progression, CDKs are involved in events including gene transcription or responses to changes in environmental conditions. The CDK Pho85p of *S. cerevisiae* is the functional homolog of the mammalian cyclin-dependent kinase Cdk5 (Huang et al., 1999) and has multiple functions, as suggested by the pleiotropic phenotype caused by its deletion (Lenburg and O'Shea, 1996; Tennyson et al., 1998). Although Pho85p is not essential for viability, a deletion of *PHO85* causes various phenotypes including abnormal morphogenesis, hypersensitivity to several chemical treatments and the constitutive expression of phosphate-starvation-dependent genes (Lee et al., 1998; Lenburg and O'Shea, 2001; Tennyson et al., 1998; Wickert et al., 1998). Its involvement in several regulatory pathways depends on the association with one of ten cyclin partners, which have been divided into two subfamilies according to their sequence homology and functional relationship (Measday et al., 1997).

The Pho80-like subfamily consists of the cyclins Pcl6p, Pcl7p, Pcl8p, Pcl10p, and Pho80p (Measday et al., 1997) postulated to function in metabolic regulation (Andrews and Measday, 1998). Pcl6p and Pcl7p are involved in the control of glycogen storage in a Pho85p-dependent manner and a deletion of *PCL6* and *PCL7* restores the accumulation of glycogen but paradoxically activates both glycogen synthase and phosphorylase (Wang et al., 2001). Furthermore, defects in carbon source utilization have been described as phenotypes of *pcl6* and *pcl7* mutants (Lee et al., 2000). The expression of *PCL7* fluctuates during the cell cycle with a peak in mid to late S phase and a Pho85p/Pcl7p complex has kinase activity dependent on the availability of phosphate and the cyclin-dependent kinase inhibitor Pho81p (Lee et al., 2000). The cyclins Pcl8p and Pcl10p also play a role in the glycogen metabolism by directing Pho85p to the glycogen synthase Gsy2p for downregulation. This is suggested by the hyperaccumulation of glycogen caused by their deletion (Huang et al., 1998). Specificity of Pho85p for the basic transcription factor Pho4p in a phosphate-rich environment is mediated by the first identified cyclin, Pho80p, resulting in Pho4p phosphorylation and inactivation (O'Neill et al., 1996). Under phosphate limiting conditions, Pho4p phosphorylation is decreased and therefore Pho4p increases the transcription of its target genes (Kaffman et al., 1998). PHOA and PHOB of *A. nidulans* are highly related to yeast Pho85p modulating differentiation in response to environmental conditions including limited phosphate (Bussink

and Osmani, 1998; Dou et al., 2003). By manual annotation and genome analysis of various Aspergilli, homologs for the genes for the ten different yeast Pho85p cyclins were found, which show relatively low similaritiy (Gallagan et al., 2005). Only *A. nidulans* PHO80 was identified as the counterpart of the *S. cerevisiae* cyclin Pho80p, which regulates Pi acquisition through a mechanism not involving the PHOA or PHOB CDKs. In addition, PHO80 is important for the developmental program in *A. nidulans* (Wu et al., 2004) (Figure 1).

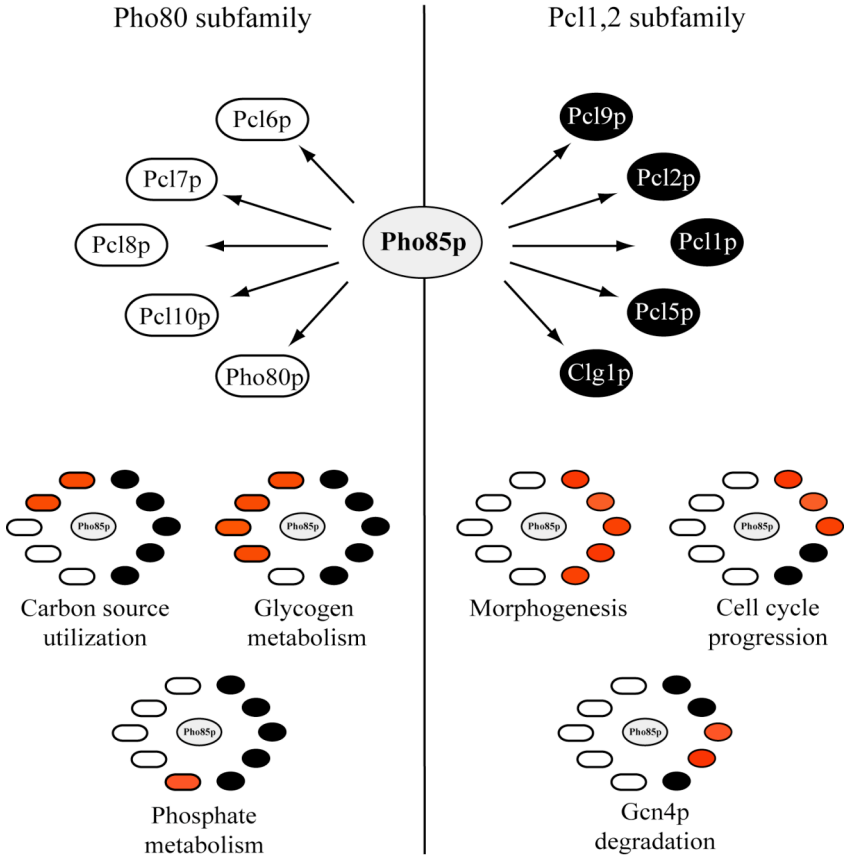

Figure 1: The kinase Pho85p, its ten different cyclins and their functions are shown. The Pho85p cyclins are divided into the Pho80 subfamily (Pcl6p, Pcl7p, Pcl8p, Pcl10p, and Pho80p) and the Pcl1,2 subfamily (Pcl9p, Pcl2p, Pcl1p, Pcl5p, and Clg1p). In each small figure the cyclins participating in the listed functions are shown in red (modified from Carroll and O'Shea, 2002).

The second Pho85p-cyclin family consists of the proteins Pcl1p, Pcl2p, Pcl5p, Pcl10p, and Clg1p and is termed Pcl1,2 subfamily (Measday et al., 1997). Pronounced morphological abnormalities were observed after deletion of members of Pcl1,2 class of genes comparable to those seen in *pho85* mutants (Measday et al., 1997). As mentioned before, when complexed with the G1 cyclins, Pcl1p and Pcl2p, Pho85p is required for initiation of the cell cycle in the absence of the Cdc28-dependent cyclins Cln1p and Cln2p (Lenburg and O'Shea, 2001). Furthermore, Pho85p is proposed to be involved in cell cycle events by regulating the

transcription factor Swi5p. Swi5p regulates the transcription of cell cycle genes including *PCL2* and *PCL9* (Aerne et al., 1998; Tennyson et al., 1998). In addition, Pho85p forms a functional kinase complex with Pcl9p, and a role for Pho85p CDKs at the M/G1 boundary has been suggested (Tennyson et al., 1998). Pho85p has also been shown to be involved in controlling the stability of proteins including the Cdc28p/Clb inhibitor Sic1p and the transcription factor Gcn4p. Both proteins undergo phosphorylation- and ubiquitination-dependent degradation (Meimoun et al., 2000; Skowyra et al., 1997). Gcn4p is the central transcription factor of the general amino acid control and is required for the induction of several hundred genes upon amino acid starvation. This environmental signal causes the increase of the Gcn4p level in the cell by translational de-repression of *GCN4* mRNA and by decreasing the degradation of Gcn4p protein (Hinnebusch, 2005). Whereas the Pho85p/Pcl1p complex is capable of Gcn4p phosphorylation *in vitro* (Meimoun et al., 2000), the cyclin Pcl5p has specifically been identified to be required for Gcn4p degradation (Shemer et al., 2002) (described in more detail in chapter 3.4 and 3.5) (Figure 1).

In summary, through its binding to a family of ten cyclins, the CDK Pho85p plays an important role in different signal transduction pathways as a response to changes in environmental conditions.

1.2 CDK regulatory mechanisms:

Cyclin abundance, cyclin-specific interactions and sub-cellular localization

The functional specificity of CDKs depends on the temporal and spatial regulation of cyclin expression and localization and furthermore on the ability of cyclins to mediate interactions between the kinase/cyclin complex and substrates, inhibitors and activators. The way in which cyclin subunits confer their specificity on the corresponding CDK has been extensively studied.

Cyclin levels are often controlled by gene transcription and cyclin proteolysis. Therefore cyclins were originally defined as proteins with fluctuating levels over a time period of a cell cycle (Evans et al., 1983). In *S. cerevisiae* the mRNA oscillations are best understood in case of the G1 cyclins (Nasmyth, 1996). At the start of the cell cycle, Cdc28p/Cln3p complexes activate the transcription of G1 cyclins *CLN1* and *CLN2* and of the S phase cyclins *CLB5* and *CLB6*. The increasing levels of Clb result in the repression of G1 cyclins and in the activation of G2/M phase cyclins (Breeden, 1996; Dirick et al., 1995; Levine et al., 1996; Stuart and Wittenberg, 1995). The control of cyclin degradation is most important at the end of mitosis, where proteolysis of mitotic B-type cyclins are mediated by the Anaphase Promoting

Complex (APC) (Irniger et al., 1995; King et al., 1996). The G1 cyclins of *S. cerevisiae* are highly unstable proteins independent of the cell cycle, which are degraded by the SCF-ubiquitin ligase pathway (Bai et al., 1996; Willems et al., 1996).

Some cyclins identified later show only little variation throughout the cell cycle leading to a new definition as a group of structurally related proteins that are able to bind and activate the kinase (Morgan, 1995). Therefore, besides the regulation of cyclin abundance, cyclins confer specificity on CDKs by specific protein interactions including CDK-inhibitory subunits. Inhibitors of CDKs are able to turn off the CDK catalytic subunit. In *S. cerevisiae* three different CDKs have been identified: Pho81p, Sic1p, and Far1p. The Clb5,6 inhibitor Sic1p is regulated at the levels of both transcription and protein stability depending on the different cell cycle phases (King et al., 1996; Nasmyth, 1996). The second CDK, Pho81p, is required for Pho85p/Pho80p inhibition in low phosphate. Binding of Pho81p to the complex occurs under both low and high phosphate conditions, whereas the kinase activity is only inhibited when cells are starved for phosphate (Hirst et al., 1994; Schneider et al., 1994). Furthermore, Pho81p itself is phosphorylated by the Pho85p/Pho80p complex, which seems to be a prerequisite for a stable interaction with cyclin Pho80p and therefore for inhibiting Pho85p activity (Knight et al., 2004; Waters et al., 2004). The inhibition function of Pho81p is mediated by a novel inhibitory domain consisting of the 80 amino acids C-terminal to the ankyrin repeats (Huang et al., 2001). This inhibitory domain is conserved in the mammalian neuronal regulator protein C42, which decreases the kinase activity of Cdk5 (Ching et al., 2002). The yeast Pho85p/Pho80p complex has been shown to be the functional counterpart of the mammalian Cdk5/p35 kinase (Huang et al., 1999). A truncated version of p35, termed p25, leads to a deregulation of Cdk5 activity that has been implicated in Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and Parkinson's disease (Lau and Ahlijanian, 2003; Nguyen and Julien, 2003; Smith et al., 2003). Furthermore, a high sequence homology of *Neurospora crassa* and *A. nidulans* CKIs Nuc-2 and AN4310 has been shown to yeast Pho81p (Poleg et al., 1996; Galagan et al., 2005).

In mammals two classes of CKIs are described, the Cip/Kip and the INK4 family, both involved in cell cycle control (Hengst and Reed, 1998; Pavletich, 1999). Mammalian CKI inhibition can be mediated by binding to different components of CDK/cyclin complexes (Carnero and Hannon, 1998; Correa-Bordes and Nurse, 1995; Pavletich, 1999; Russo et al., 1996; Serrano et al., 1993). In addition, Rum1p of *S. pombe* inhibits the Cdc2p/Cdc13p complex during G1 preventing premature mitosis (Correa-Bordes and Nurse, 1995).

An additional mechanism that contributes to the functional specificity of CDK/cyclin complexes is control of sub-cellular localization (Pines, 1999). The sub-cellular localization determines the access of cyclins to a subset of proteins, so that the control mechanism of cyclin localization is linked to the control of cyclin-protein interactions. Cyclins have to be at the right place at the right time for their predicted function (Diehl and Sherr, 1997; Hood et al., 2001). Strong evidence for the importance of sub-cellular localization for cyclin specificity comes from the cyclins B1 and B2 of higher eukaryotes. These cyclins are localized to microtubules and the Golgi apparatus respectively. Draviam et al., (2001) investigated that cyclin B1, when localized to the cytoplasm, reorganizes the cytoskeleton, whereas Golgi-localized cyclin B2 dissassembles the Golgi apparatus. By changing the N-terminal domains, but not the potential substrate binding regions of these cyclins, the new chimera proteins also change their sub-cellular localization, with cyclin B1 being directed to the Golgi and cyclin B2 to the cytoplasm. Furthermore, cyclin B2 takes on the function of cyclin B1 by reorganizing the cytoskeleton, whereas cyclin B1 function is restricted to assembling the Golgi apparatus. This indicates that the activity of these cyclins is influenced by their localization and not by the substrate targeting (Draviam et al., 2001).
In budding yeast, the G1 cyclins Cln2p and Cln3p exemplify the importance of sub-cellular localization. A shift in the localization pattern of Cln3p out of the nucleus into the Cln2p-like cytoplasmic localization allows this cyclin to take on Cln2p-like functions (Miller and Cross, 2000).

2 Regulation of protein stability

2.1 Ubiquitin-dependent protein degradation

In many eukaryotic organisms, distinct cellular processes, including signal-transduction, cell cycle progression, differentiation and development are regulated by rapid protein degradation. Therefore proteins involved in these biological events such as cyclins, cyclin-dependent kinases, transcription factors or membrane proteins have fluctuating stability depending on environmental changes (Hershko, 2005). The main eukaryotic degradation machinery includes the 26S proteasome that specifically destructs ubiquitinated proteins. Ubiquitination is carried out by the ubiquitin system ensuring that only those proteins marked with a 76kDa protein termed ubiquitin are degraded. The covalent transfer of ubiquitin to the substrate requires the activity of three different enzymes and can therefore be divided into three steps. First an ubiquitin activating enzyme (E1) activates the C-terminal Gly residue of ubiquitin in

an ATP-dependent manner, followed by transfer to an active Cys residue of a ubiquitin conjugating enzyme E2. Subsequently ubiquitin is linked to ε-amino groups of lysine residues of the substrate by an ubiquitin protein ligase, E3 (Figure 2) (Hershko and Ciechanover, 1998). Before polyubiquitinated substrates are recognized and degraded by the 26S proteasome, ubiquitin proteins are thought to de-attached from their substrates and can be directly used for the next ubiquitination (Hochstrasser, 2002).

Whereas there is only a single E1 enzyme, various types of E2 and E3 multiprotein complexes have been identified. In *S. cerevisiae* 13 genes have been described that encode E2-like enzymes, from which the E2 Cdc34p specifically associates with Cdc53p and Cdc4p, subunits of the E3 SCF ubiquitin ligase, which are involved in the degradation of the transcription factor Gcn4p (Meimoun et al., 2000). E3 ubiquitin protein ligases play an important role in substrate specificity and ubiquitination regulation. There are four types of E3 ubiquitin protein ligaes that are further subdivided into two classes: RING finger-containing E3 enzymes, which do not covalently bind to the activated ubiquitin, and ligases containing a HECT domain for direct transfer of ubiquitin to the substrate. The most prominent multi-subunit ubiquitin ligases are the RING finger SCF (Skp1/Cdc53/F-box) complexes (Deshaies, 1999; Jackson and Eldridge, 2002). A distantly related SCF complex is the Anaphase Promoting Complex/Cyclosome (APC/C) belonging to the same RING finger family. Whereas the APC/C consists of at least eleven subunits, the SCF ligase is generally composed of four major subunits: The cullin (Cul1p/Cdc53p in *S. cerevisiae*) together with the RING-H2 protein (Rbx1p, Roc1p, Hrt1p) is required for ubiquitin transfer from E2 to the substrate (Seol et al., 1999). Both proteins are linked to a special F-box protein, which receives the substrate for the Skp1p subunit (Figure 2). The SCF function is determined by the association of one of several F-box proteins, which harbor a N-terminal F-box for interaction with Skp1p and a C-terminal substrate-binding domain. Therefore, substrates are directly bound to the F-box proteins, which is followed by recruitment to the catalytic center of the core complex (Li and Johnston, 1997; Skowyra et al., 1997).

Polyubiquitinated proteins are finally degraded by the 26S proteasome, which consists of a 20S core subunit mediating peptidase activity and a 19S regulatory particle required for substrate specificity (Richmond et al., 1997).

2.2 Regulation of SCF

The activity of SCF complexes is regulated by different mechanisms. SCF activity concerning the stability of its substrate is regulated via the substrate phosphorylation status by the

corresponding kinases. For example, the transcription factor Gcn4p must be phosphorylated by the kinases Pho85p and Srb10p in complex with their appropriate cyclins to be a target for the SCF complex (Meimoun et al., 2000). Furthermore, the ubiquitination activity of SCF itself can be regulated by a process, termed neddylation. In this process the ubiquitin-related protein NEDD8 (Rub1p in *S. cerevisiae*) is reversibly conjugated onto the cullin subunit. This cycle of neddylation and deneddylation is required for E2-E3 complex formation (Figure 2) (Kawakami et al., 2001). It is also necessary for cullin-dependent polyubiquitination of SCF target proteins (Podust et al., 2000).

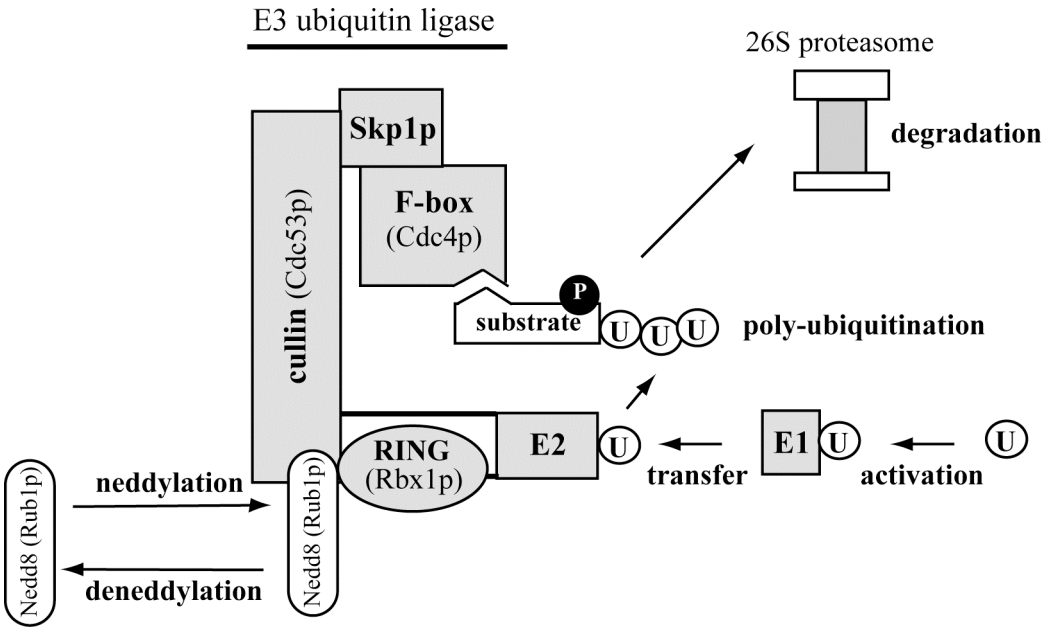

Figure 2: Model of Ubiquitin-dependent protein degradation. The ubiquitination of target substrates follows an enzymatic cascade of a ubiquitin activating enzyme (E1), a ubiquitin conjuganting enzyme (E2) and a ubiquitin ligase (E3). The ligase is composed of four subunits: Skp1p, the cullin, the RING finger-containing domain and the F-box protein. Regulation of the SCF ubiquitination activity is mediated by reversible conjugation of NEDD8 (Rub1p in *S. cerevisiae*) on the cullin subunit, termed neddylation and deneddylation. Poly-ubiquitinated substrate is degraded by the 26S proteasome. The corresponding *S. cerevisiae* proteins for degradation of the transcription factor Gcn4p are given in brackets.

An additional mechanism for regulating SCF-mediated ubiquitination is the instability of the F-box proteins themselves. They are also degraded within the SCF complex in an ubiquitin-dependent autocatalytic manner. One explanation for the instability of F-box proteins is a rapid exchange on SCF in response to changing environmental conditions.

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

To secure a sufficient amino acid supply, fungi are able to take them up from the environment, to recycle them by protein degradation or to synthesize all 20 amino acids *de novo*. In contrast to bacteria, where starvation for a single amino acid leads to activation of the corresponding biosynthetic pathway producing this amino acid, in numerous fungi the synthesis of amino acids is checked by an extensive regulatory network (Braus et al., 2003).

These regulatory networks were first identified in the filamentous fungi *A. nidulans* and *N. crassa* (Carsiotis and Jones, 1974; Carsiotis et al., 1974; Piotrowska et al., 1980) and are known as cross-pathway control (CPC). In the model organism *S. cerevisiae*, this well characterized network is called 'general amino acid control' (GAAC). The central transcription factor of this system is Gcn4p, which activates the transcription of more than 70 amino acid biosynthetic genes of 12 different pathways, pathway specific activators and genes encoding diverse aminoacyl-tRNA synthetases (Figure 3) (Hinnebusch, 2005).

The recognition of the extra- as well as intracellular concentrations of amino acids is a prerequisite for an appropriate response to limitation conditions. The sensor kinase Gcn2p of *S. cerevisiae* is attached to the ribosome and detects a limited amino acid amount in an indirect way about uncharged tRNA molecules resulting in the activation of the general amino acid control (Wek et al., 1995). Beside this regulatory network, there are further control systems that are required for facilitating an efficient translation after monitoring amino acid availability. The two highly conserved kinases Tor1p and Tor2p are part of the Target Of Rapamycin (TOR) pathway that is important for cellular growth and proliferation in response to nutrient availability (Schmelze and Hall, 2000). Furthermore, the TOR pathway regulates amino acid uptake by affecting the stability of different amino acid permeases in the plasma membrane. Thus, degradation of the amino acid transporter Tat1p is mediated by the TOR system, whereas the amino acid permease Gap1p gets stabilized in the presence of a poor nitrogen source under amino acid starvation conditions and is also controlled by Gcn4p (Beck et al., 1999; Schmidt et al., 1998; Natarajan et al., 2001).

3.1 The general control of amino acid biosynthesis in *S. cerevisiae*

The general amino acid control network in the baker's yeast is induced when the biosynthesis of amino acids is inhibited by antimetabolites or by a mutation in a biosynthetic enzyme. Besides amino acid deprivation and amino acid imbalance, various further environmental stimuli such as starvation or stress conditions also cause the activation of the general amino acid control network (Hinnebusch, 1992; Niederberger et al., 1981). This includes limitation

of purines (Mösch et al., 1991), tRNA synthetases (Meussdoerffer and Fink, 1983) or glucose (Yang et al., 2000). Furthermore, stress circumstances such as UV radiation (Engelberg et al., 1994), high salinity (Goossens et al., 2001) and treatment with rapamycin or methyl methanesulfonate (MMS) (Natarajan et al., 2001; Valenzuela et al., 2001) lead to induction of the general amino acid control. Activation of the GAAC response is not triggered by growth on minimal medium because yeast cells have a high basal expression level of many amino acid biosynthesis genes to produce all 20 amino acids themselves. Therefore amino acid biosynthesis must be inhibited using amino acid analogs such as 3-amino-triazole (3AT) (Klopotowski and Wiater, 1965), 5-methyl-tryptophan (5MT) (Schürch et al., 1974) or 8-aza-adenine (8azA) (Rolfes and Hinnebusch, 1993). Alternatively, the mutation of a biosynthetic enzyme leading to auxotrophic or bradytrophic mutant strains can cause the activation of the GAAC (Kornitzer et al., 1994).

Numerous key trans-acting factors required for the GAAC were identified by genetic analyses (Hinnebusch, 1988). The corresponding genes are subdivided into two groups. Recessive mutations in positive regulatory *GCN* genes (general control non-derepressable) result in hypersensitivity to amino acid inhibitors such as 3AT and therefore in a loss of transcriptional activation of all biosynthetic genes encoding enzymes subjected to the GAAC. In contrast, mutations in the negative regulator *GCD* genes (general control derepressed) lead to a Gcd^- phenotype with constitutively active general amino acid control (Harashima and Hinnebusch, 1986).

The global transcription factor of this regulatory network is the protein Gcn4p in the yeast *S. cerevisiae*, the functional homolog of the *A. nidulans* CPCA protein and the human JUN protein. Gcn4p regulates the transcription of more than several hundred target genes in response to different environmental stimuli (Figure 3) (Kleinschmidt et al., 2005; Natarajan et al., 2001). A genome wide transcriptional analysis of the adhesion-deficient laboratory strain S288c showed that the transcription of 539 genes is stimulated by Gcn4p in response to 3AT treatment. 176 genes belong directly or indirectly to the amino acid and nitrogen metabolism, of which 78 genes encode amino acid or purine biosynthesis enzymes. All amino acid biosynthetic pathways are directly or periphally under the control of Gcn4p. Effectively, ca. 10% of the yeast genome in amino acid starved cells are induced by Gcn4p, including as well the above mentioned genes as genes encoding vitamin and co-factor biosynthetic enzymes, peroxisomal components, mitochondrial carrier proteins, amino acid transporters, and autophagy proteins (Figure 3) (Natarajan et al., 2001).

In addition, transcriptional profiling experiments of adhesion-capable Σ1278b cells subjected to amino acid starvation conditions identified 22 novel genes inducible by amino acid starvation and 72 genes of different functional groups requiring Gcn4p for full transcriptional activation in response to amino acid starvation. Furthermore, many genes were identified in this study which are induced by amino acid deprivation in a Gcn4p-independent way (Kleinschmidt et al., 2005).

Besides this metabolic response, Gcn4p has been shown to be required for regulation of adhesion and differentiation in *S. cerevisiae* and for filamentous growth in *Candida albicans* (Braus et al., 2003; Tripathi et al., 2002). Thus, in amino acid starved *S. cerevisiae* cells, Gcn4p induces *FLO11*, which encodes a cell-surface flocculin necessary for haploid invasive growth and diploid pseudohyphal development. Therefore a direct connection between metabolic and developmental processes is shown in *S. cerevisiae*.

The transcription factor Gcn4p itself is regulated at the levels of translation initiation, transcription and protein stability (for details see text of section 3.3 and 3.4) (Figure 3).

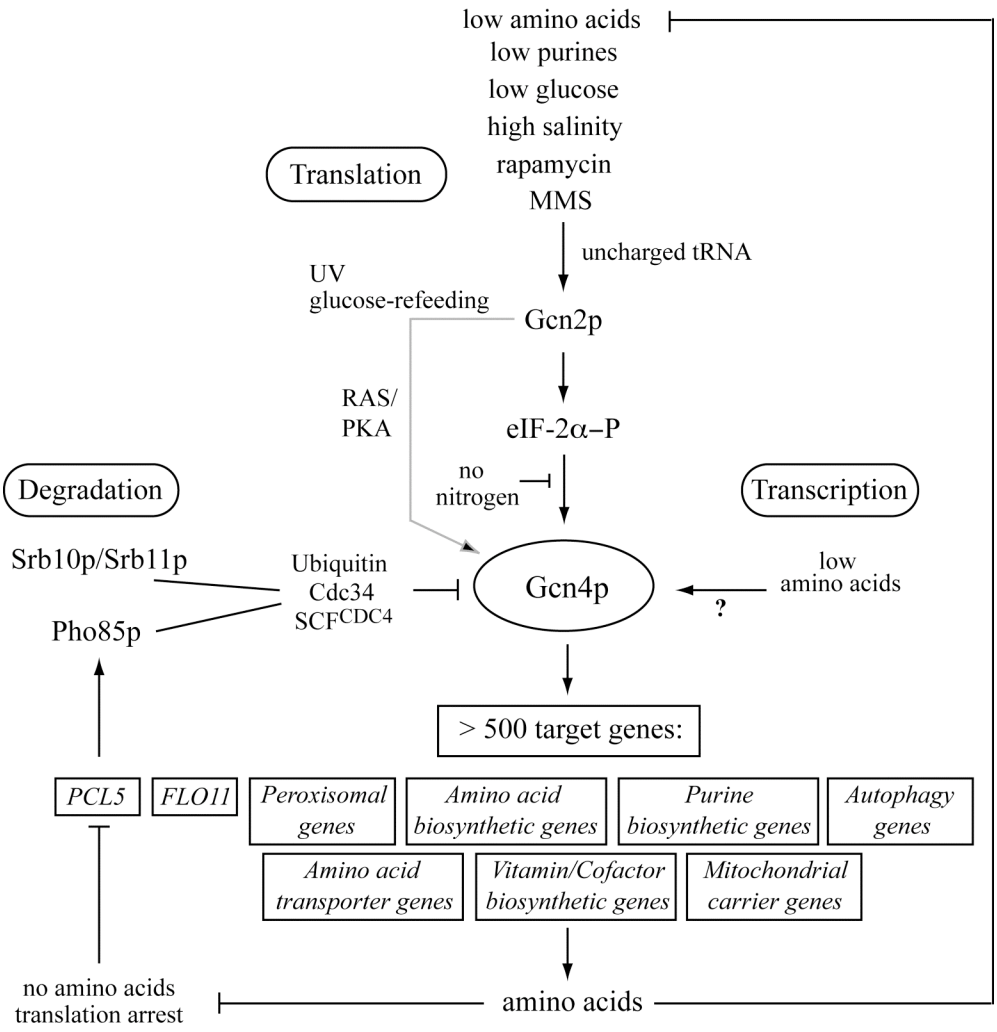

Figure 3. Model of the major mechanisms regulating Gcn4p levels and GAAC target genes in *S. cerevisiae* cells. Gcn4p is controlled by different signals and factors at the level of translation, transcription or protein degradation resulting in transcriptional regulation of a huge number of target genes from different biosynthetic pathways (see text for details).

3.2 Functional dissection of yeast Gcn4p

Gcn4p represents the central element of the general amino acid control. This transcription factor consists of 281 amino acids with a molecular weight of 31 kDa (Hinnebusch, 1984; Thireos et al., 1984). Due to its C-terminal part Gcn4p represents the prototype of the group of alkaline leucine zipper transcription factors (Weiss et al., 1990). The 60 C-terminal amino acids of Gcn4p include the Leucine Zipper domain ((LZ) aa249-289) and the basic DNA Binding Domain ((DB) aa221-249) sufficient for dimerization and DNA binding (Figure 4) (Hope and Struhl, 1986). Leucine zippers are characterized as motifs consisting of four repeats of leucine residues separated by seven amino acids (Landschulz et al., 1988). Unexpectedly, the leuzine zipper of *A. niger* cpcA does not contain the characteristic leucine

residues (Hinnebusch, 1984; Hope and Struhl, 1986). In contrast to the mammalian homologous proteins JUN and FOS, which can form homo- as well as heterodimers (Turner and Tjian, 1989), yeast Gcn4p can only bind as a homodimer (Paluh and Yanofsky, 1991) to a specific 9bp palindromic nucleotide sequence (5'-ATGA(C/G)TCAT-3') termed GCn4-protein Response Elements (GCRE) (Hope and Struhl, 1986; Oliphant et al., 1989). Furthermore, it was shown recently that Gcn4p also binds with high affinity to GCRE half sites *in vitro* (Hollenbeck and Oakley, 2000).

DNA binding of Gcn4p is followed by the transcriptional activation of appropriate target genes mediated by the activation domain (Figure 4) (Drysdale et al., 1995). This activation domain spans approximately half the protein and can be divided into the N-Terminal Activation Domain ((NTAD) aa17-98) and the Central Acidic Activation Domain ((CAAD) aa107-144) with almost identical activation potential. Between these two activation domains, Gcn4p contains a so-called PEST region named according to characteristic amino acids (Rechsteiner and Rogers, 1996). This region consists of the amino acids 99-106 and is amongst others responsible for the instability of Gcn4p in sated cells (Kornitzer et al., 1994). The regulation of Gcn4p protein stability is explained in more detail in section 3.4.

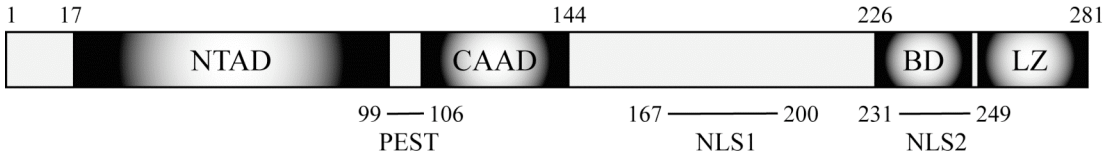

Figure 4. Functional dissection of *S. cerevisiae* Gcn4p. The transcription factor consists of 281 amino acids and is divided into a N-Terminal Activation Domain ((NTAD) aa17-98), a Central Acidic Activation Domain ((CAAD) aa107-144), a DNA Binding Domain ((BD) aa226-249) and a Leucine Zipper ((LZ) aa249-281). Between the two activation domains lies a PEST region (aa99-106), which causes the instability of the transcription factor. The Nuclear Localization Sequences NLS1 (aa167-200) and NLS2 (aa231-249) are required for nuclear import of Gcn4p.

For the transcriptional activation function of Gcn4p this protein has to be transported into the nucleus. Gcn4p nuclear import is mediated by two different Nuclear Localization Sequences (NLS) (Pries et al., 2002). NLS1 consists of the amino acids 167-200 and NLS2 is located in the DNA binding domain and consists of the amino acids 231-249 (Figure 4).

3.3 Gcn4p nuclear import

3.3.1 General protein transport in and out of the nucleus

In eukaryotic organisms the nucleus is enclosed by an endoplasmatic reticulum associated nuclear membrane resulting in a sub-cellular compartmentalization between the nucleus and the cytoplasm. For this reason, the spatial separation of transcriptional and translational processes leads to a bidirectional intracellular transport of macromolecules. Whereas nuclear proteins have to be transported into the nucleus after they have been synthesized in the cytoplasm, t- and mRNAs are exported out of the nucleus into the cytoplasm (Görlich and Mattaj, 1996). The places of exchange are the nuclear pores, which are present as elongated structures in the nuclear envelope (Kaffman and O'Shea, 1999). Dependent on their size proteins can enter the nucleus by passive diffusion or in an active manner. Smaller molecules are competent for passive diffusion, in contrast to proteins with a molecular weight higher than 40 kDa, which need to be transported in a regulated way mediated by specific transport proteins termed karyopherins. In *S. cerevisiae* 14 different karyopherins are known, of which Srp1p is the only importin α, in contrast to at least five different α-karyopherins in higher mammals (Hubner et al., 1997). There are at least thirteen different importin β homologs in yeast. So far four of these have been characterized as export and nine as import receptors respectively (Kaffman and O'Shea, 1999).

Classical signal-mediated nuclear import is initiated by the formation of a cytosolic α/β-importin heterodimer of Srp1p/Kap95p, which specifically recognizes canonical NLSs in a cargo protein. This is followed by translocation into the nucleus through the nuclear pore complex (NPC). The cargo protein is released in the nucleus and the importins must then be transported out of the nucleus for the next round of translocation (Görlich and Mattaj, 1996). In contrast, each of the Kap-β family members binds directly without importin α to a cognate signal in a transport substrate, followed by docking of the whole complex to a subset of nucleoporins (Nups) (Aitchison et al., 1996; Senger et al., 1998).

The described signal-mediated transport requires NLS motifs as well as GTP hydrolysis by RAN and soluble factors (Görlich and Mattaj, 1996; Moore and Blobel, 1993). Three classes of conventional NLSs are known, of which two are highly basic in nature. The first resembles the monopartite NLS of the SV40 large tumor antigen including five basic amino acids within a seven amino acid segment (Kalderon et al., 1984). The bipartite motif in nucleoplasmin is characterized by two clusters of basic amino acids separated by a spacer of 10-12 amino acids (Robbins et al., 1991). The third type of NLS resembles those of the proto-oncogene c-myc

where a proline and an aspartatic acid span the central basic cluster (Makkerh et al., 1996). These three classes of NLSs have been shown to be recognized by the α/β-importin heterodimer in different species (Briggs et al., 1998; Hubner et al., 1997; Smith et al., 1997). Besides the α/β-heterodimer, different importin β isoforms are also able to carry out signal-mediated transport (Palmeri and Malim, 1999; Truant and Cullen, 1999). Furthermore, an overlapping substrate specificity of the different caps results in a non-essential phenotype of several karyopherins, although these necessary for the transport of substrates that are essential for viability (Rout et al., 1997; Schlenstedt et al., 1997). As well as NLS sequences, nuclear export sequences (NES) are known as trafficking signals, which are less conserved and often leucine rich (Kaffman and O'Shea, 1999).

3.3.2 Nuclear localization of Gcn4p

Different biosynthetic pathways are regulated at the level of nuclear import and export mechanisms in yeast. As a central transcription factor of phosphate metabolism Pho4p is nuclear localized under low phosphate conditions. In contrast, a sufficient supply of phosphate results in phosphorylated Pho4p and the export of this protein to the cytoplasm, indicating that Pho4p harbors an NLS and NES motif (Kaffman and O'Shea, 1999). As a key transcriptional regulator of the general amino acid control Gcn4p must be transported into the nucleus to fulfill its function. This nuclear import is independent of the availability of amino acids and therefore a constitutive process (Pries et al., 2002). To ensure nuclear localization Gcn4p harbors two signal sequences recognized by the transport apparatus. NLS1 consists of the Gcn4p amino acid residues 167-200 and does not correspond to a canonical sequence. The second, termed NLS2, is represented by the DNA binding domain amino acids 231-249 and resembles a classical bipartite NLS motif consisting of two basic clusters separated by a seven amino acid spacer region (Pries et al., 2002). Both motifs must be deleted for Gcn4p cytoplasmic localization. An amino acid alignment between the NLS motifs of Gcn4p and the deduced sequences of the corresponding proteins of other filamentous fungi showed that only NLS2 is conserved among fungi. Indeed, NLS1 is able to target a cytoplasmic yeast protein into the nucleus but seems not to be present in filamentous fungi. Furthermore, NLS2-dependent nuclear import specifically requires the importin α Srp1p and the importin β Kap95p, whereas nuclear uptake mediated by Gcn4p NLS1 is impaired by defects in genes for several different karyopherins (Pries et al., 2004). In summary, NLS1 may act only as an unspecific or ancillary motif and NLS2 is the specific and essential nuclear transport signal of yeast Gcn4p.

3.4 Regulation of *GCN4* expression

Multiple mechanisms in the cell control the amount of the transcription factor Gcn4p. In response to amino acid starvation, the translation of *GCN4* is increased within about 20 minutes, whereas a two-fold transcriptional induction of *GCN4* mRNA level can be observed after 3-4 hours of starvation indicating that Gcn4p activation is a biphasic process (Albrecht et al., 1998). However, the main control mechanism of *GCN4* expression operates at the level of *GCN4* mRNA translation.

In the yeast *S. cerevisiae* protein biosynthesis is inhibited when cells are exposed to starving or stress conditions. In this way nutrient resources are saved and the cell division process is reduced. This reduced protein synthesis is mediated by phosphorylation of the eukaryotic translational Initiation Factor 2 (eIF2). When cells are subjected to amino acid starvation conditions the C-terminal histidyl-tRNA synthetase (HisRS) related domain of the sensor kinase Gcn2p is activated by the accumulation of uncharged tRNAs in the cell (Lanker et al., 1992). Furthermore, the interaction of the N-terminus of Gcn2p and the Gcn1p-Gcn20p complex is required for full activation of Gcn2p (Sattlegger and Hinnebusch, 2005). The activated kinase domain of Gcn2p then phosphorylates the α-subunit of eIF2-GDP on Ser51 (Wek et al., 1995; Zhu et al., 1996). This leads to the inhibition of the guanine nucleotide exchange factor eIF2B and therefore to a reduced concentration of eIF2 bound to GTP. Consequently, the amounts of ternary complexes consisting of the eIF2, GTP, and the initiation tRNA Met-$tRNA_i^{MET}$ are reduced. Therefore starvation for amino acids leads to a reduced protein synthesis in general but to an increased *GCN4* mRNA translation in *S. cerevisiae*. This contradictory response is caused by four small upstream Open Reading Frames (uORFs) in the 5' untranslated region of *GCN4* mRNA (Hinnebusch, 2005; Hinnebusch and Natarajan, 2002).

In sated cells high levels of eIF2-GTP are recycled by the guanine nucleotide exchange factor eIF-2B leading to a sufficient amount of ternary complexes (eIF2-GTP-$tRNA^{MET}$). The 40S ribosomal subunit forms a 43S preinitiation complex together with a ternary complex. Near the 5' end of the *GCN4* mRNA this preinitiation complex starts downstream migration and searches for the first start codon. At the AUG of uORF I a 80S ribosome is formed that initiates translation at this start codon. During this process the GTP bound to eIF2 is hydrolized resulting in inactive eIF2-GDP. The 80S ribosome dissociates from the mRNA at the uORF I stop codon and about half of the small subunits remain attatched to the mRNA. Before the 40S ribosomal subunit reaches the uORF IV, eIF2-GDP must be recycled to eIF2-GTP by the guanine nucleotide exchange factor eIF2B for further reinitiation of translation at

uORF IV. The translation of uORF IV is followed by a further dissociation of the 80S ribosome, preventing translation of the *GCN4* ORF (Figure 5).

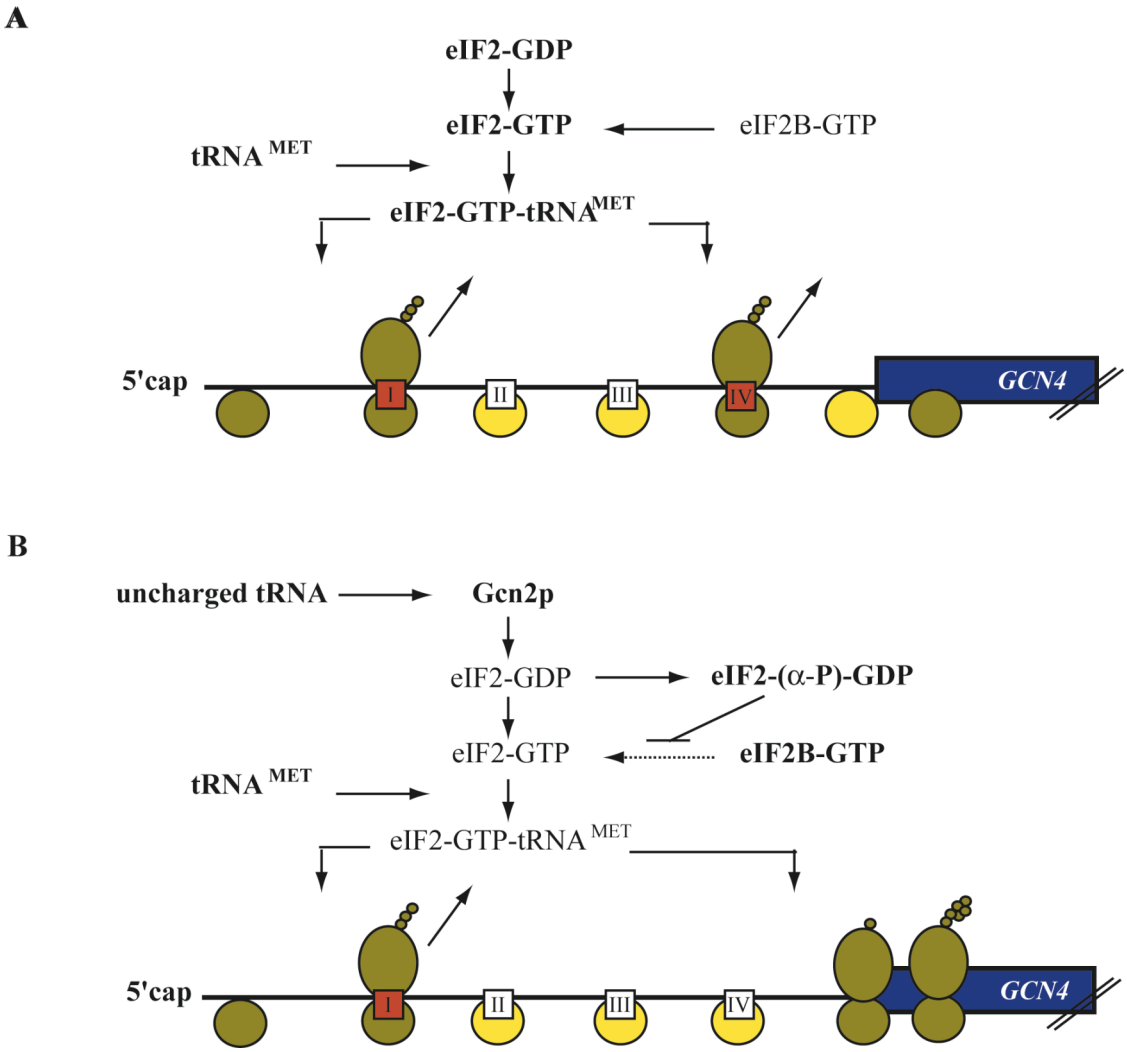

Figure 5: Translational control of *GCN4* mRNA in *S. cerevisiae*. **(A)** Under non-starvation conditions high levels of eIF2-GTP are recycled by the guanine nucleotide exchange factor eIF2B leading to a sufficient amount of ternary complexes (eIF2-GTP-tRNA^{MET}) and therefore competent 40S ribosomal subunits in the cell. A 80S ribosome is formed at uORF I that initiates translation at the start codon of this uORF and dissociates from the uORF I mRNA at its stop codon. High levels of ternary complexes allow reinitiation of translation at uORF IV followed by a further dissociation of the 80S ribosome after translation of this uORF. Because of the lack of competent 40S subunits the *GCN4* ORF is not translated. Therefore the four short open reading frames (uORFs) in the 5'untranslated region of *GCN4* mRNA prevent efficient *GCN4* translation in sated cells. **(B)** In response to amino acid starvation, uncharged tRNAs accumulate in the cell and are recognized by the sensor kinase Gcn2p. Stimulated Gcn2p thus phosphorylates the α-subunit of eIF2-GDP inhibiting eIF2B and therefore the formation of high amounts of ternary complexes in the cell. Consequently, reinitiation of translation fails at the start codon of uORF IV leading to the assembly of a 80S ribosome and therefore to initiation of translation at the *GCN4* start codon.

In contrast, under amino acid starvation the levels of eIF2-GTP and therefore of ternary complexes in the cell are very low. Thus, many 40S ribosomal subunits scan the distance between uORF I and uORF IV without rebinding a ternary complex, and reinitiation of translation takes place at the *GCN4* start codon (Figure 5).

3.5 Regulation of Gcn4p protein stability

Besides Gcn4p synthesis the cellular amount of Gcn4p is also controlled at the level of protein degradation (Kornitzer et al., 1994). In nutrient-sated yeast cells, Gcn4p is a short-lived protein with a half-life of only a few minutes, whereas under amino acid starvation degradation occurs more slowly leading to an increased Gcn4p half-life of about 20 minutes. For stabilization of Gcn4p a more severe amino acid limitation is required. This is achieved by culturing auxotrophic cells in medium lacking the required amino acid, which also results in arrested cell growth. In contrast, continued cell growth is achieved when prototrophic cells are treated with a conventional concentration of inhibitors such as 3AT or methyl sulfometuron that do not affect the stabilization of Gcn4p. Gcn4p contains a PEST region between its two activation domains, which, together with the phosphorylation and ubiquitination sites is responsible for the instability of this transcription factor (Kornitzer et al., 1994; Meimoun et al., 2000), which is finally degraded in the 26S proteasome. Therefore, a deletion of the PEST region results in stabilization of the protein (Kornitzer et al., 1994).

Rapid degradation of Gcn4p in sated cells is initiated by phosphorylation of specific residues by the cyclin-dependent protein kinases Pho85p and Srb10p, which act in an additive manner. Thus, even in non-starved cells stabilization of Gcn4p was observed in mutant strains impaired in *PHO85* or *SRB10* (Chi et al., 2001; Meimoun et al., 2000). Phosphorylation of Gcn4p marks the protein for poly-ubiquitination by the E2 ubiquitin-conjugating enzyme Cdc34 together with the E3 SCF^{CDC4} RING ubiquitin ligase (Kornitzer et al., 1994; Meimoun et al., 2000). Finally it becomes a target of the 26S proteasome where it is ultimately degraded (Figure 6) (Kornitzer et al., 1994). Accordingly, mutations in genes encoding the specific ubiquitin conjugation enzyme Cdc34p or the F-box protein Cdc4p result in stabilization of Gcn4p (Kornitzer et al., 1994). Furthermore, it has been reported recently, that mutations in the E3 SCF^{CDC4} RING ubiquitin ligase or inhibition of the proteasome resulted in a reduced transcription of Gcn4p targets suggesting that Gcn4p turnover might stimulate its gene expression activity (Lipford et al., 2005).

Pho85p/Pcl5p phosphorylation of Gcn4p is dependent on the availability of amino acids. Therefore, the stabilization of Gcn4p in response to amino acid deprivation results from

decreased Pho85p-dependent phosphorylation at Gcn4p residue Thr165 (Meimoun et al., 2000). Pcl5p is one of the ten cyclins that are able to activate the kinase Pho85p. It has been specifically identified as being required for Gcn4p degradation (Figure 6) (Shemer et al., 2002). The disappearance of Pcl5p in starved cells is suggested to be the reason for Gcn4p stabilization under these conditions (Shemer et al., 2002). Furthermore, *PCL5* is a target gene of Gcn4p and therefore the *PCL5* mRNA level is increased under amino acid limitation conditions (Jia et al., 2000), but the protein does not accumulate in the cell. The reasons for this are the reduced general protein biosynthesis under amino acid starvation and also the inherent instability of Pcl5p with a half-life of 2 minutes in both sated and starved cells. In response to amino acid replenishment conditions, high levels of *PCL5* mRNA lead to rapid production of Pcl5p and therefore to accelerated degradation of pre-existing Gcn4p (Shemer et al., 2002). Based on this model, Gcn4p stimulates its own rapid destruction in response to replenished amino acid levels.

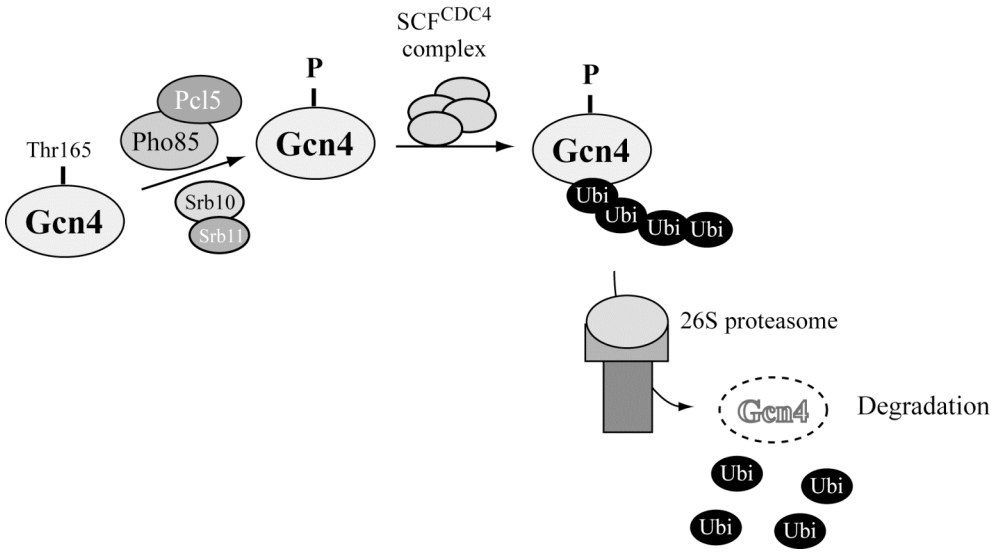

Figure 6: Proposed model of Gcn4p degradation (modified from Meimoun et al., 2000). In sated cells Gcn4p is phosphorylated by the kinase/cyclin complex Pho85p/Pcl5p at Thr165. Phosphorylated Gcn4p is ubiquitinated by the SCF^{CDC4} ubiquitin-ligase complex and finally degraded in the 26S proteasome. Constitutively phosphorylation of Gcn4p is mediated by the kinase/cyclin complex Srb10p/Srb11p.

The Srb10-dependent Gcn4p phosphorylation is a constitutive process and provides an additional putative way in which Gcn4p may stimulate its own degradation. Srb10p is a component of the RNA polymerase II mediator complex required for transcriptional activation of Gcn4p target genes and therefore recruited by Gcn4p to the promoters of these genes (Natarajan et al., 1999; Swanson et al., 2003; Zhang et al., 2004). Thus, the recruitment of Srb mediator by Gcn4p to promoter regions could animate phosphorylation and subsequent

degradation of Gcn4p and has been suggested to be part of the promoter clearing after one round of transcriptional activation (Chi et al., 2001).

According to this hypothesis, Gcn4p stability regulation occurs exclusively in the yeast nucleus (Pries et al., 2002). Furthermore, Pho85p is mainly nuclear localized and Gcn4p nuclear localization is independent of the availability of amino acids and the proteins Pho85p and Srb10p (Pries et al., 2002).

In summary, the principal mechanisms controlling Gcn4p function in yeast are the cytoplasmic translational control of *GCN4* mRNA and the proteosomal degradation of Gcn4p in the nucleus (Figure 6).

4 Regulation of adhesion in *S. cerevisiae*

The direct connection between metabolic and developmental processes is less pronounced in *S. cerevisiae*, but described for various multicellular organisms. In *C. albicans*, there is an intimately linkage between the response to the availability of amino acids and developmental processes such as morphogenesis and biofilm formation (Brega et al., 2004; Brown et al., 2001; Tripathi et al., 2002). Furthermore, amino acid biosynthetic genes were induced in *C. albicans* in response to phagocytosis by human neutrophils (Rubin-Bejerano et al., 2003). The *GCN4* homolog *cpcA* encodes the central transcription factor of the cross-pathway control in *A. nidulans*, which mediates an arrest in cleistothecia formation in amino acid starved cells. Thus, a linkage between metabolism and sexual development is shown in filamentous fungi (Hoffmann et al., 2000). Furthermore, in mammalian cells the Gcn4p like ATF4 mediates the appropriate response to amino acid availability and is also important for an intact long-term memory and food selection (Costa-Mattioli et al., 2005; Hao et al., 2005)

4.1 Medical relevance of adhesion

Adhesion plays a major role in the medical field. It is the initial step of cell-cell and cell-surface interactions and prevents cells from being removed from a nourishing environment by physical forces such as wind or water. Furthermore, adhesion allows cells to form biofilms as a protection against hazardous conditions. The dimorphic yeast *C. albicans* is able to cause disseminated systemic infection in immuno-compromised individuals. The virulence of this organism is increased because of the switch from the unicellular yeast form to a hyphal form (Lo et al., 1997). In addition, prostheses and catheters can serve as carriers for *C. albicans* biofilms leading to extremely drug-resistant infective cells (Kojic and Darouiche, 2004). Besides pathogenic fungi, bacteria such as *Pseudomonas aeruginosa* or *Salmonella*

typhimurium also achieve pathogenicity by adherence resulting in an effected gastrointestinal tract.

The budding yeast *S. cerevisiae* is a non-pathogenic fungus that is also able to switch between a unicellular and a multicellular growth type in response to different environmental stimuli in an adhesion-dependent manner. Therefore, *S. cerevisiae* is a good model organism for adherence-dependent fungal infections.

4.2 Cell surface proteins in fungi

Glycosyl-phosphatidylinositol (GPI)-linked cell surface glycoproteins are a prerequisite for fungal pathogenesis and belong to the family of fungal 'adhesins' or 'flocculins'. They have a common structure with a C-terminal GPI-anchor for adhesion to the cell wall and N-terminal signal peptides sticking out from the cell surface (Bony et al., 1997; Kapteyn et al., 1999; Rigden et al., 2004). The middle part is characterized by multiple serine- and threonine-rich repeats required for generation of new adhesin alleles (Stratford, 1992). In contrast to the common domain structure, different families of adhesions are expressed in different yeast species. Furthermore, to enable the cells to express only the appropriate adhesin in different situations, each cell contains different specialized adhesins (Guo et al., 2000; Sheppard et al., 2004).

The pathogen *C. albicans* expresses genes of the ALS adhesin family permitting this organism to adhere to mammalian host tissues (Sundstrom, 2002). In contrast, the baker's yeast *S. cerevisiae* contains a family of cell wall proteins encoded by the genes *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*, of which only the protein Flo11p is required for adhesion to substrates. This is termed haploid invasive growth and diploid pseudohyphal development (Lambrechts et al., 1996; Lo and Dranginis, 1998; Verstrepen et al., 2004). Flo1p, Flo5p, Flo9p and Flo10p are called flocculins because of their ability to promote cell-cell adhesion (Caro et al., 1997; Teunissen and Steensma, 1995). In the Σ1278b genetic background only *FLO11* is expressed, in contrast to the other *FLO* genes which are transcriptionally silenced (Guo et al., 2000).

4.3 Induction of adhesion by different environmental stimuli

In the yeast *S. cerevisiae*, two different mating types exist called *MATa* and *MATα*. Conjugation of two haploid cells with different mating types results in a genetic switch from haploid to diploid a/α cells. Furthermore, diploid yeast cells can sporulate resulting in haploid

cells. Both haploid and diploid cells can grow vegetatively in the yeast form, switch to an invasive and adhesive growth modus or arrest growth in the stationary phase.

The nutrient availability is a large control system for developmental possibilities in the life cycle of *S. cerevisiae*. On media containing a fermentable carbon source such as glucose and sufficient nitrogen, haploid and diploid cells favor growth in the yeast form characterized by a unicellular ellipsoid morphology and an axial (haploid) or bipolar (diploid) budding pattern.

In haploid cells, starvation for glucose induces the change from the budding modus to a unipolar distal pattern and cells became adhesive and form multicellular complexes. In contrast, nitrogen limitation does not result in haploid invasive growth (Cullen and Sprague, 2000). Biofilm formation in yeast is also caused by starvation for glucose (Reynolds and Fink, 2001).

When diploid cells are starved for nitrogen - even in the presence of a fermentable carbon source - they are induced to develop pseudohyphae. Pseudohyphae cells exhibit a long and thin morphology with a larger surface area for a more efficient absorption of nutrients. Furthermore, the unipolar distal budding pattern of pseudohyphal cells result in multicellular filaments allowing the cells to leave the colony. Standard amounts of ammonium, arginine, glutamine, glutamate, or a mixture of proline and histidine in the media represses the dimorphic switch from yeast to pseudohyphae, whereas low ammonium levels are tolerant for pseudohyphal development. The sensor system for differentiation of the different nitrogen components is not yet understood in complete detail (Figure 7).

Besides glucose or nitrogen starvation, amino acid limitation is also a nutritional signal that activates adhesive growth. Haploid and diploid cells became adhesive in response to amino acid starvation even in the presence of the known adhesion repressors glucose and ammonium (Braus et al., 2003). Furthermore, two elements of the general amino acid control system (Hinnebusch and Natarajan, 2002) are required for amino acid starvation-induced adhesive growth. The key regulator of this genetic network is Gcn4p, which is also required for pseudohyphal development induced by nitrogen starvation. The second player is represented by the sensor kinase Gcn2p that regulates the synthesis of Gcn4p by sensing uncharged tRNA molecules leading to an increased translation of *GCN4* mRNA in response to amino acid limitation (Dever et al., 1992). Cells impaired in *GCN2* expression are no more able to grow adhesively under amino acid limitation (Braus et al., 2003).

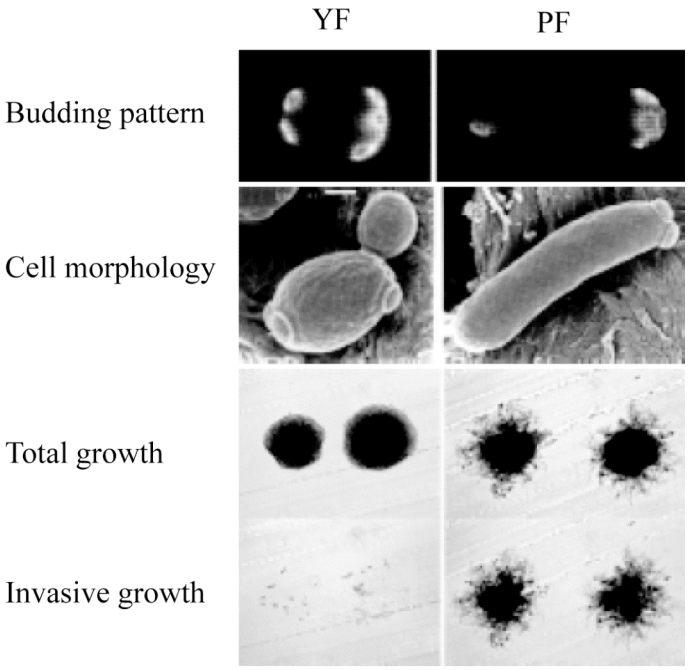

Figure 7. Comparison of budding pattern, cell morphology and growth behavior of cells growing as yeast form (YF) or as pseudohyphae form (PF) (modified from Mösch, 2002). In diploid cells, starvation for nitrogen induces the changing of the bipolar budding modus in the YF to a unipolar distal pattern of pseudohyphae cells. This was determined by calcofluor staining of the bud scars followed by visualization by fluorescence microscopy. Cellular morphology differences are demonstrated by electron microscopy of typical unicellular ellipsoid YF and elongated pseudohyphae cells. Yeast form cells grow on the agar plate surface whereas pseudohyphae cells invade in the agar, termed invasive growth. This is demonstrated by a wash test.

4.4 Controlling of adhesion by several signaling pathways

Adhesion in yeast is under the control of different signaling pathways. The pseudohyphal mitogen-activated protein kinase (MAPK) cascade and the cAMP-dependent protein kinase A (PKA) pathway are the main signal transduction pathways required for regulation (Gancedo, 2001; Gustin et al., 1998; Kronstad et al., 1998; Mösch, 2000; Palecek et al., 2002).

The small GTP-binding protein Ras2p represents the central switch of filamentation in both pathways. In general, MAPK modules are characterized as highly conserved cascades consisting of protein kinases acting in sequence, which ultimately activate transcription factors. In yeast five different MAPK pathways are known, which are involved in developmental processes such as mating or filamentous growth (Banuett, 1998; Gustin et al., 1998). In the case of the pseudohyphal MAPK cascade Ras2p affects filamentous growth via the small GTP-binding protein Cdc42 (Mösch et al., 1996). This cascade includes the protein kinases Ste20p (MAPKKKK), Ste11p (MAPKKK), Ste7p (MAPKK) and Kss1p (MAPK). The transcription factor Ste12p is activated by Kss1p-dependent phosphorylation in response to environmental stimuli (Liu et al., 1993; Madhani and Fink, 1997). Ste12p induces the

activation of its target genes together with the transcription factor Tec1p required for diploid pseudohyphal formation and haploid invasive growth (Burglin, 1991; Gavrias et al., 1996; Madhani and Fink, 1997; Mösch and Fink, 1997). They bind specifically as heterodimer to filamentous response elements (FREs) in the promoter regions of their target genes, e.g. *FLO11* (Lo and Dranginis, 1998). Furthermore, Tec1p can activate its target genes by binding to Tec1p binding sites (TCE elements) in their promoters when no Ste12p is available (Köhler et al., 2002). In the absence of signals permissive for filamentous growth, Kss1p binds as an unphosphorylated protein to the transcription factor Ste12p and therefore prevents Ste12p-dependent activation of *FLO11* expression or other target genes (Bardwell et al., 1998) (Figure 8).

In addition to the pseudohyphal MAPK pathway, stimuli-activated Ras2p transmits signals for filamentation by stimulating the adenylate cyclase Cyr1p leading to increased intracellular cAMP levels. High levels of cAMP activate protein kinase A (PKA), which is composed of one of three catalytic subunits Tpk1, Tpk2p, or Tpk3p and an inhibitory subunit termed Byc1p (Broach, 1991). Only Tpk2p is required for pseudohyphal development (Robertson and Fink, 1998), whereas cells impaired in *TPK1* or *TPK3* are hyper-filamentous, indicating that Tpk1p and Tpk3 are inhibitors of pseudohyphal growth (Nikawa et al., 1987; Pan and Heitman, 1999). The transcription factors Sfl1p and Flo8p are known targets of the PKA, both of which regulate the expression of the cell surface flocculin Flo11p. This protein is required for haploid invasive growth and diploid pseudohyphal development (Lambrechts et al., 1996; Lo and Dranginis, 1998). Sfl1p itself is repressed by the PKA and also negatively regulates *FLO11* transcription (Robertson and Fink, 1998). In contrast, Flo8p is also necessary for filamentous growth and therefore acts as a positive regulator of *FLO11* (Pan and Heitman, 1999). At this point it is important to mention that many laboratory strains, e.g. the S288c or the W303 strains, harbor a occurring *flo8* mutation and have therefore lost the ability to develop filaments (Liu et al., 1993). This differs from the genetic background of wild-type strains like Σ1278b that are appropriate for the study of adhesion, differentiation or morphogenesis processes in yeast.

The importance of the transcriptional activator Mss11p for *FLO11* expression has recently been shown (van Dyk et al., 2005). Overexpression studies of *TPK2*, *FLO8* or *TEC1* with respect to transcriptional activation of *FLO11* revealed that a functional *MSS11* gene is required. This demonstrates the central role of Mss11p in regulation of *FLO11* transcription. Additionally, the activity of Mss11p is independent of the main signal transduction cAMP or MAPK pathways. In *Saccharomyces diastaticus* it has been shown that the heterodimer of

Flo8p and Mss11p can activate expression of *STA1*, harboring the identical promoter as *S. cerevisiae FLO11* (Kim et al., 2004).

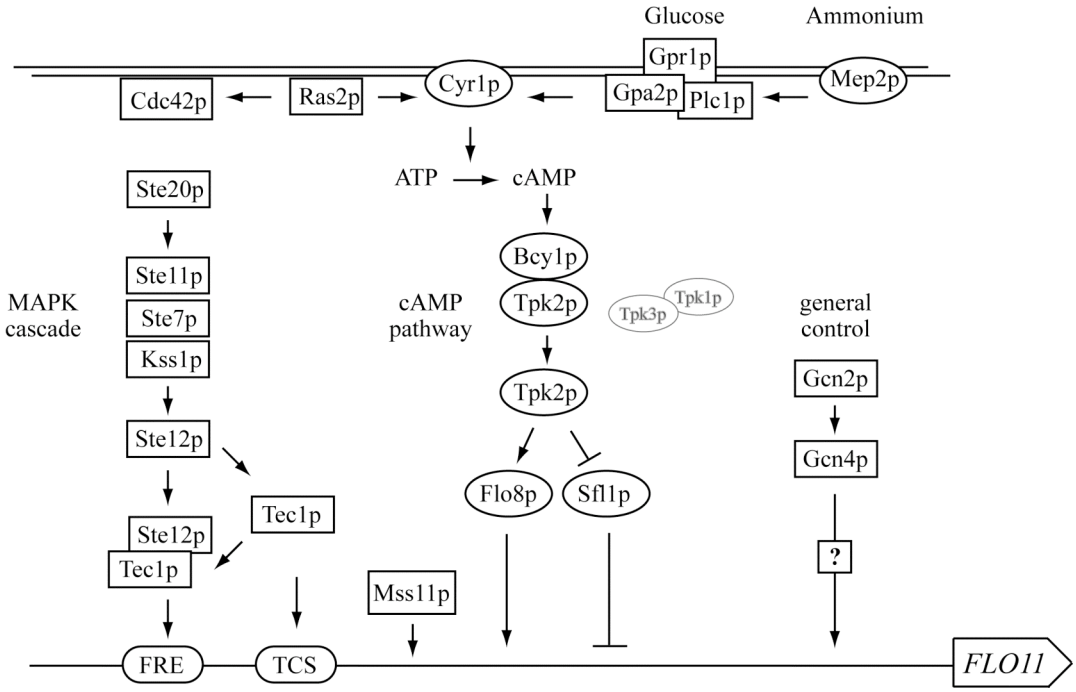

Figure 8. Model of signaling pathways regulating adherence and pseudohyphal growth in *S. cerevisiae* (see text for details).

The general amino acid control represents an additional signaling pathway for regulation of adhesion (Braus et al., 2003). This genetic network is activated in response to amino acid starvation and activates numerous genes encoding enzymes of various amino acid or purine biosynthetic pathways (Hinnebusch, 1992; Mösch et al., 1991). Furthermore, amino acid starvation is a nutritional signal activating haploid invasive growth and diploid pseudohyphal development in a Flo11p-dependent manner, even in the presence of the known suppressors glucose and ammonium. Amino acid starvation-induced adhesion is independent of the pseudohyphal MAPK module, but requires the proteins Tpk2p and Flo8p, the two central elements of the cAMP pathway. In addition, the sensor kinase Gcn2p and the key regulator Gcn4p of the general amino acid control system are necessary for adhesion and *FLO11* expression in response to amino acid limitation. This nutritional signal leads to an accumulation of uncharged tRNAs in the cell. Gcn2p detects these molecules and inactivates the eukaryotic translation initiation factor 2, eIF2, by phosphorylation, leading ultimately to derepression of *GCN4* translation (Hinnebusch, 1997) (described in more detail in chapter 3.4). As a global transcription factor Gcn4p regulates the expression of more than 500 target genes (Kleinschmidt et al., 2005; Natarajan et al., 2001). So far, Gcn4p has been suggested as

controlling *FLO11* expression in an indirect way or in concert with other transcription factors (Braus et al., 2003) (Figure 8).

5 Aim of this work

The protein stability of the JUN like yeast transcriptional factor Gcn4p is highly controlled in the nucleus by a complex network of various regulatory proteins. One aim of this work was to elucidate the molecular mechanisms of the amino acid-dependent Gcn4p stability regulation and to identify further proteins involved in this process. Therefore, the assembly and disassembly, the protein stability and the sub-cellular localization of novel identified proteins were investigated depending on the availability of amino acids. The unstable cyclin Pcl5p confers specificity on the nuclear cyclin-dependent kinase Pho85p for initiating the degradation of Gcn4p by phosphorylation. Deletion and transfer experiments of Pcl5p and Pcl5p/Pho80p chimera were carried out to analyze the control of the correct sub-cellular localization of the Pho85p cyclin Pcl5p as an important mechanism contributing to the functional specificity of the CDK-cyclin complex. Subsequently, the karyopherins required for the sub-cellular localization of Pcl5p should be identified using a set of *S. cerevisiae* mutant strains defective in the corresponding importins.

In addition, a limited supply of amino acids induces an adherence growth phenotype, which also depends on the expression of *GCN4* and the cell-surface flocculin encoding gene *FLO11*. A further aim of this work was to analyze the connection between the stability status of Gcn4p and its resulting activity. Therefore, a strong stabilized Gcn4p was generated by *PCL5* deletion or mutational analysis of *GCN4* and the resulting Gcn4p activity was measured on the basis of amino acid-dependent adhesion.

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CHAPTER II

Yeast Gcn4p stabilization is initiated by the dissociation of the nuclear Pho85p/Pcl5p complex

Abstract

Protein stability of the JUN like yeast bZIP transcriptional activator Gcn4p is exclusively controlled in the yeast nucleus. Phosphorylation by the nuclear Pho85p cyclin-dependent protein kinase, a functional homolog of mammalian Cdk5, initiates the Gcn4p degradation pathway in complex with the cyclin Pcl5p. We show that the initial step in Gcn4p stabilization is the dissociation of the Pho85p/Pcl5p complex. Pcl7p, another nuclear and constantly present cyclin, is required for Gcn4p stabilization and is able to associate to Pho85p independently of the activity of the Gcn4p degradation pathway. In addition, the nuclear cyclin-dependent Pho85p kinase inhibitor Pho81p is required for Gcn4p stabilization. Pho81p only interacts with Pcl5p when Gcn4p is rapidly degraded but constitutively interacts with Pcl7p. Our data suggest that Pcl7p and Pho81p are antagonists of the Pho85p/Pcl5p complex formation in a yet unknown way, which are specifically required for Gcn4p stabilization. We suggest that dissociation of the Pho85p/Pcl5p complex as initial step in Gcn4p stabilization is a prerequisite for a shift of equilibrium to an increased amount of the Pho85p/Pcl7p complexes and subsequently results in decreased Gcn4p phosphorylation and therefore increased stability of the transcription factor.

Introduction

Cyclin-dependent kinases (CDKs) play a crucial role in the regulation of eukaryotic cell cycle progression (Morgan, 1997), gene transcription and various cellular processes including sub-cellular localization and trafficking or interaction with other proteins, respectively. Activation of the kinases requires specific cyclin subunits, which mediate the specificity for targeting the kinase to the respective substrates (Huang et al., 1998; Jeffrey et al., 1995; Wilson et al., 1999). Deregulation of cyclin-dependent kinases as Cdk5 in humans are assumed to promote neurodegenerative processes e.g. in Alzheimer's disease (Patrick et al., 1999). The activities of numerous kinases are modulated by various CDK inhibitors (CKIs), which are able to interact with the kinase-cyclin complexes (Mendenhall, 1998).

In the eukaryotic model *Saccharomyces cerevisiae* there are six different cyclin-dependent kinases of which Pho85p is the functional homolog of the mammalian Cdk5 cyclin-dependent protein kinase (Huang et al., 1999) and capable to interact with ten different cyclin partners (Measday et al., 1997). Therefore, a deletion of *PHO85* results in a pleiotropic phenotype (Lenburg and O'Shea, 1996; Tennyson et al., 1998).

The more complex filamentous fungus *Aspergillus nidulans* carries the three CDKs $NIMX^{cdc2}$, PHOA and PHOB, and among them PHOA and PHOB are highly related to the *S. cerevisiae* Pho85p (Bussink and Osmani, 1998; Dou et al., 2003). Manual annotation and genome analysis of different *Aspergilli* revealed the presence of homologs of ten different yeast Pho85p cyclins exhibiting relatively low similarities (Galagan et al., 2005).

The cyclins that bind and activate the *S. cerevisiae* kinase Pho85p to perform different functions have been divided into two subfamilies according to their sequence homology and functional relationship (Measday et al., 1997). Members of the Pcl1,2 subfamily as Pcl1p, Pcl2p, Pcl5p, Pcl9p or Clg1p are involved in association with Pho85p in cell cycle control (Measday et al., 1997; Tennyson et al., 1998) as well as in the regulation of cell wall maintenance (Andrews and Measday, 1998).

The Pho80 subfamily consisting of Pho80p, Pcl6p, Pcl7p, Pcl8p and Pcl10p is functionally involved in distinct metabolic pathways (Andrews and Measday, 1998). The two cyclins Pcl6p and Pcl7p participate in carbon source utilization (Lee et al., 2000; Wang et al., 2001), whereas Pcl7p-dependent Pho85p activity is regulated by the cyclin-dependent kinase inhibitor (CKI) Pho81p (Lee et al., 2000; Measday et al., 1997). Besides Sic1p and Far1p, Pho81p is one of the three CKIs identified in *S. cerevisiae* (Mendenhall, 1998).

The Pho85p/Pho80p kinase phosphorylates the basic transcription factor Pho4p in phosphate rich environment resulting in its reduced activity (O'Neill et al., 1996). In response to phosphate starvation, phosphorylation of Pho4p is inhibited and Pho4p is able to increase the transcription of its target genes (Kaffman et al., 1998). The reduced kinase activity of Pho85p/Pho80p in low phosphate is mediated by the CKI Pho81p (Huang et al., 2001) that binds stably to Pho85p/Pho80p under both high and low phosphate conditions, yet it only inhibits when cells are starved for phosphate (Schneider et al., 1994).

The *S. cerevisiae* inhibitor domain of Pho81p (Huang et al., 2001) is conserved in the mammalian protein C42, a neuronal regulator protein that displays an inhibitory effect on Cdk5 kinase activity (Ching et al., 2002). An increased Cdk5 activity has been implicated in Alzheimer's and Parkinson's disease (Lau and Ahlijanian, 2003; Smith et al., 2003). In addition, the *Neurospora crassa* and *A. nidulans* CKIs Nuc-2 and AN4310 show high sequence homology to yeast Pho81p (Poleg et al., 1996; Galagan et al., 2005).

The *S. cerevisiae* Pho85p cyclin Pcl5p is specifically required for phosphorylation of the transcription factor Gcn4p in sated cells (Shemer et al., 2002). The JUN like Gcn4p is a global key regulator of the appropriate cellular response to starvation of amino acids, purines or various drugs as rapamycin (Hinnebusch and Fink, 1983). This genetic network is known as the 'general control' (GC) system of amino acid biosynthesis (Natarajan et al., 2001) and is conserved from yeast to man. In mammalian cells, the Gcn4p like ATF4 is the central activator of the GC, that functions also to guide food selection, learning and memory (Costa-Mattioli et al., 2005; Hao et al., 2005). Activity of the yeast *GCN4* gene product is regulated via control of protein synthesis in the cytoplasm and control of protein degradation in the nucleus. Starvation for amino acids results in an increased *GCN4* mRNA translation, mediated by phosphorylation of the general translation initiation factor eIF2α by the kinase Gcn2p (Dever et al., 1992; Hinnebusch, 1984). In addition, protein stability of the highly unstable Gcn4p increases in response to amino acid starvation (Kornitzer et al., 1994). Two CDKs are involved in Gcn4p degradation: Pho85p and Srb10p. Srb10p phosphorylation of Gcn4p occurs constitutively and independently of the availability of amino acids. As a component of the RNA polymerase II holoenzyme, Srb10p might be part of promoter clearing after activation of transcription and is also required for the activation function of Gcn4p (Chi et al., 2001).

The initial and committing step of the Gcn4p degradation pathway is its phosphorylation at the specific residue Thr165 by the kinase Pho85p/Pcl5p (Meimoun et al., 2000). This step is regulated and depends on the presence or absence of amino acids. Phosphorylated Gcn4p is

then poly-ubiquitinated by the E2 ubiquitin-conjugating enzyme Cdc34 together with the E3 SCF^{CDC4} RING ubiquitin ligase (Kornitzer et al., 1994; Meimoun et al., 2000). Gcn4p stability regulation depends on its phosphorylation and occurs exclusively in the yeast nucleus (Pries et al., 2002). Nuclear import of Gcn4p is triggered by the α-importin Srp1p and the β-importin Kap95p (Pries et al., 2004).

In this work, we elucidated the molecular mechanisms of the amino acid-dependent Gcn4p stability regulation. Our data present the first evidence of a novel regulatory pathway within the fine tuned network of Gcn4p stability regulation, namely the dissociation and disassembly of the Pho85p/Pcl5p complex in response to amino acid starvation. The CKI Pho81p and the cyclin Pcl7p are identified to be specifically required for Gcn4p stabilization. We propose a molecular mechanism for the stabilization of Gcn4p where the Pho81p or Pho85p association to Pcl5p is disrupted and replaced by Pho81p/Pcl7p and Pho85p/Pcl7p complexes.

Materials and Methods

S. cerevisiae strains and growth conditions

All yeast strains used in this study are listed in Table 1. They are either congenic to *S. cerevisiae* S288c (RH1168) or the W303 genetic background. Standard methods for genetic crosses and transformation were used as described (Ito et al., 1983). Yeast strains RH3237 and RH3238 were obtained by replacing the mutant *his3-11* allele of yeast strains KY346 and KY826 by a wild-type *HIS3* allele using *Bam*HI linearized plasmid B1683 (Table 2).

Yeast strain RH3306 was obtained by PCR-based C-terminal tagging of chromosomal *GCN4*-ORF (Janke et al., 2004). Primers were designed for amplification of the *9Myc-natNT2*-module from plasmid pYM21. Yeast strain RH2712 was transformed with the PCR product and transformants were selected on YEPD with 100 mg/l natNT2, nourseothricin (ClonNAT, Werner BioAgents, Jena-Cospeda, Germany). Transformants were replica-plated onto the same medium and the correct integration of the 9Myc-tag was confirmed by Western hybridization. Yeast strain RH3307 was created by PCR-based N-terminal promoter exchange of *GCN4* (Janke et al., 2004). The *kanMX4-GALL*-module was amplified from plasmid pYM-N27 using designed primers with homologous sequences to the *GCN4*-ORF. RH3306 was transformed with the PCR product and plated onto rich medium supplemented with 200 μg/ml G418 (Geneticin, Gibco). Transformants were replica-plated and the correct integration was confirmed by Western hybridization.

Yeast strain RH3255 (*pcl7Δ::kanMX4*) was constructed by PCR-mediated gene replacement (Longtine et al., 1998). Primers were designed specifically for amplification of *pcl7Δ::kanMX4* with chromosomal DNA of the Euroscarf strain EY1443 (*pcl7Δ::kanMX4)* (Brachmann et al., 1998). The PCR product was transformed into strain RH3237 and plated on rich medium supplemented with 200 μg/ml G418 (Geneticin, Gibco). Transformants were replica-plated and deletions were confirmed by Southern hybridization.

The yeast strain RH2977 was obtained by PCR-based C-terminal tagging of chromosomal *PCL7*-ORF (Knop et al., 1999). Primers were designed for PCR amplification of the *9Myc-k1TRP1*-module from plasmid pYM6. The PCR product was transformed into the yeast strain RH3237 to be introduced at the desired chromosomal location via homologous recombination. Tryptophan auxotrophic cells were plated on medium without tryptophan. Transformants were replica-plated onto the same medium and the correct integration of the 9Myc-tag was confirmed by Southern hybridization.

The strains were grown in standard yeast extract-peptone-dextrose (YPD: 1% yeast extract, 2% peptone, 2% dextrose) and minimal yeast nitrogen base media (YNB: 1,5 g/l yeast nitrogen base lacking amino acids and ammonium sulfate, 4,5 g/l ammonium sulfate, 2% dextrose or galactose and supplemented with the appropriate amino acids).

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
RH1168	*MATa, leu2-3, ura3-52, gal2*	our collection
RH2711	*MATa, ade2, trp1, leu2-3, his3, ura3-52*	(O'Neill et al., 1996)
RH2712	*MATa, ade2, trp1, leu2-3, his3, ura3-52, pho81Δ::TRP1*	(O'Neill et al., 1996)
RH3306	*MATa, ade2, trp1, leu2-3, his3, ura3-52, pho81Δ::TRP1, GCN4-9myc-natNT2*	this study
RH3307	*MATa, ade2, trp1, leu2-3, his3, ura3-52, pho81Δ::TRP1, kanMX4-GALLprom-GCN4-9Myc-natNT2*	this study
RH2977	*MATa, ura3-1, can1-100, leu2-3, trp1-1, PCL7-9Myc-k1TRP1*	this study
RH3237	*MATa, ura3-1, can1-100, leu2-3, trp1-1*	this study
RH3238	*MATa, ura3-1, can1-100, leu2-3, trp1-1, pcl5::hisG*	this study
RH3241	*MATa, ade2, trp1, leu2-3, his3, ura3-52, pho81Δ::HIS3*	(Ogawa et al., 1995)
RH3255	*MATa, ura3-1, can1-100, leu2-3, trp1-1, pcl7Δ::kanMX4*	this study
KY346	*MATa, ura3-1, can1-100, leu2-3, trp1-1,his3-11*	Kornitzer, pers. comm.
KY826	*MATa, ura3-1, can1-100, leu2-3, trp1-1,his3-11; pcl5::hisG*	Kornitzer, pers. comm.

Plasmid constructions

All plasmids used in this study are listed in Table 2. Construction of plasmid KB294 is described in Pries et al., 2002. Plasmid pME2228 expressing *GFP-PHO81* was obtained by amplifying the 750 bp *GFP*-ORF with *Pfu*-Polymerase from plasmid pBAD-GFP (Clontech, Heidelberg, Germany). The *GFP*-ORF was introduced as a *Bgl*II-fragment into *Bam*HI restricted p426MET25. The *PHO81*-ORF was amplified with *Pfu*-Polymerase and ligated as a *Cla*I-fragment behind the *GFP*-ORF. Plasmid pME2230 expressing *GFP-PCL7* was constructed by amplifying the *PCL7*-ORF followed by introduction via *Bam*HI/*Hin*dIII into p426MET25. In front of the *PCL7* coding region, *GFP* was introduced as a *Bgl*II-fragment into the *Bam*HI restricted plasmid. pME2564, pME2933 and pME2863 expressing *GFP*, *PCL7* and *PHO81* were constructed by amplifying the *GFP*-ORF, the *PCL7*-ORF and the *PHO81*-ORF with *Pfu*-Polymerase and introducing them as a *Sma*I/*Cla*I-fragment (*GFP*) into p426MET25 (pME2564), as *Bam*HI/blunt-*Sma*I (*PCL7*), or *Cla*I-fragments (*PHO81*) into

p424MET25 (pME2933, pME2863). Plasmid pME2865 expressing a nine-fold epitope tagged version of *PCL5* was obtained by amplifying *PCL5* with *Pfu*-Polymerase and inserting it into p425GAL1 as *Sma*I/*Hin*dIII-fragment. A 360 bp *Bgl*II-fragment carrying myc^{9} was introduced into a *Bgl*II restriction site in front of the third amino acid of Pcl5p. Plasmids pME2866 and pME2867 expressing *GST-PHO85* or *GST-PHO81* were constructed by amplifying the *PHO85*-ORF or *PHO81*-ORF with *Pfu*-Polymerase. The ORFs were inserted via *Spe*I/*Sma*I (pME2866) or *Spe*I/*Sal*I (pME2867) into pYGEX-2T.

Protein analysis

Shut-off-Western procedure. Yeast cells were pre-grown in selective minimal medium with glucose as the carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express myc^{3}*-GCN4* from the *GAL1* promoter. After 3h, the cells were collected via centrifugation and half of these *leu2*-deficient cells were starved for leucine by shifting them to minimal medium lacking leucine. 2% glucose was added to shut off the promoter after half an hour of leucine starvation. In case of GFP-Pho81p or GFP-Pcl7p, leucine auxotrophic cells were collected after pre-growing in selective minimal medium and half of them were shifted to a medium lacking leucine to induce the ‚general control of amino acid biosynthesis'. After half an hour of starvation, 1 mM methionine was added to reduce the *MET25* promoter activity down to 10% of the induced level. Samples were analyzed at the indicated time points after promoter-shut-off (0-min time point).

Purification of GST-fusions. Yeast strains expressing *GST*, *GST-PHO85* or *GST-PHO81* together with a myc-tagged version of *PCL5* or *PCL7* were pre-grown in selective minimal medium containing raffinose as the carbon source. 2% galactose was added to induce the expression of the *GAL1*-driven fusions. After 3h of induction, a bigger part of the cells was collected by centrifugation and shifted to minimal medium lacking tryptophan for half an hour to stabilize Gcn4p. Protein extracts were prepared exactly as previously described (Roberts et al., 1997). Extracts were incubated with glutathione-agarose overnight at 4°C and the beads were repeatedly washed and collected to purify GST-fusions and any associated proteins. Samples were denatured by heating at 65°C for 15 min in SDS loading dye and equal amounts of each sample were analyzed by Western hybridization.

Table 2. Plasmids used in this study

Plasmid	Description	Source
pBKSII®	2.96 kb vector, *Amp*^{R} (*bla*), *lacZ*, *ori*	Stratagene (La Jolla, USA)
pRS424	*TRP*, *2µm*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pRS425	*LEU2, 2µm*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pRS426	*URA, 2µm*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pYM6	*9Myc-k1TRP1*-module	(Knop et al., 1999)
pYM21	*9Myc-natNT2*-module	(Janke et al., 2004)
pYM-N27	*kanMX4-GALL* promoter-module	(Janke et al., 2004)
p424MET25	pRS424 containing *MET25* promoter ,*CYC1* terminator	(Mumberg et al., 1994)
p425GAL1	pRS425 containing *GAL1* promoter, *CYC1* terminator	(Mumberg et al., 1994)
p426MET25	pRS426 containing *MET25* promoter, *CYC1* terminator	(Mumberg et al., 1994)
pYGEX-2T	*GAL1-10prom-GST-CYC1term, URA3, 2µm*	(Pries et al., 2002)
KB294	*GAL1-10prom-myc*^{3}*-GCN4*-fusion in *URA3*-marked *2µm* vector	(Pries et al., 2002)
pME2228	*MET25prom-GFP-PHO81*-fusion in p426MET25	this study
pME2230	*MET25prom-GFP-PCL7*-fusion in p426MET25	this study
pME2564	*MET25prom-GFP*-fusion in p426MET25	this study
pME2933	*MET25prom-PCL7*-fusion in p424MET25	this study
pME2863	*MET25prom-PHO81*-fusion in p424MET25	this study
pME2865	*GAL1-10prom-myc*^{9}*-PCL5* fusion in p425GAL1-10	this study
pME2866	*GAL1-10prom-GST-PHO85*-fusion in pYGEX-2T	this study
pME2867	*GAL1-10prom-GST-PHO81*-fusion in pYGEX-2T	this study
B1683	1720 bp *HIS3* gene in pBKSII®	Hill, pers. comm.

Whole-cell extracts of S. cerevisiae. Extracts were prepared from yeast cultures grown to exponential-phase. Cells were washed in ice-cold buffer B (50 mM Tris-HCl pH 7,5, 1 mM EDTA, 50 mM dithiothreitol), lysed with glass beads in 200 µl of buffer B + PIM (1 mM each phenylmethylsulfonyl fluoride, tosyl-L-lysine-chloromethylketone, tosyl-L-phenylalanine-chloromethylketone, *p*-aminobenzamidine–HCl and *o*-phenanthroline) + 3% Triton X-100 + 0,8% SDS at 4°C and spun at 3500 rpm for 15 min to remove glass beads and large cell debris. Extracts (10 µl) were removed to determine total protein concentration using a protein assay kit from Bio-Rad (München, Germany). Proteins were denatured in SDS loading dye by heating at 65°C for 15 min and were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes. GFP, GST and the myc-fusion proteins, Cdc28p, eIF2p and eIF2α-Pp were detected using ECL technology (Amersham, UK). For the first incubation, monoclonal mouse anti-GFP (Clontech, Heidelberg, Germany), polyclonal rabbit anti-GST

(Santa Cruz Biotechnologies, Santa Cruz, CA), monoclonal mouse anti-myc (9E10), polyclonal rabbit anti-Cdc28p, polyclonal rabbit anti-eIF2p or anti-eIF2α-Pp (Biosource, Nivelles, Belgium) antibodies were used. Peroxidase-coupled goat anti-rabbit or goat anti-mouse IgG were used as secondary antibodies (Dianova, Hamburg, Germany). Gcn4p protein bands were quantified using the KODAK 1D Image Analysis Software.

GFP fluorescence microscopy

Yeast strains harbouring plasmids encoding GFP-Pho81p or GFP-Pcl7p were grown to early log-phase and analyzed under sated and starved conditions. Leucine or tryptophan starvation was induced by transferring these *leu2*-deficient yeast cells from minimal medium containing leucine to minimal medium lacking leucine for 1 hour. 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) or in case of 4',6-diamidino-2-phenylindole (DAPI) staining, a standard DAPI filter set. DAPI staining was used for visualization of nuclei. Cells were photographed using a Hamamatsu-Orca-ER digital camera and the Improvision Openlab software (Improvision, Coventry, UK).

Growth tests

Yeast strains were pre-cultured to the same optical densities (OD_{600}= 1) and spotted onto selective YNB medium supplemented with or without 100 mM 3AT. Five-fold dilution experiments started with $3x10^4$ cells per 20 µl. After 3 days of growth the spotted strains were photographed under white light.

Results

The CKI Pho81p is involved in the control of yeast Gcn4p stabilization

In sated yeast cells, rapid Gcn4p decay is initiated by phosphorylation at residue Thr165 by the kinase/cyclin complex Pho85p/Pcl5p (Shemer et al., 2002). This marks the transcription factor for ubiquitination by the SCF^{CDC4} mediated pathway and guarantees subsequent Gcn4p degradation at the 26S proteasome. Amino acid-dependent Gcn4p degradation is restricted to the yeast nucleus (Pries et al., 2002). In response to amino acid starvation, Gcn4p is stabilized from a half-life of five minutes up to 20 minutes (Irniger and Braus, 2003). Our aim was to analyze the stability regulation of Gcn4p with respect to the molecular mechanisms and interactions of the involved proteins.

Stabilization of yeast Gcn4p requires the CKI Pho81p. Several inhibitors of cyclin-dependent kinases are described. In case of the kinase Pho85p, the cyclin-dependent kinase inhibitor Pho81p is known to inhibit the Pho85p activity in response to phosphate starvation when this kinase associates with another cyclin, Pho80p (Kaffman et al., 1998). We asked whether Pho81p fulfills a similar function for the Pho85p/Pcl5p activity in an amino acid limiting environment resulting in an altered Gcn4p stability.

Therefore, we analyzed the requirement of Pho81p for Gcn4p stabilization during amino acid starvation. Gcn4p stabilization was induced in a *PHO81* wild-type strain and compared to the corresponding *pho81* mutant strain. High copies of *myc^3-GCN4* driven from an inducible *GAL1* promoter were expressed and the amounts of myc^3-Gcn4p were analyzed after *GAL1* promoter-shut-off in sated and amino acid starved cells. Our data show that the *pho81* mutant strain is impaired in stabilizing Gcn4p in starved cells when compared to the wild-type *PHO81* strain suggesting that Pho81p is required for tuning down the protein degradation pathway (Figure 1A). These data were confirmed with endogenous *GCN4-myc^9* expressed from the less active *GALL* promoter. Before it was shown that expression of *GCN4-myc^9* from the authentic *GCN4* promoter in glucose containing medium is similar to the level of *GCN4-myc^9* driven from the *GALL* promoter in galactose containing medium (Figure 1B).

We next asked whether an overexpression of *PHO81* affects the stability of Gcn4p in sated and amino acid starved cells. Therefore, *GCN4* was expressed from the *GAL1* promoter together with *MET25* driven *PHO81* in leucine auxotrophic *pho81* cells. A promoter-shut-off experiment was performed as described above. Figure 1C shows that Gcn4p is rapidly degraded in sated cells resulting in a half-life of only a few minutes similar to *PHO81* wild-

type cells. Furthermore, the stabilization of Gcn4p under leucine starvation conditions indicates the functional complementation of the *pho81* mutation by Pho81p.

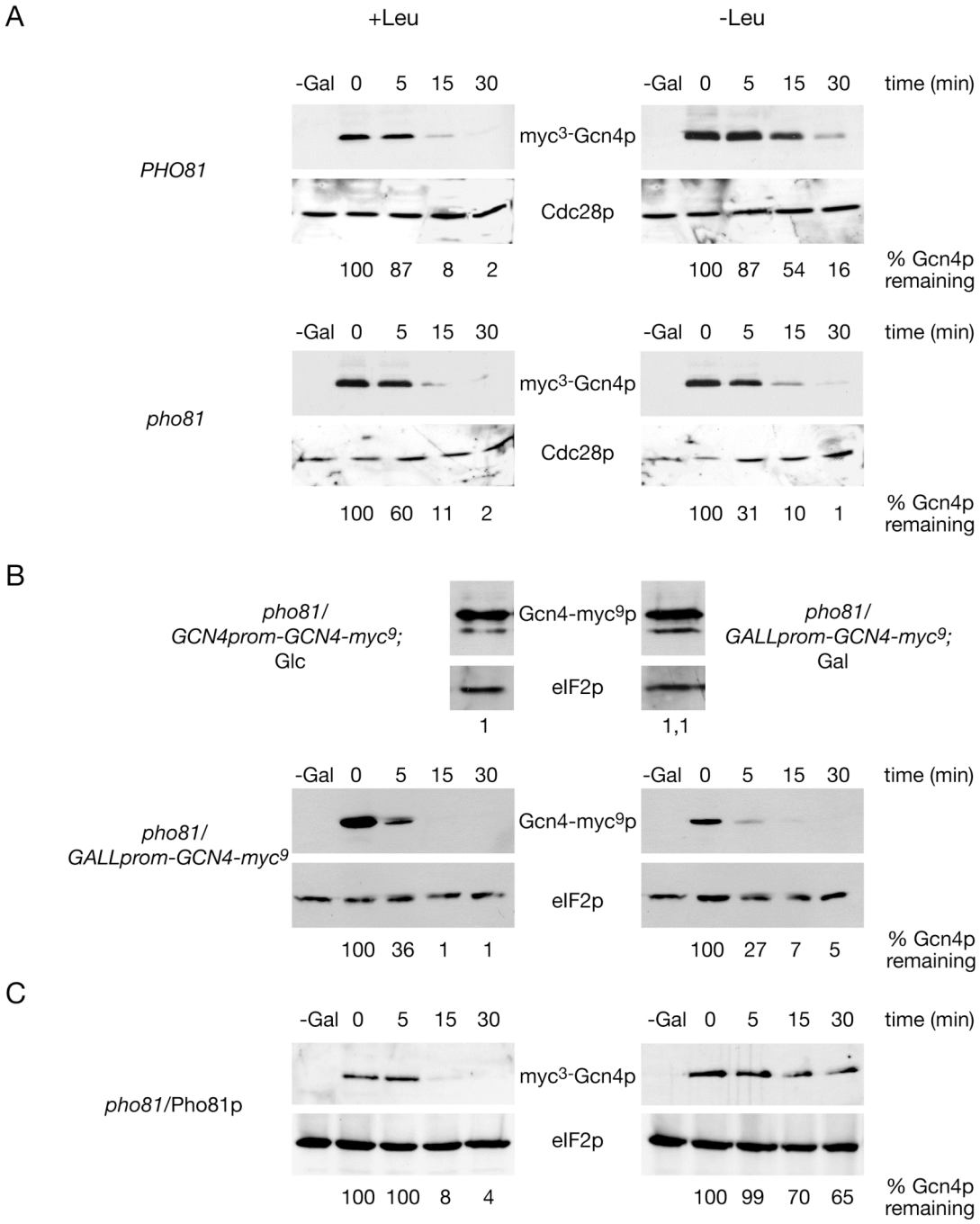

Figure 1. A *pho81* mutation leads to unstable Gcn4p in amino acid starved yeast cells. The isogenic *S. cerevisiae* strains RH2711 (*PHO81*) and RH2712 (*pho81*) were transformed to express the *GAL1*-driven *myc*^{3}*-GCN4* on plasmid KB294 (A). The *pho81* mutant strains RH3306 and RH3307 express endogenous *GCN4-myc*^{9} from the *GCN4* promoter under glucose conditions or from the less active *GALL* promoter in galactose medium (B). In addition, *GCN4* was expressed from the *GAL1* promoter (KB294) together with *MET25* driven *PHO81* (pME2863) in the *pho81* mutant strain RH3241 (*pho81*/Pho81p) (C). Protein levels of myc^3-Gcn4p, Gcn4-myc^9 and Cdc28p or eIF2p as loading control were determined in sated (+Leu) and amino acid starved (-Leu) cells after the *GAL1* promoter-shut-off. A two-fold protein amount was loaded for overexpressed *PHO81* to obtain similar amounts of Gcn4p at time point 0. Numbers given below indicate remaining Gcn4p-percentages when

compared to Cdc28p or eIF2p as internal standard quantified by image station of the gel shown.

In summary, our data underline a novel and specific role for the CKI Pho81p, which is required for the stabilization of the short-lived transcription factor Gcn4p. Therefore, this inhibitor might be able to modulate the Pho85p/Pcl5p activity dependent on the presence or absence of amino acids. We compared the sub-cellular localization, the stability and protein-protein interaction of Pho81p under conditions where Gcn4p is either unstable or stabilized to establish the mechanism by which amino acid availability regulates Gcn4p stability.

Pho81p is a nuclear protein in *S. cerevisiae* independent of the stability of Gcn4p. Pho81p is required for stabilization of Gcn4p. Since the regulation of Gcn4p degradation occurs exclusively in the yeast nucleus (Pries et al., 2002), we investigated the sub-cellular localization of Pho81p as a putative tool for Gcn4p stability regulation. Therefore, a *pho81* mutant strain was transformed to express chimeric *GFP-PHO81* from the efficient *MET25* promoter instead of the weak native *PHO81* promoter. Localization of GFP-Pho81p was monitored in living yeast cells under leucine starvation or non-starvation conditions by fluorescence microscopy. Figure 2A illustrates, that Pho81p is enriched in the nucleus under conditions when Gcn4p is unstable due to a sufficient supply of amino acids as well as under amino acid starvation conditions when Gcn4p is stabilized. DAPI staining confirms the nuclear enrichment of Pho81p.
In summary, we have shown that Pho81p is necessary for Gcn4p stabilization within the yeast nucleus and that Pho81p is a nuclear protein under both Gcn4p degrading and stabilizing conditions. Accordingly, Pho81p-dependent regulation of Gcn4p stabilization most likely also takes place in the yeast nucleus.

Pho81p is a stable protein in *S. cerevisiae* independent of the stability of Gcn4p. The requirement of Pho81p for the stabilization of Gcn4p leads to the question whether the stability of this inhibitor is affected by amino acid starvation, conditions where Pho85p/Pcl5p activity is reduced. We used the *MET25* promoter, which can be repressed by methionine to analyze the stability of Pho81p. *GFP-PHO81* was expressed in exponentially growing yeast cells and subsequently the *MET25* promoter was shut off by the addition of methionine resulting in a halted Pho81p expression. Since Gcn4p stabilization occurs within a time window of about 30 minutes (Kornitzer et al., 1994), samples were collected 20 and 40 min after the promoter-shut-off. Figure 2B demonstrates that no significant differences in the

amount of the chimeric GFP-Pho81 protein and therefore in the rate of Pho81p-degradation were observed under conditions when Gcn4p is rapidly degraded or stabilized. This indicates that amino acid starvation does not affect the stability of Pho81p.

Interaction of Pcl5p with Pho81p and Pho85p is disrupted when Gcn4p is stabilized

The Gcn4p degradation pathway is initiated by the kinase activity of Pho85p/Pcl5p. The activity of CDKs is predominantly regulated by the presence or absence of specific cyclin subunits mediating the specificity for targeting the kinase to the respective substrate (Huang et al., 1998; Jeffrey et al., 1995; Wilson et al., 1999). We wanted to know whether additional mechanisms are essential for the regulation of Gcn4p degradation and therefore analyzed the association of Pho81p with the Pho85p/Pcl5p complex under conditions when Gcn4p is either unstable or is stabilized.

Pho81p/Pcl5p complex dissociates under conditions when Gcn4p is stabilized. Pho81p forms a stable ternary complex with Pho85p/Pho80p independently of the kinase activity of Pho85p. This occurs by the recognition and binding of Pho81p to the Pho80p cyclin subunit (Schneider et al., 1994) which leads to decreased Pho85p activity in low phosphate. Since the inhibitor, Pho81p, is involved in Gcn4p stabilization, we asked whether Pho81p is able to interact with the Pho85p/Pcl5p complex by binding to the unstable cyclin Pcl5p.

Pho81p/Pcl5p interaction was investigated by an *in vivo* co-precipitation assay under conditions when cellular Gcn4p is unstable due to a sufficient supply of amino acids. Functional versions of *GST-PHO81* and *myc*^{9}*-PCL5* were expressed from the *GAL1* promoter. The protein-fusions were induced and purified with glutathione beads to isolate the GST-fusion and its associated proteins. Figure 3A shows that myc^{9}-Pcl5p co-purifies with GST-Pho81p under conditions when Gcn4p is rapidly degraded.

Next, we were interested whether there is any difference in the association of Pho81p with Pcl5p, when Gcn4p is stabilized. The half-life of Gcn4p is increased from 3-5 minutes up to 20 minutes when cells are starved for tryptophan (data not shown). Under conditions when Gcn4p is stabilized, both Pho81p and Pcl5p are clearly detectable in the protein extract, but myc^{9}-Pcl5p does not co-purify with Pho81p (Figure 3A).

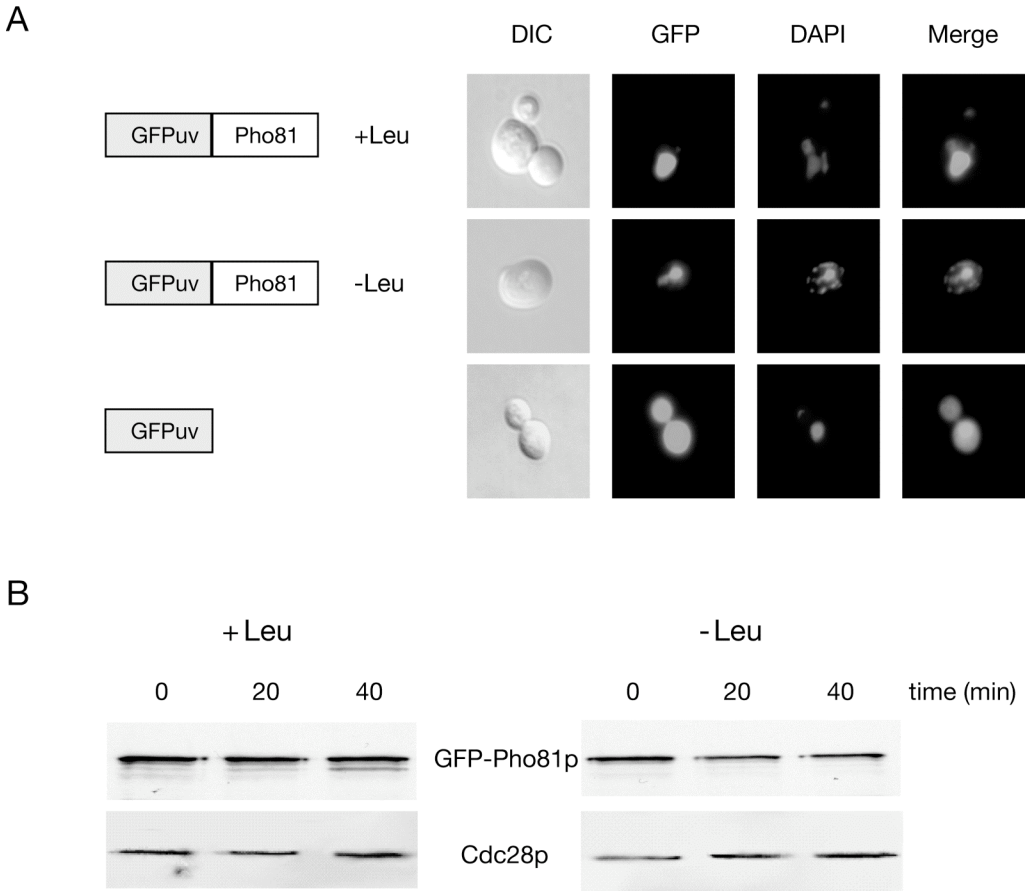

Figure 2. Nuclear localization and stability of Pho81p are unaffected by the availability of amino acids. **(A)** Nuclear import of Pho81p is independent of the presence or absence of amino acids. Yeast *pho81* mutant strain RH2712 was transformed to express *GFP-PHO81* driven from the *MET25* promoter in high amounts (pME2228). Cells were analyzed under sated (+Leu) and starved (-Leu) conditions. On the left, DIC microscopy is shown and on the right, GFP and DAPI signals are merged (Merge). **(B)** Amino acid starvation results in unchanged GFP-Pho81p protein stability. The *pho81*-deficient yeast strain RH2712 was transformed to express *GFP-PHO81* from the repressible *MET25* promoter on a *2µm* plasmid (pME2228). Protein levels of GFP-Pho81p and Cdc28p as loading controls were determined in sated (+Leu) and amino acid starved (-Leu) cells after the *MET25* promoter-shut-off.

Our data show that Pho81p interacts with the Pho85p/Pcl5p complex by binding to the cyclin under conditions when Gcn4p is rapidly degraded, whereas dissociation occurs in response to stabilization of Gcn4p in tryptophan starved cells.

Pho85p and Pcl5p physically interact and this interaction is impaired when Gcn4p is stabilized. An interaction between Pho85p and Pcl5p had only been shown genetically by the yeast two-hybrid system (Measday et al., 1997). Since we successfully monitored the *in vivo* interaction between Pho81p and the unstable cyclin Pcl5p, we focused on the *in vivo* physical interaction between kinase Pho85p and the cyclin Pcl5p. A fusion between glutathione S

transferase (GST) and *PHO85* was constructed (as described in Huang et al., 2001; Measday et al., 1997) and expressed from the *GAL1* promoter together with *myc*^{9}*-PCL5*. Fusion proteins were induced in sated cells under conditions when Gcn4p is unstable. Protein extracts with physiologically activated Pho85p/Pcl5p complexes were prepared. We analyzed the interaction of co-expressed Pho85p and Pcl5p and found that myc^{9}-Pcl5p co-purifies with GST-Pho85p under conditions when Gcn4p is rapidly degraded, i.e., in the presence of sufficient amino acids (Figure 3B).

Under amino acid starvation, i.e., conditions that stabilize Gcn4p, we found that the Pho81p/Pcl5p interaction is disrupted. Therefore, we wanted to know whether the kinase Pho85p and the cyclin Pcl5p are still interacting under these conditions. Gcn4p stabilization was induced by tryptophan starvation and both Pho85p and Pcl5p were expressed and detectable in protein extracts prepared under these conditions. However, myc^{9}-Pcl5p was not co-purified together with GST-Pho85p, when Gcn4p is stabilized and therefore this interaction was disrupted.

Stabilization of Gcn4p was also induced using the histidine analogue 3-amino-triazole to corroborate the Pho85p/Pcl5p-dissociation effect under conditions when Gcn4p degradation is decreased. The use of high concentrations of 3AT (100 mM) also resulted in the stabilization of Gcn4p (data not shown) and a decreased interaction between GST-Pho85p and myc^{9}-Pcl5p compared to sated conditions (data not shown).

These findings show that Pho85p interacts with Pcl5p when the Gcn4p degradation pathway is initiated. Under conditions when this transcription factor is required, the Pho85p/Pcl5p complex is disassembled and therefore allowing Gcn4p stabilization.

Pcl5p is not detectable in a *pho81* mutant under amino acid starvation conditions. We have shown that the protein-protein interaction of Pcl5p with Pho81p and Pho85p is disrupted under conditions when Gcn4p is stabilized**,** i.e., in response to amino acid starvation. Furthermore, Pho81p is required for this Gcn4p stabilization. Now we asked whether Pho81p is required for disruption of the Pho85p/Pcl5p complex under amino acid starvation conditions leading to a stabilized Gcn4p. To test this, expression of GST tagged *PHO85* and myc^{9} epitope tagged *PCL5* was induced in sated and amino acid starved *pho81* mutant cells. Induction of amino acid starvation, protein purification and detection were accomplished as described before. We found that myc^{9}-Pcl5p co-purifies with GST-Pho85p in sated cells when Gcn4p is instable. Under amino acid starvation conditions a reduced amount of the cyclin Pcl5p in a *pho81* mutant strain is detectable in the protein extract. Therefore, myc^{9}-Pcl5p did

not purify with GST-Pho85p under these conditions compared to sated *pho81* mutant cells (Figure 3C). These data suggest that although no interactions between the CKI Pho81p and Pcl5p were recognized under conditions when Gcn4p is stabilized this inhibitor is required to obtain a certain Pcl5p-level under these conditions.

Cyclin Pcl7p participates in Gcn4p stability regulation

Yeast Gcn4p stabilization requires the cyclin Pcl7p. The CDK Pho85p is known to bind at least ten different cyclins. One of these cyclins is Pcl7p, which is involved in the regulation of glycogen biosynthesis and catabolism. Additionally, it is known that besides Pho85p, the CKI Pho81p is also able to interact with the cyclin Pcl7p (Lee et al., 2000; Measday et al., 1997). Furthermore, depending on phosphate availability, Pho81p is suggested to regulate the activity of both Pho85p/Pho80p and Pho85p/Pcl7p complexes (Lee et al., 2000). We have shown here that Pho81p is required for Gcn4p stabilization under amino acid starvation conditions. The disassembly of the Pho81p/Pcl5p and Pho85p/Pcl5p complexes during Gcn4p stabilization prompted us to analyze whether other cyclins such as Pcl7p are also involved in the control of Gcn4p stability.

Therefore, we analyzed stability regulation of Gcn4p in a *pcl7* mutant strain and the corresponding wild-type strain expressing *myc*^{3}*-GCN4* driven from the inducible *GAL1* promoter. Protein levels of myc^3-Gcn4p were analyzed after *GAL1* promoter-shut-off in sated and amino acid starved cells. Western hybridization analysis revealed that a *pcl7* mutant strain is unable to stabilize Gcn4p in contrast to wild-type cells (Figure 4A).

Amino acid starvation induces within the cell the phosphorylation of the translation initiation factor eIF2α. The presence of eIf2α-P was verified as evidence that the general control signal transduction pathway controlling Gcn4p synthesis is not impaired. Therefore, these data strongly support for the first time that the cyclin Pcl7p is required for Gcn4p stabilization under amino acid starvation (Figure 4A).

The *PCL7* overexpression phenotype was determined by expressing *GCN4* from the *GAL1* promoter together with *MET25* driven *PCL7* in *pcl7* cells. Promoter-shut-off experiments were carried out indicating that Gcn4p is rapidly degraded in sated cells with a half-life of only a few minutes like in *PCL7* wild-type cells. A strong stabilization of Gcn4p under leucine starvation conditions indicates proper functioning of expressed Pcl7p protein (Figure 4B).

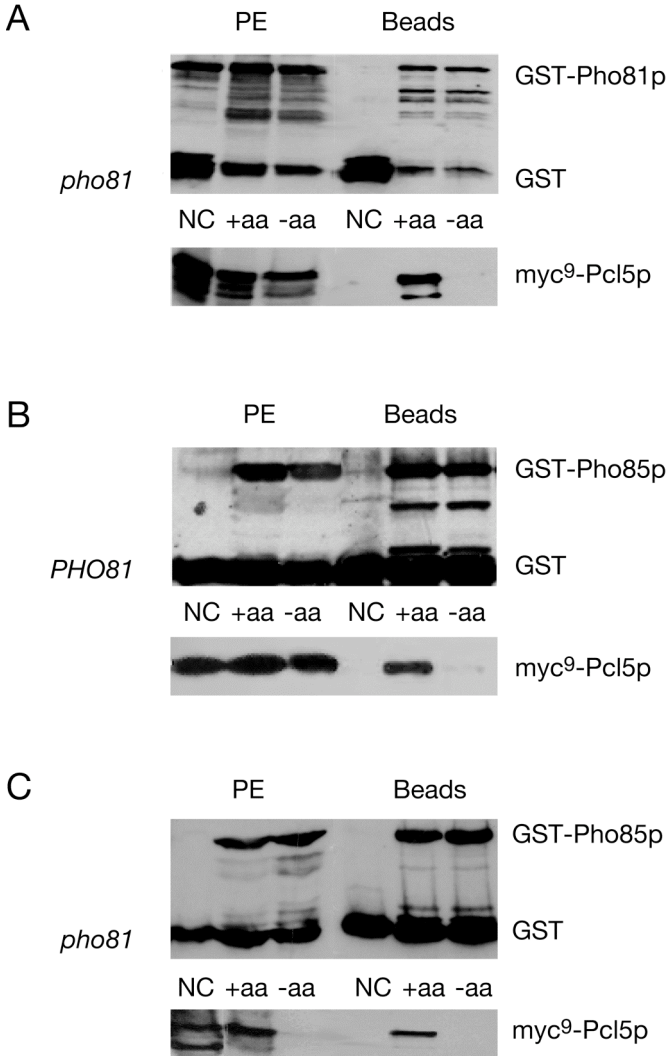

Figure 3. Protein-protein interaction of Pcl5p with Pho81p and Pho85p are disrupted in *S. cerevisiae* when Gcn4p is stabilized. The strain RH3241 (*pho81*) (A) was transformed to express either *myc*^{9}*-PCL5* (pME2865) with glutathione S transferase (*GST* on pYGEX-2T) as negative control (NC), or *myc*^{9}*-PCL5* (pME2865) together with *GST-PHO81* (pME2867). In addition, yeast strains RH3238 *(PHO81)* (B) and RH3241 (*pho81*) (C) were transformed to express either *myc*^{9}*-PCL5* (pME2865) with glutathione S transferase (*GST* on pYGEX-2T) as negative control, or *myc*^{9}*-PCL5* (pME2865) together with *GST-PHO85* (pME2866). Protein levels of the fusion proteins were determined in sated (+aa) and amino acid starved (-aa) cells. The left part represents the GST, GST-Pho81p, GST-Pho85p and myc^{9}-Pcl5p prior to GST-agarose incubation to ensure that the initial protein extracts (PE) contain similar amounts of the fusion proteins. On the right, the elutions of the glutathione beads are shown (Beads). The two-fold amount of protein extract and elution of the glutathione beads of amino acid starved cells were loaded in case of the myc-antibody reaction in the *pho81* mutant strain (Figure 3A and 3C) to obtain similar amounts of myc^{9}-Pcl5p in the initial protein extract (Figure 3A).

For further characterization studies, wild-type *(PCL7), pcl7* mutant cells and cells overexpressing *PCL7* were spotted in five-fold dilutions on minimal medium (YNB), YNB with 100 mM 3AT and YNB with 100 mM 3AT and histidine. Plates were incubated at 30°C for three days. As shown in Figure 4C, yeast cells overexpressing *PCL7* are unable to grow in

the presence of 100 mM 3AT, whereas growth can be restored by the addition of histidine. Growth of *pcl7* mutant is slightly reduced on media containing 100 mM 3AT. In the case of 10 mM 3AT all strains are able to grow like the wild-type control (data not shown). These data indicate that a *pcl7* deletion as well as an overexpression of *PCL7* leads to sensitivity against amino acid starvation further corroborating the importance of Pcl7p in the control of Gcn4p degradation.

Pcl7p is a predominantly nuclear protein. Stability regulation of yeast Gcn4p is restricted to the nucleus (Pries et al., 2002) and Pcl7p is involved in this process. Therefore, the localization of Pcl7p was monitored *in vivo* by expressing *GFP-PCL7* from the repressible *MET25* promoter. Localization of GFP-Pcl7p was examined by fluorescence microscopy in *pcl7* mutant cells grown in sated and amino acid starved cells. GFP-Pcl7p was localized mainly in the yeast nucleus under both conditions with an additional weak cytoplasmic localization (Figure 5A) indicating that the Gcn4p stability regulation is independent of the sub-cellular localization of this cyclin.

Pcl7p protein stability is not influenced by amino acid starvation in yeast cells. Since Gcn4p could hardly be stabilized in *pcl7* mutant cells under amino acid starvation conditions, we asked whether varying amounts of Pcl7p might be the cause for rapid Gcn4p turnover. Most cyclins are known to undergo rapid synthesis and turnover processes according to the cellular requirements. The cyclin Pcl5p is affected by the availability of amino acids on two levels. Although, *PCL5* is transcriptionally induced by Gcn4p in response to amino acid starvation (Jia et al., 2000), the levels of the constitutively unstable protein Pcl5p are decreased in amino acid starved cells (Shemer et al., 2002). To analyze whether Pcl7p is affected by the amino acid availability, the stability of Pcl7p was investigated.
Promoter-shut-off experiments were carried out to analyze the relative turnover rates of GFP-Pcl7p in sated and amino starved cells. A functional *GFP-PCL7*-fusion was expressed from the repressible *MET25* promoter in exponentially growing yeast cells. After *MET25* promoter-shut-off, analysis of GFP-Pcl7p showed similar protein levels of Pcl7p under both conditions and a highly stable protein independent of the availability of amino acids (Figure 5B). These data indicate a tentative difference between Pcl7p and Pcl5p, with Pcl7p being not regulated at its protein level. Therefore, the function of Pcl7p in the Gcn4p stability control seems to be independent on regulating the amount of Pcl7p within the cell.

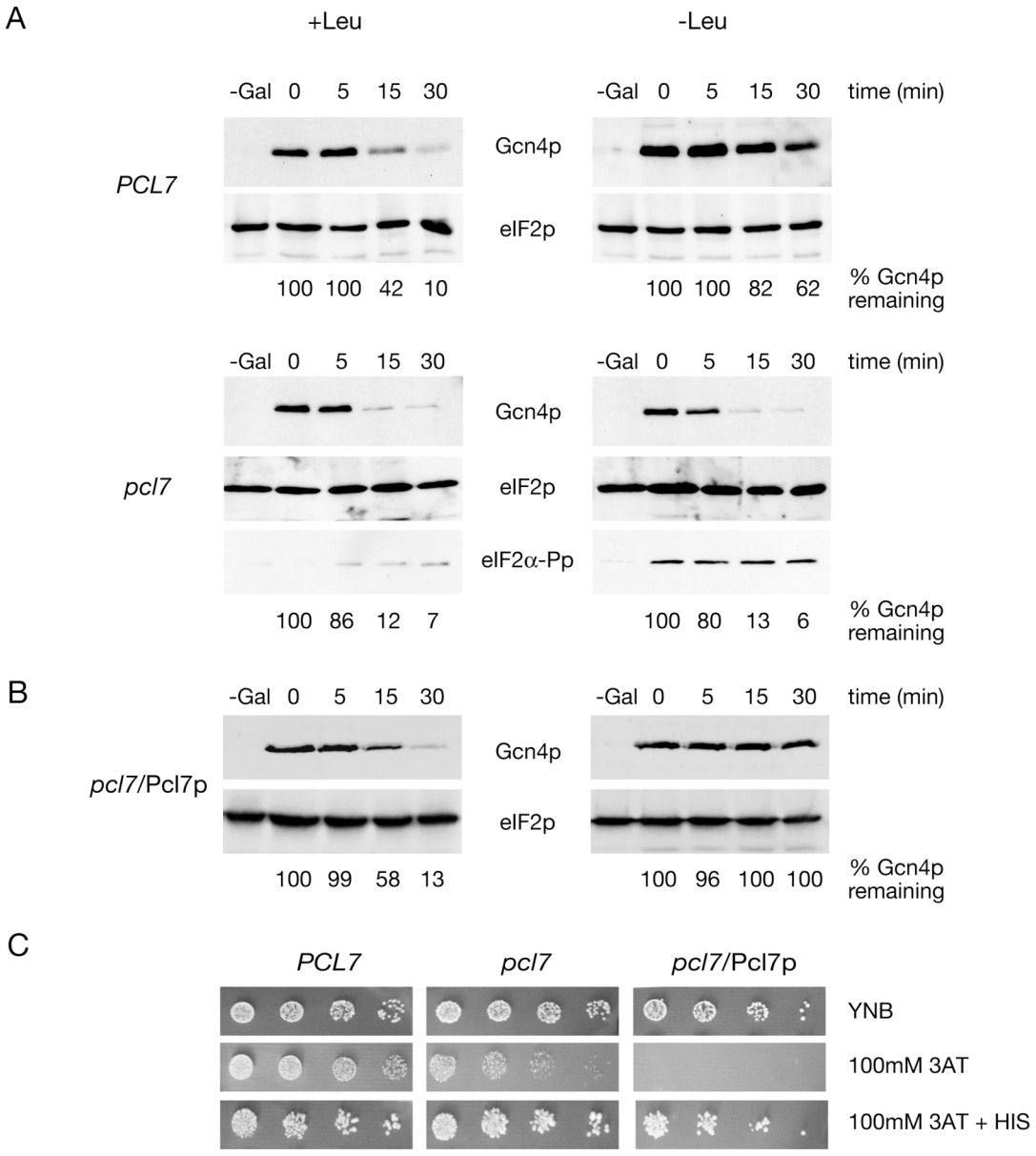

Figure 4. A *pcl7* mutation leads to unstable Gcn4p in amino acid starved yeast cells. The isogenic yeast strains RH3237 (*PCL7*) and RH3255 (*pcl7)* were transformed to express *myc*^{3}-*GCN4* from the *GAL1* promoter from the high-copy number plasmid KB294 (A). In addition *GCN4* was expressed from the *GAL1* promoter (KB294) together with *MET25* driven *PCL7* (pME2933) in the *pcl7* mutant strain RH3255 (*pcl7*/Pcl7p) (B). Protein levels of myc^3-Gcn4p and Cdc28p or eIF2p as loading control were determined in sated (+Leu) and amino acid starved (-Leu) cells after the *GAL1* promoter-shut-off. In addition, phosphorylated eIF2α-Pp confirms amino acid starvation. Numbers given below indicate remaining Gcn4p-percentages when compared to eIF2p as internal standard quantified by image station of the gel shown. **(C)** Overexpression of *PCL7* results in sensitivity towards amino acid starvation. Yeast strains RH3237 (*PCL7*), RH3255 (*pcl7)* and *pcl7* mutant cells expressing *PCL7* (pME2933) from the *MET25* promoter on *2μm* plasmids (*pcl7*/Pcl7p) were spotted in five-fold dilution on minimal medium (YNB), YNB with 100 mM 3AT and YNB with 100 mM 3AT and histidine. Plates were incubated at 30°C for three days.

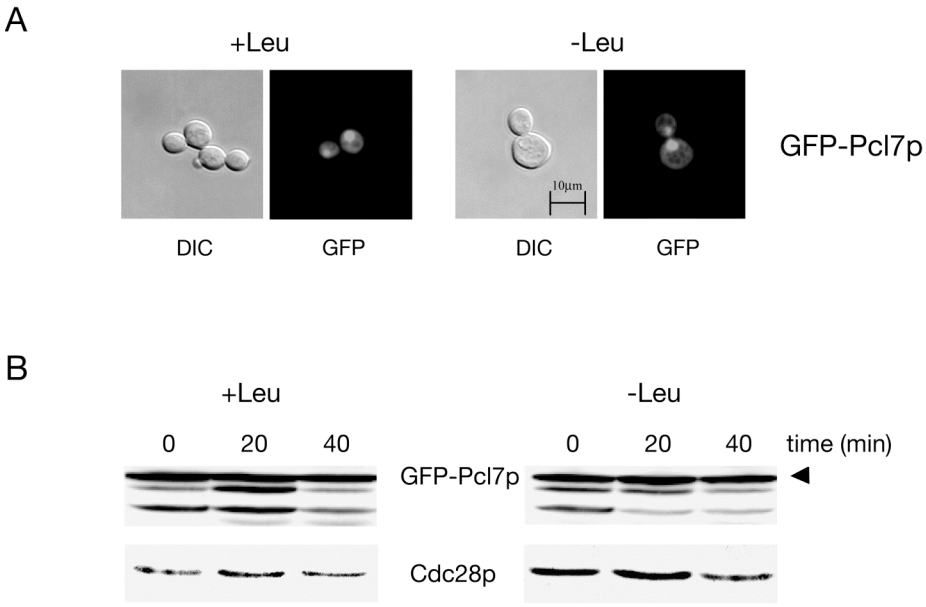

Figure 5. Nuclear localization and stability of cyclin Pcl7p are unaffected by the availability of amino acids. **(A)** The GFP-Pcl7-fusion is predominantly enriched in the yeast nucleus in sated and amino acid starved cells. *pcl7* mutant cells (RH3255) expressing *GFP-PCL7* from the *MET25* promoter (pME2230) were analyzed under sated (+Leu) and starved (-Leu) conditions by DIC microscopy and fluorescence microscopy (GFP). **(B)** GFP-Pcl7p is a stable yeast protein independently of the availability of amino acids. The yeast strain RH1168 was transformed to express *GFP-PCL7* (pME2230). Protein levels of GFP-Pcl7p and Cdc28p as loading control were determined in sated (+Leu) and amino acid starved (-Leu) cells after the *MET25* promoter-shut-off. The GFP antibody recognizes additional bands of different sizes, which might be products of premature translation termination or protein degradation.

Pcl7p interacts with Pho81p and Pho85p independently of Gcn4p stability. We investigated the complex formation of both Pho81p/Pcl7p and Pho85p/Pcl7p under conditions when Gcn4p is rapidly degraded in sated yeast cells and when Gcn4p is stabilized under amino acid starvation conditions. Therefore, *GST-PHO81* or *GST-PHO85* were expressed from the *GAL1* promoter and transformed into a strain containing a genomic fusion of *PCL7-myc*[9]. Fusion proteins were expressed in sated and amino acid starved cells, whereas amino acid starvation was induced as described above. The GST-fusion protein with its associated proteins were purified by glutathione agarose beads and analyzed by Western hybridization. Figure 6A shows that the interaction of Pcl7p with Pho81p significantly differs from the interaction of the other relevant cyclin, Pcl5p. Whereas Pcl5p is unable to assemble with Pho81p in starved cells, a similar amount of Pcl7-myc[9]p is co-precipitated with the GST-Pho81p in sated and starved cells. Similarly, Pcl7p interacts constitutively with Pho85p - as opposed to Pcl5p that only interacts with Pho85p under sated conditions (Figure 6B). These results verify the *in vivo* interaction between the cyclin Pcl7p with the CKI Pho81p and the kinase Pho85p but hint that Pcl7p plays an auxiliary role during Gcn4p stability control. Pcl7p

interactions are independent of the amino acid concentration in the medium, whereas the Pcl5p interactions correlate to the amino acid availability. Therefore, both Pho81p and Pho85p are able to distinguish between different cyclins in response to amino acid starvation.
In addition, we analyzed the complex formation of Pho85p/Pcl5p in a *pcl7* mutant strain to examine whether Pcl7p is involved in the dissociation of Pho85p/Pcl5p in low amino acids. Complex formation was not significantly affected compared to the analyzed *pcl5* mutant strain (Figure 3B). Pho85p and Pcl5p are not interacting in amino acid starved *pcl7* mutant cells (Figure 6C) indicating that Pcl7p is not required for direct disruption of the Pho85p/Pcl5p interaction in amino acid starved cells.

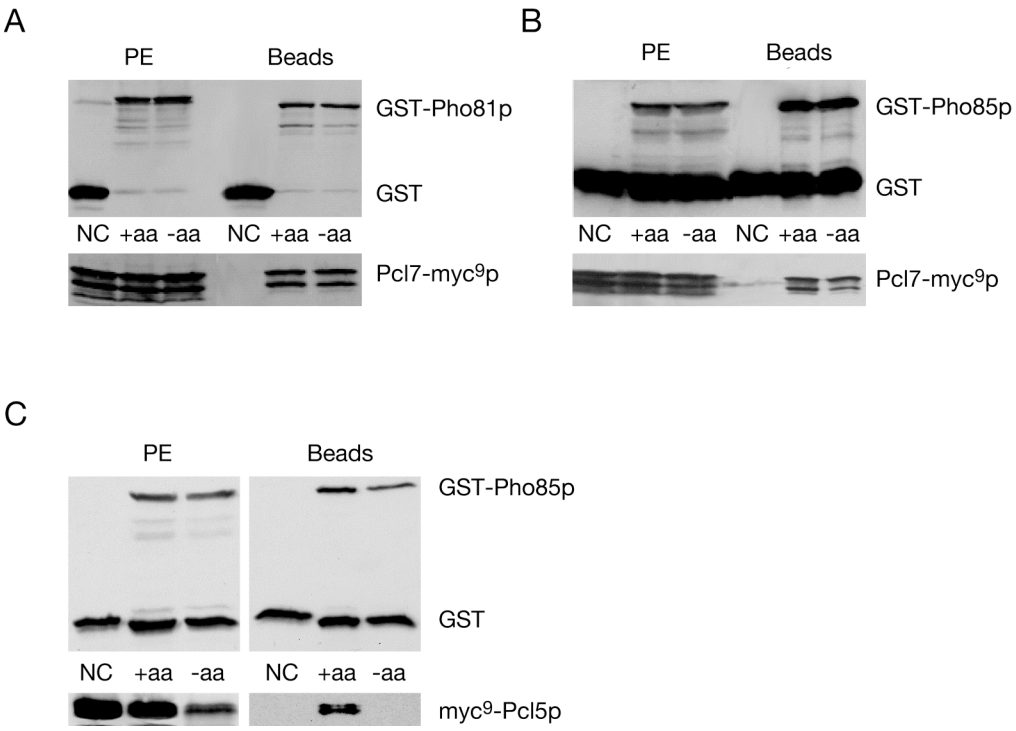

Figure 6. Pcl7p interacts with Pho81p and Pho85p independently of the stability of Gcn4p. The *S. cerevisiae* strain RH2977 containing *PCL7-myc*^{9} behind its endogenous promoter expresses either *PCL7-myc*^{9} with glutathione S transferase (*GST* on pYGEX-2T) as negative control (NC), *PCL7-myc*^{9} together with *GST-PHO81* (pME2867) (A) or *PCL7-myc*^{9} together with *GST-PHO85* (pME2866) (B). In addition, yeast strain RH3255 (*pcl7)* was transformed to express either *myc*^{9}*-PCL5* (pME2865) with glutathione S transferase (*GST* on pYGEX-2T) as negative control, or *myc*^{9}*-PCL5* (pME2865) together with *GST-PHO85* (pME2866) (C). Protein levels of the fusion proteins were determined in sated (+aa) and amino acid starved (-aa) cells. The left part represents GST, GST-Pho81p, GST-Pho85p and Pcl7-myc^{9}p prior to GST-agarose incubation (PE). On the right, the elutions of the glutathione beads are shown (Beads).

Discussion

It is generally assumed that the presence or absence of specific cyclins is a major prerequisite for controlling the activity of a cyclin-dependent protein kinase in a eukaryotic cell. Accordingly, decreasing levels of the highly unstable cyclin Pcl5p were assumed to be the reason for reduced phosphorylation of Gcn4p by the kinase Pho85p during amino acid starvation (Irniger and Braus, 2003; Shemer et al., 2002). Rapid Gcn4p decay in the nucleus (Pries et al., 2002) is initiated in sated yeast cells by phosphorylation of Thr165 by the kinase cyclin complex Pho85p/Pcl5p (Shemer et al., 2002) whereas in response to a reduced amino acid supply Gcn4p is stabilized. In this work, we present evidence that a new mechanism including additional proteins is essential to control amino acid-dependent Gcn4p stabilization. The Pho85p/Pcl5p complex dissociates when Gcn4p is required under amino acid starvation and the Gcn4p degradation pathway therefore has to be inhibited. The cyclin Pcl7p is able to interact constitutively with Pho85p and Pho81p independently of the presence or absence of amino acids. The CKI Pho81p behaves like Pho85p and is therefore only able to associate with Pcl5p in sated yeast cells. Our findings suggest that there has to be a yet unknown molecular mechanism by which Pho81p and Pcl7p affect the Pho85p/Pcl5p activity and therefore Gcn4p stability.

Pho81p and Gcn4p stability regulation

Previous studies have shown that Pho81p inhibits another Pho85p complex, the Pho85p/Pho80p activity, by binding to the Pho80p cyclin subunit. Pho81p is only activated as inhibitor under phosphate starvation but forms a stable complex with Pho85p/Pho80p under both high and low phosphate conditions (Schneider et al., 1994). Huang et al., 2001 proposed an increased or altered affinity of the Pho81p/Pho80p interaction in low phosphate, leading to an inhibited kinase activity. We show here that binding and release seems to be an issue for the Pho81p/Pcl5p interaction, because Pho81p interacts with Pcl5p only in sated cells. The fact that the interaction of Pho81p with Pcl5p is lost precisely under conditions where Pho85p/Pcl5p becomes inactive contradicts an inhibitory role of Pho81p in the process. When we analyzed the possibility whether Pho81p and Pcl5p interact only as part of a complex with Pho85p, we found that Pho81p/Pcl5p interaction still occurs in cells impaired in *PHO85* (data not shown) indicating that Pho85p is not required for binding of Pho81p to Pcl5p. Therefore, we can exclude a putative model where Pho81p/Pcl5p interaction can only occur in a ternary

complex with Pho85p, which is disrupted in response to amino acid starvation leading to separated Pho81p and Pcl5p.

We have excluded as possible function of Pho81p in the regulation of Gcn4p stability that Pho81p is required to disrupt directly the Pho85p/Pcl5p complex in amino acid starved cells (Figure 3C). When we analyzed the role of Pho81p in the dissociation of Pho85p/Pcl5p in low amino acids, we found surprisingly that myc^9-Pcl5p is no more detectable in amino acid starved cells impaired in *PHO81* (Figure 3C). The N-terminal myc^9-tagged version of Pcl5p resembles native instable Pcl5p concerning its half-life of only a few minutes. In contrast, a GFP at the C-terminus of Pcl5p leads to a more stabilized cyclin. Only this stabilized Pcl5-GFP-fusion can be expressed in sated as well as amino acid starved *pho81* mutant cells (data not shown). Therefore, we assume that Pho81p has an important function in the regulation of Pcl5p stability.

We have shown that Gcn4p is constitutively degraded in *pho81* or *pcl7* mutant cells, even under amino acid limitation conditions (Figure 1A; 4A), where Pcl5p is either absent or depleted (Figure 3C; 6C). Since Pcl5p is required for rapid Gcn4p turnover (Shemer et al., 2002), these results point to the existence of an additional Pho85p/Pcl5p-independent Gcn4p degradation. One possible explanation is that Gcn4p is being targeted for degradation by other Pho85p/cyclin complexes, including Pho85p/Pcl1p or Pho85p/Pho80p, which were previously shown to be able to promote Gcn4p phosphorylation (Meimoun *et al*., 2000). Furthermore, the kinase Srb10p has been shown to phosphorylate Gcn4p independently of the availability of amino acids (Chi et al., 2001) and therefore is able to initiate the turnover of Gcn4p. We have investigated these possibilities by measuring Gcn4p half-lives in *pho81/pho85* and *pho81/srb10* double knockouts under sated and amino acid starved conditions resulting in no difference of Gcn4p stability compared to *pho85* and *srb10* single mutants (data not shown).

Another important question is, how Pho81p itself is regulated in response to the availability of amino acids. Activation might include phosphorylation because it was shown that Pho81p phosphorylation is required for the inhibition of Pho85p kinase activity (Knight et al., 2004). It was proposed that Pho81p inhibitor activity is regulated by Pho85p mediated phosphorylation of Pho81p (Waters et al., 2004). The Pho85p/Pcl5p complex differs from the Pho85p/Pho80p complex because Pho81p binds to the cyclin of Pho85p/Pcl5p only in sated cells. This interaction is not required for the degradation of Gcn4p because under sated conditions Gcn4p is similarly degraded in the presence or absence of functional Pho81p (Figure 1A). It is an attractive model that under amino acid starvation, Pho81p is

posttranslationally modified, e.g. by phosphorylation or dephosphorylation resulting in an activated Pho81p. This leads to the question, why are Pho81p and Pcl5p interacting under conditions when Gcn4p is rapidly degraded, i.e., in the presence of sufficient amino acids. One possibility is that Pho81p binds to Pcl5p in sated cells as prerequisite or support for a strong binding between Pho85p and Pcl5p.

Pcl7p and Gcn4p stability regulation

We show that a second cyclin, Pcl7p, is involved in the control of Gcn4p stability besides the cyclin Pcl5p. Pcl7p is required for the stabilization of Gcn4p in low amino acids and is able to interact constitutively with Pho85p or Pho81p. A possible mechanism of Pcl7p function in Gcn4p stability control is antagonistic binding to the kinase Pho85p and therefore a competition for binding to Pho85p between Pcl7p and Pcl5p. Amino acid starvation induces the disruption of Pho85p/Pcl5p complexes and therefore a shift in the equilibrium towards an increased number of Pho85p/Pcl7p complexes. Since Pcl7p is also more stable than Pcl5p, Gcn4p remains preferentially unphosphorylated and stable under these conditions (Figure 7). Analyses of the Pho85p-Pcl5p complex formation in *pcl7* mutant cells revealed, that Pcl7p is not required to disrupt directly the Pho85p/Pcl5p interaction in low amino acids (Figure 6C).

A more detailed analysis indicated that a *pcl7* deletion as well as an overexpression of *PCL7* leads to sensitivity against amino acid starvation induced by 100 mM 3AT (Figure 4C). A *gcn4* mutant is hypersensitive to 10mM 3AT (Hinnebusch, 1992). In contrast, a *pcl5* strain is more able to deal with an induced general control, indicating the negative role of Pcl5p on Gcn4p activity (Shemer *et al*., 2002). Based on the constitutive Gcn4p degradation in *pcl7* cells together with the fact that Gcn4p is required for 3AT resistance, the decreased growth of *pcl7* cells on 100 mM 3AT suggests a positive effect of Pcl7p on Gcn4p activity and therefore an antagonistic role to Pcl5p. An overexpression of *PCL7* leads to a strong stabilization of Gcn4p under amino acid starvation and also to a hypersensitivity to high amounts of 3AT (Figure 4B, C). One possible explanation for the 3AT-sensitive phenotype of overexpressed *PCL7* is a strong stabilized Gcn4p under 100 mM 3AT with a decreased activity that is not more able to mediate full 3AT-resistance.

In summary, our data illustrate that the stability of the global transcriptional activator Gcn4p is highly controlled in yeast by a complex network of various regulatory proteins including two cyclins, Pcl5p and Pcl7p, the CDK Pho85p and the inhibitor Pho81p. The Gcn4p stability network is only part of a still larger number of proteins, which are orchestrating the modulation of Gcn4p activity on additional levels like protein-synthesis or interaction with

the transcriptional machinery to secure proper Gcn4p function within the 'general control of amino acid biosynthesis', a control mechanism which is conserved from yeast to man (Costa-Mattioli et al., 2005; Hao et al., 2005).

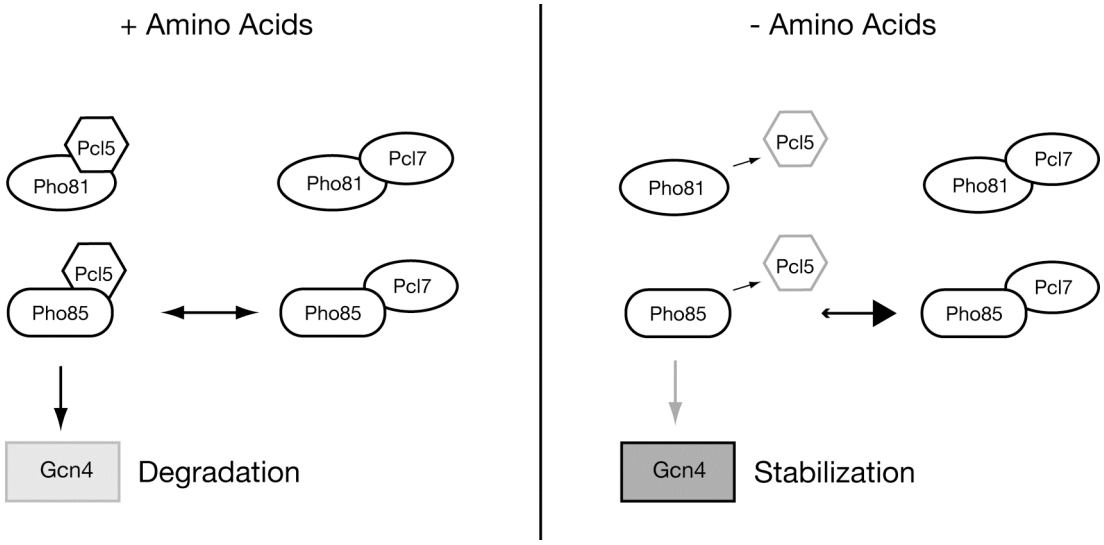

Figure 7. Model for Gcn4p stability regulation. In sated cells, when amino acids are available (+Amino Acids), both cyclins, Pcl5p and Pcl7p, compete for binding to the kinase Pho85p. This results in a possible equilibrium of both complexes with sufficient amounts of Pho85p/Pcl5p for Gcn4p phosphorylation and therefore rapid protein degradation. In response to amino acid starvation (-Amino Acids), Gcn4p is stabilized because the Pho85p/Pcl5p complex is dissociated, and Pcl5p is replaced by Pcl7p which is present in high amounts. The decrease of Pho85p/Pcl5p complexes results in less phosphorylation of Gcn4p and subsequently in its stabilization.

Acknowledgments

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CHAPTER III

Degradation of yeast transcription factor Gcn4p requires a central specificity domain and a C-terminal nuclear localization signal in the cyclin Pcl5p

Abstract

The unstable cyclin Pcl5p is the specificity factor for the Pho85p kinase to initiate the degradation of the *Saccharomyces cerevisiae* general amino acid control transcriptional activator Gcn4p. The cyclin Pcl5p is predominantly localized in the nucleus due to the β-importin Kap95p and independently of the presence or absence of amino acids or the proteins Pho85p, Gcn4p and Pho81p respectively. A mislocalization of Pcl5p into the cytoplasm results in constitutively stabilized Gcn4p underlining that nuclear localization of Pcl5p cyclin is required for efficient Gcn4p phosphorylation by Pho85p/Pcl5p. The carboxy-terminal part of Pcl5p, which lacks a classical nuclear localization sequence is sufficient for its proper nuclear localization but does not promote Gcn4p degradation. Pho80p is another Pho85p kinase interacting cyclin, which, however, does not mediate Gcn4p degradation specificity. A Pho80-Pcl5-Pho80p hybrid protein, which includes amino acids 78-179 of Pcl5p, results in a cyclin, which promotes Gcn4p degradation. Therefore, this central region of Pcl5p includes the specificity domain of Gcn4p. In contrast to Pcl5p, which depends on a C-terminal NLS Pho80p is transported into the nucleus by a N-terminal NLS. Therefore, the nuclear localization signal of these cyclins corresponds to distinct domains, which can be exchanged.

Introduction

Cyclin-dependent protein kinases (CDKs) consist of a kinase subunit, which is physically associated to a regulatory cyclin partner (De Bondt et al., 1993; Jeffrey et al., 1995). Cyclins mediate functional specificity of CDKs leading to phosphorylation of their substrates (Miller and Cross, 2001). This phosphorylation marks the proteins, e.g. for initiation of the ubiquitin-degradation pathway or for changing the sub-cellular localization (Kornitzer et al., 1994; Kaffman et al., 1998). CDKs were originally identified because of their role in the regulation of the eukaryotic cell division cycle (Levine and Cross, 1995). They have also been shown to be involved in several processes including the control of gene transcription (Miller and Cross, 2001). Cyclins were named because of their fluctuating levels during the cell cycle (Evans et al., 1983). Little variation throughout the cell cycle of some newly identified cyclins extended the definition of a cyclin subunit to a family of structurally related proteins, which bind and activate CDKs (Morgan, 1995).

Most CDKs are able to interact with more than one cyclin resulting in different substrate specificity for the kinase. In budding yeast, six different CDKs are known, from which Pho85p is related to the mammalian Cdk5 that was shown to be involved in Alzheimer's disease (Huang et al., 1999, Patrick et al., 1999). Pho85p has been implicated in cell cycle control, but its well-established function lies also in the control of gene expression (Lenburg and O'Shea, 1996). This kinase is capable to interact with ten different cyclin partners (Measday et al., 1997), which are divided into two subfamilies because of their sequence similarities and functional relationships (Measday et al., 1997). The members of the Pcl1,2 subfamily as Pcl1p, Pcl2p, Pcl5p, Pcl9p, or Clg1p are involved in complex with Pho85p in the regulation of cell wall maintenance (Andrews and Measday, 1998) as well as in cell cycle control (Measday et al., 1997; Tennyson et al., 1998). Members of the Pho80p subfamily as Pho80p, Pcl6p, Pcl7p, Pcl8p, and Pcl10p are involved in the regulation of distinct metabolic pathways (Andrews and Measday, 1998). The Pho85p/Pho80p complex specifically phosphorylates the transcription factor Pho4p in a phosphate rich environment (O'Neill et al., 1996) resulting in reduced activity. In contrast, in response to phosphate starvation the Pho4p phosphorylation activity is inhibited (Kaffman et al., 1998). Cyclin-dependent protein stability regulation controls the amount of the JUN like transcriptional activator Gcn4p within the cell. The unstable cyclin Pcl5p is the specificity factor of the kinase Pho85p to phosphorylate Gcn4p at Thr165 (Meimoun et al., 2000; Shemer et al., 2002). Phosphorylation of Gcn4p initiates the degradation pathway and marks the protein for ubiquitination by the E3

SCF^{CDC4} ubiquitin ligase (Kornitzer et al., 1994; Meimoun et al., 2000). Finally, poly-ubiquitinated Gcn4p is degraded by the 26S proteasome (Kornitzer et al., 1994). The kinase Pho85p as well as the substrate Gcn4p are predominantly nuclear localized proteins (Pries et al., 2002). Gcn4p contains two nuclear localization signals, from which NLS2-dependent nuclear import requires the karyopherins Srp1p and Kap95p (Pries et al., 2004). The amino acid-dependent Gcn4p stability regulation occurs in the yeast nucleus (Pries et al., 2002) and therefore it is suggested that also cyclin Pcl5p has to be transported into the nucleus to fulfill its function.

CDK activity is tightly controlled and requires the same localization of CDK, specific cyclin and correct substrate in the cell (Hood et al., 2001; Maridor et al., 1993; Pines and Hunter, 1991). The same sub-cellular localization of many cyclins implies molecular mechanisms, which decide about the interacting cyclin under specific cellular conditions. The activity of Pho85p/Pcl5p, which is crucial for destabilization of Gcn4p is abolished under Gcn4p-stabilization conditions by the dissociation of Pho85p kinase subunit and the cyclin Pcl5p (Bömeke et al., 2006). Furthermore, CDK activity is modulated by interaction to additional inhibitors or activators. CDK-inhibitory subunits, which are capable to turn off the CDK catalytic subunit are known to bind specifically to different components of CDK-cyclin complexes (Morgan, 1995; Sherr and Roberts, 1999). The cyclin-dependent kinase inhibitor (CKI) Pho81p is besides Sic1p and Far1p one of the three identified CKIs in *S. cerevisiae* (Mendenhall, 1998; Peter and Herskowitz, 1994) and able to inactivate Pho85p-Pho80p complexes under low phosphate conditions (Lenburg and O'Shea, 1996). Besides its inhibiting function, Pho81p is required for the Pho85p/Pcl5p-dependent stabilization of the transcription factor Gcn4p (Bömeke et al., 2006).

So far, the substrate specificity or sub-cellular localization regions of cyclin Pcl5p have not been explored. We wanted to know, whether there is a distinct Gcn4p-specific domain of Pcl5p that confers Gcn4p substrate specificity. We were also interested, whether we can use part of Pcl5p to change the specificity of one of the other Pho85p cyclins, which normally does not mediate Gcn4p degradation, towards the substrate Gcn4p. Furthermore, we wanted to know, whether nuclear import of Pcl5p is a prerequisite for rapid Gcn4p turnover and if so in which way this cyclin is transported into the nucleus.

Materials and Methods

S. cerevisiae strains and growth conditions

All yeast strains used in this study are either congenic to *S. cerevisiae* S288c (RH3239, RH1168, RH1408) or the W303 (RH3237, RH3238, RH3241, EY0140, RH3242, RH2701, RH2702, RH2703, RH2704, RH2706, RH2707, RH2708, RH2709, RH2710 and RH3058) genetic background. Details of the strains are given in Table 1. Creation of yeast strains RH3237 and RH3238 is described in Bömeke et al., 2006. RH3242 was obtained by replacing the mutant *his3-11* allele of yeast strain EY0140 by a wild-type *HIS3* allele using *Bam*HI linearized plasmid B1683 (Table 2). Strain RH3239 expressing *GFP* tagged version of *PCL5* at endogenous levels was obtained by PCR-mediated gene tagging (Knop et al., 1999). Briefly, primers were designed specific for amplification of the *yEGFP-kanMX4*-module with homologous sequences to the *PCL5*-3'end using plasmid pYM12[6] as template. The PCR-Product was transformed directly into yeast strain RH1168 and plated onto rich medium with 200 µg/ml G418 (Geneticin, Gibco). Transformants were replica-plated onto the same medium. The *GFP* tagged *PCL5* version was confirmed by Southern hybridization. Standard methods for genetic crosses and transformation were used (Ito et al., 1983).

The strains were grown in standard yeast extract-peptone-dextrose (YPD: 1% yeast extract, 2% peptone, 2% dextrose) and minimal yeast nitrogen base media (YNB: 1,5 g/l yeast nitrogen base lacking amino acids and ammonium sulfate, 4,5 g/l ammonium sulfate, 2% dextrose or galactose and supplemented with the appropriate amino acids).

Plasmid constructions

The plasmids used in this study are listed in Table 2 and details of important primers are given in Table 3. Plasmid pME2844 expressing *PCL5-GFP* was obtained by amplifying the *PCL5*-ORF with *Pfu*-Polymerase and introducing them via *Sma*I/*Cla*I into the low copy number GFP-C-Fus vector (pME2843) (Niedenthal et al., 1996). pME2846 expressing *PCL5-GFP* was constructed by amplifying the *PCL5*-ORF with *Pfu*-Polymerase and cloning it as *Sma*I/*Hin*dIII-fragment into p426MET25. Afterwards a 750 bp *Bgl*II-fragment encoding the GFPuv variant of GFP that was amplified from plasmid pBAD-GFP (Clontech, Heidelberg, Germany) was inserted behind the *PCL5*-ORF.

Construction of plasmids pME2849, pME2850, pME2851, pME2852, pME2853, pME2854, pME2855, pME2856, pME2857, pME2858, and pME2859 expressing *GFP* fused to the 3'end of different *PCL5* fragments driven from the *MET25* promoter was started by

amplifying the *GFP*-ORF as described before and introducing it via *Sma*I/*Cla*I into p426MET25. *PCL5* fragments were fused via *Spe*I/*Sma*I to the 5'end of *GFP*. pME2950 was constructed by amplifying *PCL5*$_{bp610-654}$*-GFP* (encoding Pcl5$^{aa204-218}$-GFP) from plasmid pBAD-GFP using primer KB57 and KB16 (Table 3) and cloning them via *Sma*I/*Cla*I into p426MET25.

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
RH2701	*MATα, mtr10::HIS3, ade2, leu2, trp1, hi3, ura3*	(Senger et al., 1998)
RH2702	*MATa, kap104::HIS3, ura3, his3, trp1, leu2, lys2*	(Aitchison et al., 1996)
RH2703	*MATa, pse1-1, ura3, trp1, leu2*	(Seedorf and Silver, 1997)
RH2704	*MATα, rsl1-4(kap95ts), ura3, trp1, leu2, ade2*	(Koepp et al., 1996)
RH2706	*MATa, pse1-1, kap123::HIS3, ura3, trp1, leu2*	(Seedorf and Silver, 1997)
RH2707	*MATα, kap123::HIS3, ura3, trp1, leu2*	(Seedorf and Silver, 1997)
RH2708	*MATα, nmd5::HIS3, ura3, his3, leu2, ade2, ade8*	(Ferrigno et al., 1998)
RH2709	*MATa, pdr6::HIS3, ura3, his3, leu2, trp1*	(Lau et al., 2000)
RH2710	*MATa, sxm1::HIS3, ura3, his3, trp1, leu2*	(Seedorf and Silver, 1997)
RH3058	*MATa, kap114::HIS3, ura3, his3, trp1, leu2, lys2*	(Pemberton et al., 1999)
RH3237	*MATa, ura3-1, can1-100, GAL+, leu2-3, trp1-1*	(Bömeke et al., 2006)
RH3238	*MATa, ura3-1, can1-100, GAL+, leu2-3, trp1-1; pcl5::hisG*	(Bömeke et al., 2006)
RH1168	*MATa, leu2-3, ura3-52, gal2*	our collection
RH3239	*MATa, leu2-3, ura3-52, gal2; PCL5-yEGFP-kanMX4*	this study
RH3241	*MATa, ade2, trp1, leu2-3, his3, ura3-52, pho81::HIS3*	(Ogawa et al., 1995)
EY0140	*MATa, ade2, trp1, can1-100, leu2-3,112, his3-11,15, ura3, pho85::LEU2*	(O'Neill et al., 1996)
RH3242	*MATa, ade2, trp1, can1-100, leu2-3,112, ura3, pho85::LEU2*	this study
RH1408	*MATa; ura3-52; gal2; gcn4-103*	(Pries et al., 2002)

Table 2. Plasmids used in this study

Plasmid	Description	Source
pBKSII®	2.96 kb vector, *Amp*^{R} (*bla*), *lacZ*, *ori*	Stratagene (La Jolla, USA)
pRS415	*LEU2*, *CEN*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pRS416	*URA3*, *CEN*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pRS426	*URA3*, *2µm*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
p415GAL1	pRS415 containing *GAL1* promoter, *CYC1* terminator	(Mumberg et al., 1994)
p416GAL1	pRS416 containing *GAL1* promoter, *CYC1* terminator	(Mumberg et al., 1994)
p426MET25	pRS426 containing *MET25* promoter , *CYC1* terminator	(Mumberg et al., 1994)
pME2843	pGFP-C-FUS	(Niedenthal et al., 1996)
pME2844	*MET25prom-PCL5-GFP*-fusion in pGFP-C-FUS	this study
pME2846	*MET25prom-PCL5-GFP*-fusion in p426MET25	this study
pME2848	*GAL1prom-myc*^{3}*-GCN4*-fusion in p415GAL1	this study
pME2849	*MET25prom-GFP*-fusion in p426MET25	this study
pME2850	*MET25prom-PCL5*$_{aa1-95}$ *GFP*-fusion in p426MET25	this study
pME2851	*MET25prom-PCL5*$_{aa1-127}$ *GFP*-fusion in p426MET25	this study
pME2852	*MET25prom-PCL5*$_{aa61-127}$ *GFP*-fusion in p426MET25	this study
pME2853	*MET25prom-PCL5*$_{aa1-180}$ *GFP*-fusion in p426MET25	this study
pME2854	*MET25prom-PCL5*$_{aa61-229}$ *GFP*-fusion in p426MET25	this study
pME2855	*MET25prom-PCL5*$_{aa111-180}$ *GFP*-fusion in p426MET25	this study
pME2856	*MET25prom-PCL5*$_{aa111-229}$ *GFP*-fusion in p426MET25	this study
pME2857	*MET25prom-PCL5*$_{aa153-229}$ *GFP*-fusion in p426MET25	this study
pME2858	*MET25prom-PCL5*$_{aa61-180}$ *GFP*-fusion in p426MET25	this study
pME2859	*MET25prom-PCL5*$_{aa181-229}$ *GFP*-fusion in p426MET25	this study
pME2950	*MET25prom-PCL5*$_{aa204-218}$ *GFP*-fusion in p426MET25	this study
pME2951	*MET25prom-GFP-ARO7-PCL5*$_{aa207-215}$ fusion in p426MET25	this study
pME2860	*MET25prom-PHO80*$_{aa1-73}$*-PCL5*$_{aa79-178}$*-PHO80*$_{aa170-294}$ *GFP*-fusion in p426MET25	this study
pME2948	*MET25prom-PHO80*$_{aa1-73}$*-PCL5*$_{aa79-178}$ *GFP*-fusion in p426MET25	this study
pME2949	*MET25prom-PCL5*$_{aa79-178}$*-PHO80*$_{aa170-294}$ *GFP*-fusion in p426MET25	this study
pME2966	*MET25prom-PCL5*$^{K140A,\ E167A,\ L171A}$ *GFP*-fusion in p426MET25	this study
pME2861	*MET25prom-PCL5-GFP-NES*-fusion in p426MET25	this study
KB1360	*GAL1prom-PHO80*$_{aa1-73}$*-PCL5*$_{aa79-178}$*-PHO80*$_{aa170-294}$-fusion in p416GAL1	Kornitzer, pers. comm.

B1683	1720 bp *HIS* gene in pBKSII®	Hill, pers. comm.
pYM12[6]	*yEGFP-kan*MX4-module	(Knop et al., 1999)

The plasmid pME2951 was obtained by amplifying *GFP-ARO7-PCL5*$_{bp619-645}$ (encoding GFP-Aro7-Pcl5$_{aa207-215}$) with *Pfu*-Polymerase using primer RP14 and KB55 (Table 3) and introducing it via *Sma*I/*Eco*RI into p426MET25.

The plasmid pME2860 was constructed by amplifying the hybrid-ORF *PHO80*$_{bp1-219}$-*PCL5*$_{bp235-534}$-*PHO80*$_{bp508-882}$ (encoding Pho80$_{aa1-73}$-Pcl5$_{aa79-178}$Pho80$_{aa170-294}$-GFP) with *Pfu*-Polymerase from plasmid KB1360 and fused it via *Spe*I/*Sma*I to the 5'end of *GFP* on plasmid pME2849. To obtain pME2948 and pME2949 the hybrids *PHO80*$_{bp1-219}$-*PCL5*$_{bp235-534}$ (pME2948) and *PCL5*$_{bp235-534}$-*PHO80*$_{bp508-882}$ (pME2949) (encoding Pho80$_{aa1-73}$-Pcl5$_{aa79-178}$-GFP or Pcl5$_{aa79-178}$Pho80$_{aa170-294}$-GFP) were amplified via *Pfu*-Polymerase from plasmid pME2860 and ligated as *Spe*I/*Sma*I-fragments in front of the *GFP*-ORF of pME2849.

Construction of plasmid pME2966 was started by site directed mutagenesis of the *PCL5*-ORF via *KOD*-Polymerase using primer KB65, KB66, KB71, KB72 (Table 3) resulting in the amino acid substitutions K140A, E167A and L171A. The mutated *PCL5*-ORF was introduced as *Spe*I/*Sma*I-fragment in front of the *GFP*-ORF of plasmid pME2849.

In the *PCL5-GFP-NES*-fusion (pME2861), the NES sequence from PKI (Stade et al., 1997) was engineered at the 3'end of *PCL5-GFP* in two steps. The *PCL5*-ORF was amplified with *Pfu*-Polymerase and inserted in p426MET25 as *Sma*I/*Hin*dIII-fragment as first step. Secondly, the *GFP-NES* cassette was amplified using Primer KB5 and KB40 (encoding GFP-NESaa GMDELYKNELALKLAGLDINKTKLTLA) (Table 3) and introduced as a *Bgl*II-fragment at the 3' end of *PCL5*.

Plasmid pME2848, expressing a triple myc epitope tagged version of *GCN4* under the control of the *GAL1* promoter, was obtained by amplifying *GCN4* with *Pfu*-Polymerase and subsequent insertion in p415GAL1 as blunt/*Hin*dIII-fragment. A 120 bp *Bam*HI-fragment carrying the triple myc epitope was inserted into a *Bgl*II restriction site after the fifth amino acid of Gcn4p.

Table 3. Primer used in this study

Primer (5'-3')	Sequence	Construct
KB5	GAG AGA TCT ATG GCT AGC AAA GGA GAA GAA	pME2861
KB40	GGA AGA TCT TCC TTA CTT GTT GAT ATC GAG GCC TGC TAG TTT CAG CGC TAA TTC ATT TTT GTA CAA TTC ATC CAT GCC	pME2861
KB57	TCC CCC GGG GGA ATG GCC AAC GCT CCG GTG AAG AGA CCC AGA GAA TCA GAC AAT GAT TAT GCT AGC AAA GGA GAA GAA	pME2950
KB16	CCA TCG ATG GTT ATT TGT AGA GCT CAT CCA T	pME2950
RP14	TAA CCC GGG ATG GCT AGC AAA GGA GAA GAA	pME2951
KB55	CCG GAA TTC CGG TTA GTC TGA TTC TCT GGG TCT CTT CAC CGG CTC TTC CAA CCT TCT TAG CAA	pME2951
KB65	GCT GTC TTA TTC TAT CGC ACG CAT TTT TGA ACG	pME2966
KB66	CGT TCA AAA ATG CGT GCG ATA GAA TAA GAC AGC	pME2966
KB71	GGA TCT ATC TCT AAT GGC AAG ATG GTG TGC GGG TAA GCT GAA TTA TGG CA	pME2966
KB72	GCT CAT AAT TCA GCT TAC CCG CAC ACC ATC TTG CCA TTA GAG ATA GAT CC	pME2966

Protein analysis

Shut-off-Western procedure. Yeast cells were pre-grown in selective minimal medium with glucose as carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express *myc*^{3}*-GCN4* from the *GAL1* promoter. After 3h of induction 2% glucose was added to shut off the *GAL1* promoter. Samples were analyzed at the indicated time points after promoter-shut-off (0-min time point).

Whole-cell extracts of S. cerevisiae. Extracts were prepared from yeast cultures grown to exponential phase. Cells were washed in ice-cold buffer B (50 mM Tris-HCl pH 7,5, 1 mM EDTA, 50 mM dithiothreitol), lysed with glass beads in 200 µl of buffer B + PIM (1 mM each phenylmethylsulfonyl fluoride, tosyl-L-lysine-chloromethylketone, tosyl-L-phenylalanine-chloromethylketone, *p*-aminobenzamidine–HCl and *o*-phenanthroline) + 3% Triton X-100 + 0,8% SDS at 4°C, and spun at 3500 rpm for 15 min to remove glass beads and large cell debris. Extracts (10 µl) were removed to determine total protein concentration using a protein assay kit from Bio-Rad (München, Germany). Proteins were denatured in SDS loading dye by heating at 65°C for 15 min. Proteins were subjected to SDS-PAGE and transferred to

nitrocellulose membranes. GFP and myc-fusion proteins, Cdc28p and eIF2p were detected using ECL technology (Amersham, UK). For the first incubation, monoclonal mouse anti-GFP (Clontech, Heidelberg, Germany), monoclonal mouse anti-myc (9E10), polyclonal rabbit anti-Cdc28p or polyclonal rabbit anti-eIF2p antibodies were used. Peroxidase-coupled goat anti-rabbit or goat anti-mouse IgG were used as secondary antibodies (Dianova, Hamburg, Germany).

GFP fluorescence microscopy

Yeast strains harbouring plasmids encoding Pcl5-GFP-fusion proteins were grown to early log-phase and analyzed under sated and starved conditions. Amino acid starvation was induced by adding 10 mM of the histidine analogue 3-amino-triazole (3AT) to the cycling yeast cells for 1 hour. 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) or in case of 4',6-diamidino-2-phenylindole (DAPI) staining a standard DAPI filter set. DAPI staining was used for visualization of nuclei. Cells were photographed using a Hamamatsu-Orca-ER digital camera and the Improvision Openlab software (Improvision, Coventry, UK).

Growth tests

Yeast strains were pre-cultured to the same optical densities (OD_{600}= 1) and spotted onto selective YNB medium supplemented with or without 100 mM 3AT. Five-fold dilution experiments started with $3x10^4$ cells per 20 µl. After 3 days of growth the spotted strains were photographed under white light.

Results

Nuclear localization of yeast Pcl5p is required for Gcn4p degradation

Previous experiments have suggested that phosphorylation and ubiquitination, which are the first two steps of the Gcn4p degradation pathway, are restricted to the nucleus (Pries et al., 2002). We asked, whether nuclear localization of the cyclin Pcl5p, which is required for Gcn4p phosphoryation, is a prerequisite for efficient Gcn4p degradation in sated cells. To further analyze the compartment-specificity of Gcn4p degradation in the nucleus, we mislocalized the cyclin Pcl5p into the cytoplasm and tested the resulting impact on Gcn4p stability in sated cells.

The localization of Pcl5-GFP was manipulated by fusing a nuclear export sequence (NES) to the C-terminus of the Pcl5-GFP hybrid. The used NES sequence derived from the polypeptide inhibitor (PKI) of the cAMP-dependent protein kinase (PKA). It represents a short and hydrophobic motif with high leucine content (**L**A**L**K**L**AG**L**D**I**) (Nigg, 1997). Correct expression of the resulting Pcl5-GFP-NES chimera protein was verified by Western blot analysis of *S. cerevisiae* cell extracts using monoclonal anti-GFP antibodies and resulted in the expected size of 54 kDa (Figure 1A). Localization of this fusion was examined by fluorescence microscopy of yeast cells expressing *PCL5-GFP-NES* from the constitutive *MET25* promoter under sated conditions. Pcl5-GFP-NES was localized in the cytoplasm in contrast to the predominantly nuclear localized Pcl5p without any NES sequence. DAPI staining of the chromosomes confirmed the different localizations of Pcl5-GFP in the nucleus and of Pcl5-GFP-NES in the cytoplasm (Figure 1B).

Pcl5p cyclin function on Gcn4p degradation was tested genetically for the Pcl5-GFP-NES hybrid. Highly overexpressed *GCN4* inhibits cellular growth, possibly by the interference of Gcn4p with other transcriptional activation pathways (Tavernarakis and Thireos, 1995). A *pcl5*-deficient yeast strain is hypersensitive to even moderately overexpressed *GCN4* fused to the *GAL1* promoter (Shemer et al., 2002). When, in addition to *GCN4*, a functional *PCL5* is expressed, the Gcn4p-toxicity can be suppressed *in vivo* resulting in cells that can grow under Gcn4p producing conditions on galactose medium. The Gcn4p toxicity assay revealed that cytoplasmic Pcl5-GFP-NES is unable to suppress the Gcn4p-mediated growth inhibition, which is in contrast to nuclear Pcl5-GFP, which restores growth (Figure 1C). Furthermore, the effect of mislocalized cytoplasmic Pcl5p on Gcn4p degradation in sated cells was tested. As control, a *pcl5* mutant strain was transformed to express *GAL-myc*^{3}*-GCN4*. A promoter-shut-off experiment of the fusion gene revealed a strong stabilization of Gcn4p compared to

the *PCL5* wild-type strain. Additionally expressed nuclear localized Pcl5-GFP shows a rapid degradation of Gcn4p. In contrast, a simultaneous expression of cytoplasmic Pcl5-GFP-NES resulted in a similar strong stabilization of Gcn4p as described above (Figure 1D).

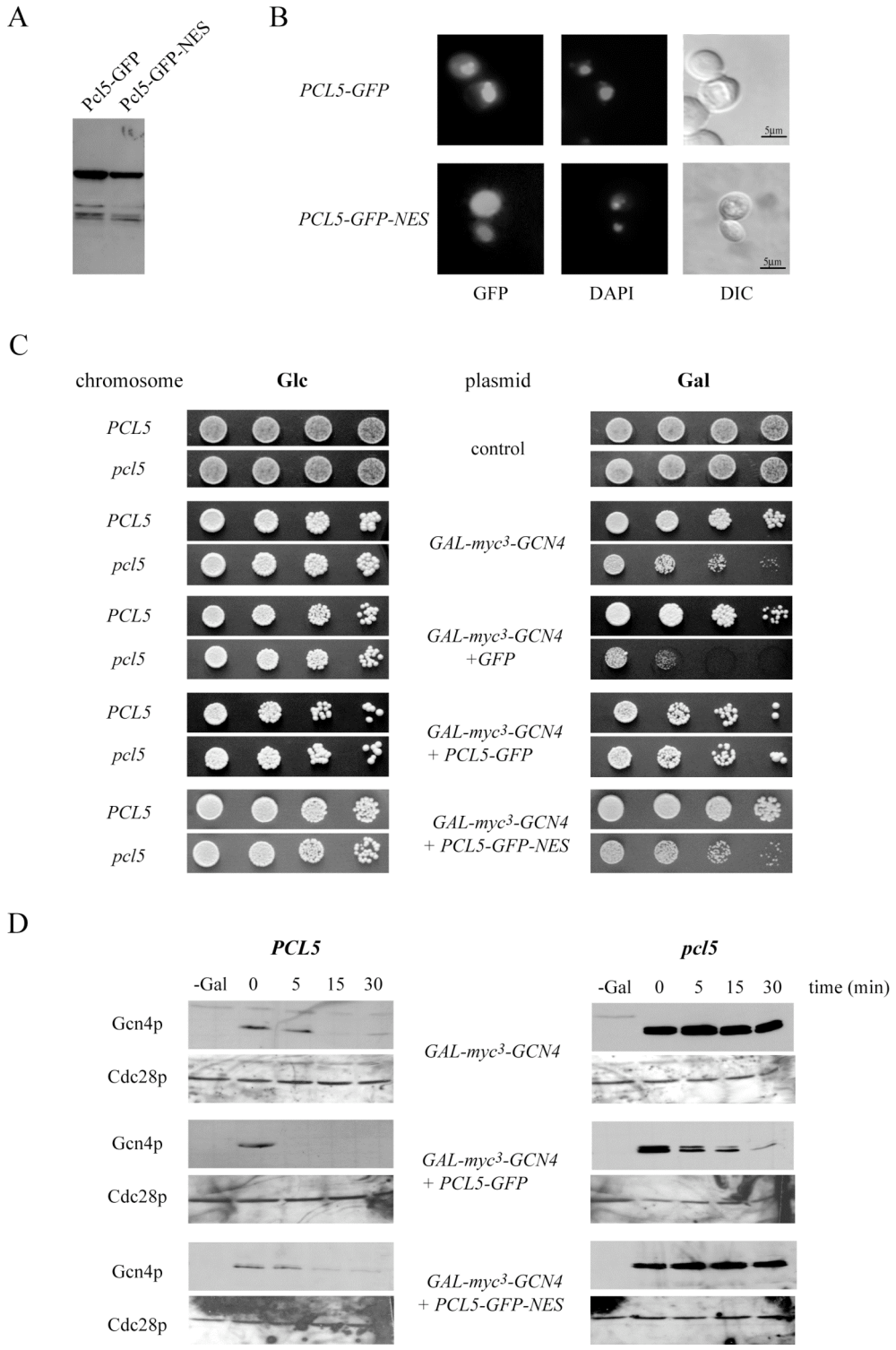


Figure 1. Nuclear localization of yeast Pcl5p is required for Gcn4p degradation. **(A)** Pcl5-GFP fused to a nuclear export sequence (NES) is correctly expressed as 54 kDa protein. Yeast strain RH3238 (*pcl5)* expressing either *PCL5-GFP* (pME2846) or *PCL5-GFP-NES* (pME2861) from the *MET25* promoter were grown to early log-phase and expression of the

fusion proteins was verified by Western blot analysis using monoclonal anti-GFP antibodies. **(B)** Pcl5-GFP-NES is transported out of the nucleus into the cytoplasm. Localization of the fusion proteins was analyzed in a *pcl5* mutant strain (RH3238) by fluorescence microscopy (GFP), DAPI staining (DAPI), and DIC (differential interference contrast) microscopy. **(C)** Pcl5-GFP-NES is uncapable to suppress the toxicity of overexpressed Gcn4p in the absence of a functional *PCL5* gene. Wild-type cells (RH3237) and *pcl5* mutant cells (RH3238) expressing *myc*^{3}*-GCN4* from plasmid pME2848 alone or together with *GFP* (pME2849), *PCL5-GFP* (pME2846) or *PCL5-GFP-NES* (pME2861) were spotted in five-fold dilutions on glucose and galactose to induce expression of *GCN4* driven from the *GAL1* promoter. As control wild-type and *pcl5* mutant cells were used without any plasmid (control). **(D)** Gcn4p degradation is not mediated by Pcl5-GFP-NES. The isogenic yeast strains RH3237 (*PCL5*) and RH3238 (*pcl5)* were transformed to express *GAL1*-driven *myc*^{3}*-GCN4* on a low copy number plasmid (pME2848) alone or together with *PCL5-GFP* (pME2846) or *PCL5-GFP-NES* (pME2861). Cells were pre-grown to early log-phase in selective medium containing glucose as carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express *myc*^{3}*-GCN4*. After 3h of induction the *GAL1* promoter was turned off by adding 2% glucose to the medium (0-min time point). Samples were analyzed at the indicated time points after the shift to glucose medium. Levels of myc^3 tagged Gcn4p were determined by immunoblotting using myc-antibodies. The kinase Cdc28p was used as loading control.

Taken together, cytoplasmic Pcl5p resulted in stabilized Gcn4p in sated cells, which suggests that nuclear localization of Pcl5p cyclin is required for efficient Gcn4p degradation in the nucleus. In addition, these experiments further support that Pcl5p plays a key role in Pho85p-mediated decay of Gcn4p (Shemer et al., 2002).

Pcl5p of *S. cerevisiae* is a predominantly nuclear protein in sated and amino acid starved cells

In the presence of sufficient amounts of amino acids, the cyclin-dependent kinase complex Pho85p/Pcl5p secures rapid Gcn4p decay; in the absence of amino acids, Pho85p and the cyclin Pcl5p dissociate and Gcn4p becomes stabilized (Bömeke et al., 2006). Since the nutrition-dependent Gcn4p stability regulation occurs in the nucleus (Pries et al., 2002), we expected a nuclear localization of the cyclin Pcl5p. Pcl5p is an unstable cyclin with a half-life of only approximately two minutes. We wondered, whether is it possible to monitor such an unstable protein in yeast *in vivo*. We hypothesized that Pcl5p-fusions with the green fluorescent protein (GFP) might stabilize the protein and might be functional resulting in Gcn4p degradation. Therefore the open reading frame for GFP was chromosomally integrated at the 3'-terminus of the *PCL5*-ORF to analyze the sub-cellular localization of the cyclin Pcl5p with respect to its authentic expression and regulation. In addition, genes for C- or N-terminal GFP tagged versions of Pcl5p were constructed on both high and low copy vectors

and driven from the repressible *MET25* promoter to increase the amount of this unstable protein and to study the localization independently of its expression.
Expression of the different Pcl5-GFP-fusion proteins of the correct size was verified by Western blot analysis of *S. cerevisiae* cell extracts using monoclonal anti-GFP antibodies (data not shown). The functionality of all Pcl5-GFP hybrids was genetically tested as described above. Whereas additionally expressed GFP is not able to suppress overexpression toxicity of Gcn4p, all tested Pcl5-GFP-fusions complement the *pcl5* mutant phenotype, suggesting functionality for initializing Gcn4p degradation (Figure 1C). A GFP-fusion of *PCL5*, which is integrated at the original locus as single copy is already sufficient to suppress Gcn4p toxicity, further corroborating that the fusion protein is functional. Complementation of the *pcl5* mutant phenotype could also be achieved by corresponding N-terminally tagged GFP-Pcl5-fusions (data not shown). This suggests that neither the N- nor the C-terminus of Pcl5p is directly involved in its function. The same results could be observed when Gcn4p was stabilized due to amino acid starvation induced by adding 10 mM 3-amino-triazole (3AT) to the medium, which acts as a competitive inhibitor of the *HIS3* gene product. All tested Pcl5-GFP hybrids are able to suppress Gcn4p toxicity like full-length-Pcl5p (data not shown).

Localization of Pcl5p was analyzed under conditions where Gcn4p is degraded due to a sufficient supply of amino acids. Chromosomally tagged Pcl5-GFP, low amounts of GFP tagged Pcl5p derived from a centromere plasmid as well as high amounts derived from a *2µm* vector were all predominantly localized within the nucleus (Figure 2A). Similar results were obtained with N-terminal GFP-fusions of Pcl5p (data not shown).
Under amino acid starvation conditions the Pho85p/Pcl5p complex formation is disrupted and therefore the kinase activity is decreased (Bömeke et al., 2006). Therefore we wondered, whether dissociation of Pho85p/Pcl5p is based on an altered localization of Pcl5p dependent on the availability of amino acid. Pcl5p localization was analyzed in cells grown under histidine starvation conditions that were induced by adding 10 mM 3AT to the medium. Figure 2B demonstrates that Pcl5p is efficiently transported into the nucleus, even under amino acid starvation conditions. These data illustrate that Pcl5p is located in the nucleus when the Pho85p/Pcl5p complex is active as well as when Pho85p and Pcl5p are dissociated.

Nuclear import of Pcl5p is independent of the substrate Gcn4p, the kinase Pho85p, or the cyclin-dependent inhibitor Pho81p

The amino acid sequence of Pcl5p contains no classical, basic NLS like the monopartite one of the SV40 large tumor antigen (Kalderon et al., 1984) or the bipartite motif of

nucleoplasmin (Robbins et al., 1991). Therefore we wondered, whether Pcl5p is directly imported into the nucleus by interaction with importins or indirectly by the help of the import of another nuclear protein. Phosphorylation of Gcn4p by the kinase/cyclin complex Pho85p/Pcl5p is counteracted by the cyclin-dependent kinase inhibitor (CKI) Pho81p, which is involved in amino acid-dependent stabilization of Gcn4p (Bömeke et al., 2006). Gcn4p, Pho85p, and Pho81p are exclusively nuclear proteins independently of the availability of amino acids (Pries et al., 2002). Gcn4p nuclear import is mediated by the classical importin α/β complex Srp1p/Kap95p (Pries et al., 2004). In addition, we have shown before that Pho85p and Pho81p interact with the cyclin Pcl5p *in vivo* (Bömeke et al., 2006). We analyzed, whether the phosphorylation substrate Gcn4p, the kinase Pho85p or the inhibitor Pho81p are required for Pcl5p nuclear import to test whether nuclear import of Pcl5p is enhanced through interaction with other proteins. Therefore the localization of Pcl5-GFP in *S. cerevisiae gcn4, pho85* or *pho81* mutant cells was examined. The cyclin was detected predominantly in the nucleus in all tested mutant strains, suggesting that nuclear import of Pcl5p is independent of the transcription factor Gcn4p, the cyclin-dependent kinase Pho85p and the CKI Pho81p (Figure 2B). Therefore we conclude that Pcl5p should possess its own nuclear localization signal (NLS) for the interaction with karyopherins.

The C-terminal part of Pcl5p directs nuclear localization

To analyze, which part of Pcl5p is responsible for its nuclear localization, we constructed a set of GFP-fusions. The first sixty N-terminal amino acids of Pcl5p are followed by the central part of the protein (aa 61-180) containing the so-called cyclin box of amino acids 127-180 (Figure 3). This central domain is conserved between the different Pho85p cyclins (Measday et al., 1997) and followed by additional 50 aa at the C-terminus. The sub-cellular localization of a set of 13 truncated Pcl5-GFP versions was analyzed to identify Pcl5 *cis*-acting sequences responsible for nuclear import mediation (Figure 3). All fusion constructs were driven from the repressible *MET25* promoter on a *2µm* plasmid to increase the amounts of presumably unstable truncated Pcl5-GFP-versions. Expression of Pcl5-GFP derivatives was verified by Western analyses of *pcl5* mutant cell extracts using monoclonal anti-GFP antibodies (data not shown).

Localization of GFP and the different Pcl5-GFP-fusion proteins was examined by fluorescence microscopy in sated *pcl5* cells. GFP alone localized throughout the cell in both the nucleus and the cytoplasm in contrast to the distinctly nuclear full-length Pcl5p-GFP-

fusion (Figure 4). N-terminal parts of Pcl5p consisting of the amino acids 1-95, 1-127 or 61-127 mislocalized to a completely different cellular location, the plasma membrane. In addition, the Pcl5aa61-127 showed aggregates covering the entire cell. Aggregation in addition to the predominantly nuclear localization was also observed for Pcl5-GFP constructs lacking the N- or C-terminal domain (Pcl5aa1-180; 61-229). In contrast, the conserved middle part of Pcl5p (aa 61-180) containing the cyclin box is visualized via GFP only as aggregates spread over the whole cell (Figure 4). This suggests that aggregate formation is connected to the Pcl5p amino acids 61-127. The Pcl5p amino acid stretch 1-180 is able to suppress the plasma membrane localization of amino acids 1-127 of Pcl5p. In contrast, the truncated Pcl5 protein devoid of the N-terminus and the cyclin box (Pcl5aa111-229, Pcl5aa153-229 and Pcl5aa181-229) was targeted into the nucleus. These data indicate that the carboxy-terminal 49 aa-residues of Pcl5p are sufficient to target GFP to the nucleus and therefore include the nuclear localization signal.

This region contains an interesting putative NLS motif (PV**KR**P**R**ESD) consisting of the Pcl5p amino acids 207-215. The structure of this motif highly resembles the c-myc NLS (PAA**KR**V**K**LD) where the proline and aspartic acid residues flanking the basic cluster play an important role in nuclear import (Makkerh et al., 1996). We tested the function of this putative NLS motif by two different approaches. The amino acids 204-218 of Pcl5p were fused to the N-terminus of GFP (pME2950) and the cytoplasmic yeast chorismate mutase (EC 5.4.99.5) was used as reporter by fusing the Pcl5aa207-215 to the C-terminus of GFP-Aro7p (pME2951), respectively. *S. cerevisiae* chorismate mutase is encoded by the *ARO7* gene, which is involved in the biosynthesis of the aromatic amino acids tyrosine and phenylalanine. We have shown that Aro7p is exclusively localized in the yeast cytoplasm (Pries et al., 2002). The Pcl5p amino acid stretches from position 204-218 fused to GFP or 207-215 fused to GFP-Aro7 are not able to trigger nuclear import of these predominantly cytoplasmic proteins. Fluorescence microscopy revealed localization of the fusion proteins in the cytoplasm (Figure 4). In summary, our data indicate, that the 207-215-motif is not sufficient for nuclear localization, but the entire C-terminus of Pcl5p, spanning the amino acids 181-229, represents a non-classical NLS.

Pcl5p nuclear transport requires the importin Kap95p

Pcl5p nuclear import is mediated by its C-terminal part and independent of the availability of amino acids. Since no classical NLS could be defined in Pcl5p, we examined whether a β-importin family member and not the classical α/β-importin heterodimer is required for Pcl5p

nuclear import. We analyzed the sub-cellular localization of Pcl5-GFP in a set of *S. cerevisiae* mutant strains that are defective in particular β-importins.

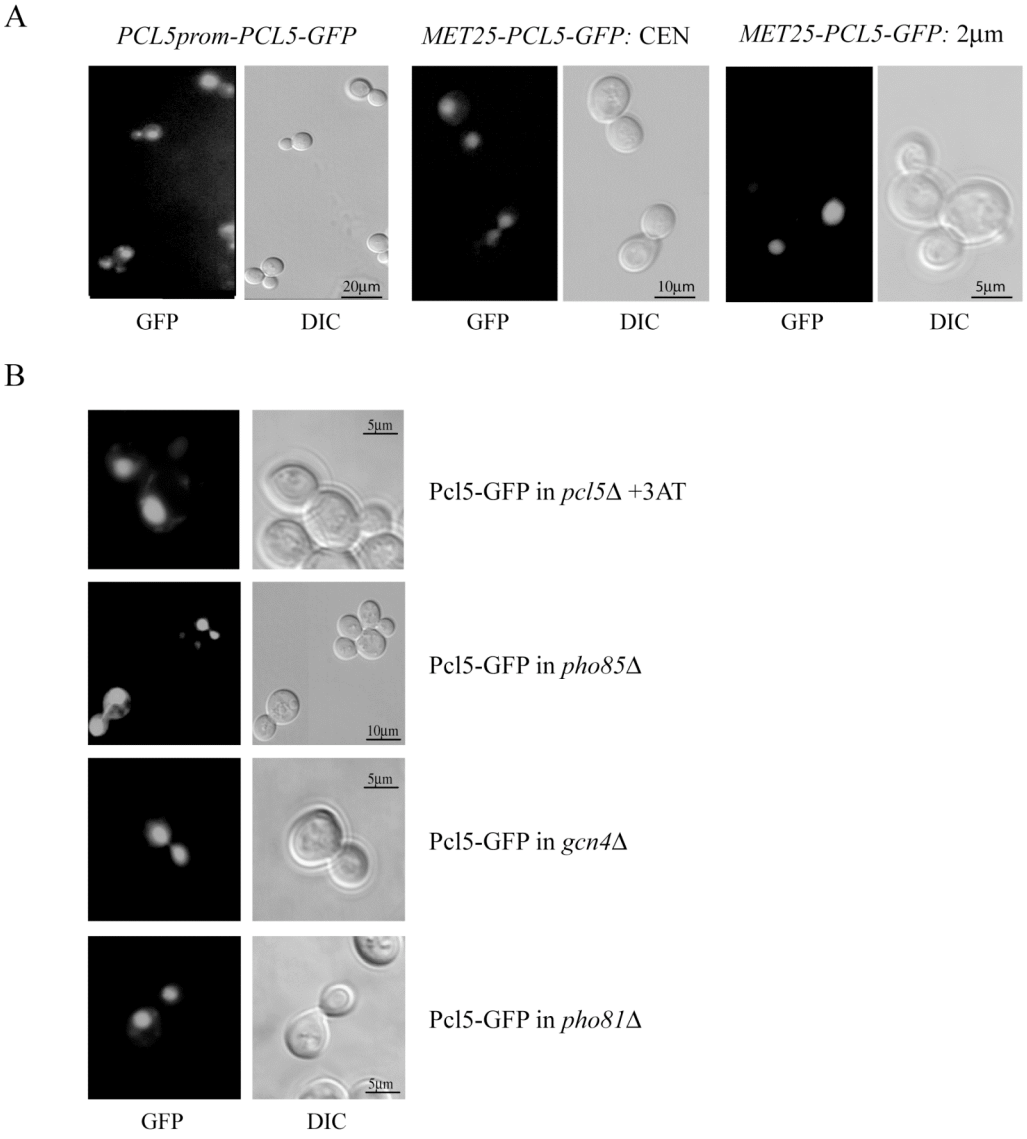


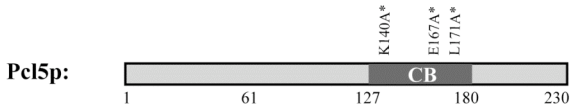
Figure 2. Pcl5-GFP is transported into the *S. cerevisiae* nucleus independently of the presence or absence of amino acids. **(A)** Nuclear localization of high and low amounts of the functional cyclin Pcl5-GFP. *S. cerevisiae* cells (RH3239) expressing *PCL5-GFP*-fusion derived from its endogenous *PCL5* promoter at the chromosomal locus or *pcl5* mutant cells (RH3238) expressing *PCL5-GFP*-fusions from the *MET25* promoter on a low-copy plasmid (pME2844) or on a high copy plasmid (pME2846) were grown to early log-phase at 30°C and analyzed by DIC microscopy (right) and fluorescence microscopy (left). **(B)** Nuclear localization of Pcl5-GFP in *pcl5* cells (RH3238) under amino acid starvation conditions (induced by the histidine analogue 3-amino-triazole (3AT)), *pho85* (RH3242), *gcn4* (RH1408), and *pho81* (RH3241) mutant strains. Yeast cells were grown to early log-phase at 30°C and analyzed by DIC microscopy (right) and fluorescence microscopy (left).

Yeast strains carrying the non-conditional importin mutations *kap114*, *kap123*, *nmd5*, *pdr6*, and *sxm1*, respectively were cultured and analyzed at a temperature of 30°C. Strains carrying

the temperature-sensitive mutations *kap95*, *mtr10*, *kap104*, *pse1*, and *pse1/kap123* were analyzed at the permissive temperature of 20°C and the restrictive temperature of 30°C.

Localization studies showed that Pcl5-GFP was similarly localized in the nucleus in the *kap114, kap123*, *nmd5*, *pdr6*, *sxm1*, *mtr10*, *kap104*, *pse1,* and *pse1/kap123* mutant strains as in wild-type cells. In contrast, a cytoplasmic accumulation of Pcl5-GFP was observed in the *kap95* mutant cells at their restrictive temperature of 30°C suggesting that Kap95p is necessary for nuclear import of yeast cyclin Pcl5p (Figure 5). These data further support that Kap95p interacts to a non-classical NLS, which is responsible for the constitutive nuclear localization of Pcl5p.

A



plasmid	Pcl5 amino acids	Localization	*pcl5* complementation	Gcn4p degradation
pME2846	1–229 GFP	N	+	+
pME2850	1–95 GFP	PM	-	-
pME2851	1–127 GFP	PM	-	-
pME2852	61–127 GFP	PM+dots	-	-
pME2853	1–180 GFP	N+dots	+	+
pME2854	61–229 GFP	N+dots	+	+
pME2855	111–180 GFP	C	-	-
pME2856	111–229 GFP	N	-	-
pME2857	153–229 GFP	N	-	-
pME2859	181–229 GFP	N	-	-
pME2950	204–218 GFP	N+C	-	-
pME2951	GFP-Aro7 207–215	N+C	-	-
pME2849	GFP	N+C	-	-
pME2858	61–180 GFP	dots	-	-
pME2860	1–73 79–178 170–294 GFP	N	+	+
pME2948	1–73 79–178 GFP	N+C	+	+
pME2949	79–178 170–294 GFP	dots	-	+
pME2966	1–229 (* at 140, * * at 167 171) GFP	N	+	-/-

B

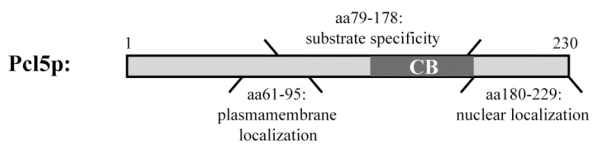


Figure 3. Domain analysis of yeast cyclin Pcl5p. **(A)** *Left half*, Pcl5p fragments generated by serial deletion fused to the N-terminus of GFP. pME2846 (Pcl5aa1-229), pME2850 (Pcl5aa1-95), pME2851 (Pcl5aa1-127), pME2852 (Pcl5aa61-127), pME2853 (Pcl5aa1-180), pME2854 (Pcl5aa61-229), pME2855 (Pcl5aa111-180), pME2856 (Pcl5aa111-229), pME2857 (Pcl5aa153-229), pME2859 (Pcl5aa181-229), pME2950 (Pcl5aa204-218), pME2849 (GFP alone), pME2858 (Pcl5aa61-180), pME2860 (Pho80aa1-73, Pcl5aa79-178, Pho80aa170-294), pME2948 (Pho80aa1-73, Pcl5aa79-178), pME2949 (Pcl5aa79-178, Pho80aa170-294), pME2966 (Pcl5aa1-229 containing the three amino acid substitutions K140A, E167A and L171A marked by asterisks). pME2951 expresses *GFP-ARO7-PCL5*$_{bp619-645}$ (encoding GFP-Aro7-Pcl5$_{aa207-215}$). The summary of the results is shown on the right. The columns indicate the sub-cellular localization of the different *PCL5* or *PCL5-PHO80* deletions (Figure 4), their ability to complement the *pcl5* phenotype of Gcn4p toxicity or to promote Gcn4p degradation (Figure 6). CB, cyclin box; N, nuclear; PM, plasma membrane; C, cytoplasm, + (smaller size) slow growing cells with hardly detectable Gcn4p, -/- slow growing cells with non-detectable Gcn4p. **(B)** Sheme of identified Pcl5p domains. The relative positions of the different domains within the full-length Pcl5p protein are shown. The amino acids 61-95 represent a putative plasma membrane binding site motif and the middle part consisting of the amino acids 79-178 is required for the right substrate specificity. The Pcl5p carboxyl terminus of amino acids 180-229 is sufficient for nuclear localization.

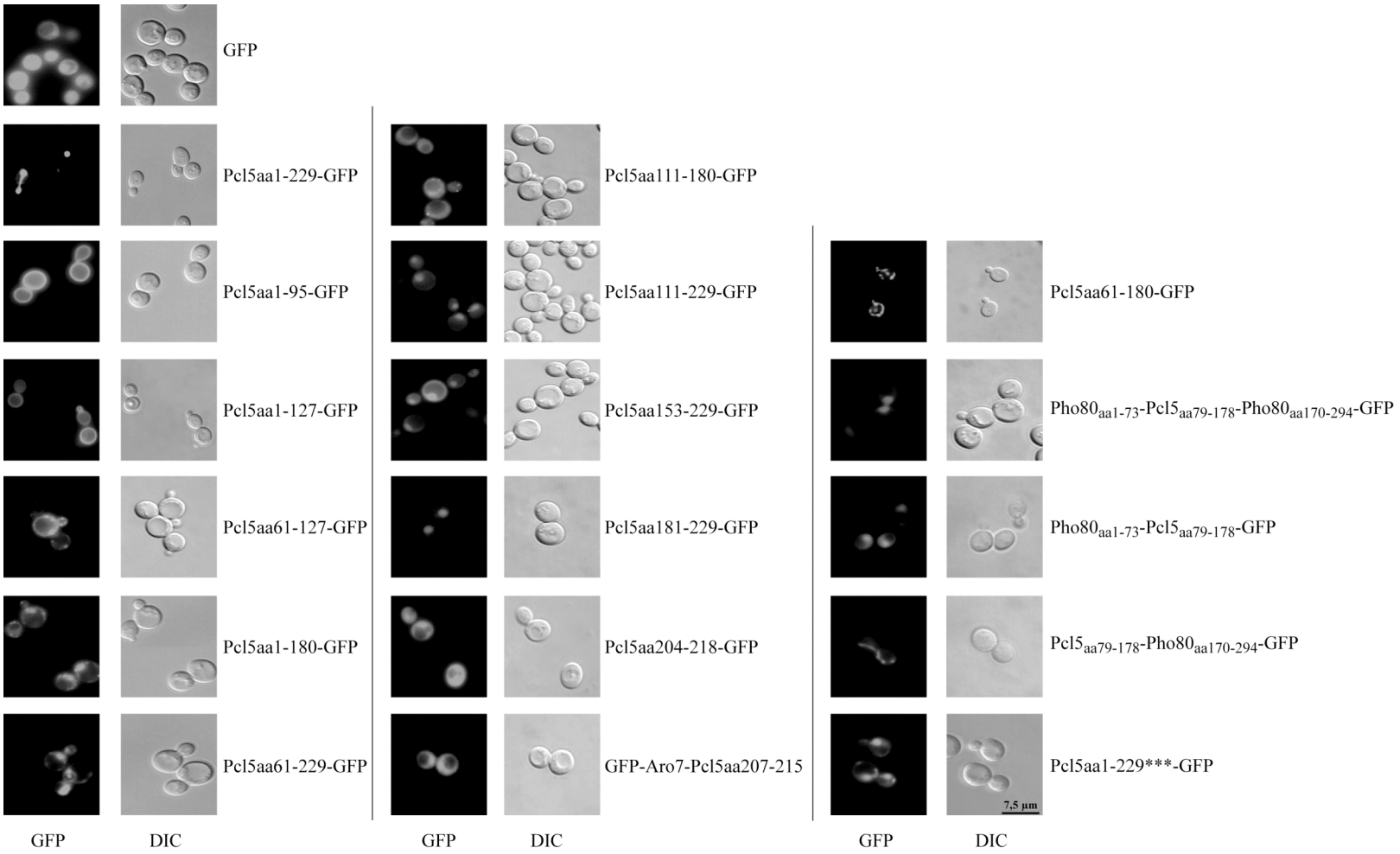


Figure 4. The C-terminal part of Pcl5p directs nuclear localization. Yeast *pcl5* mutant strain RH3238 was transformed to express either GFP alone or in N-terminal fusion with different Pcl5aa stretches, Pcl5-Pho80 hybrids or GFP-Aro7-Pcl5aa207-215 from the *MET25* promoter. GFP signals were analyzed by fluorescence microscopy (GFP) or differential interference contrast (DIC) microscopy.

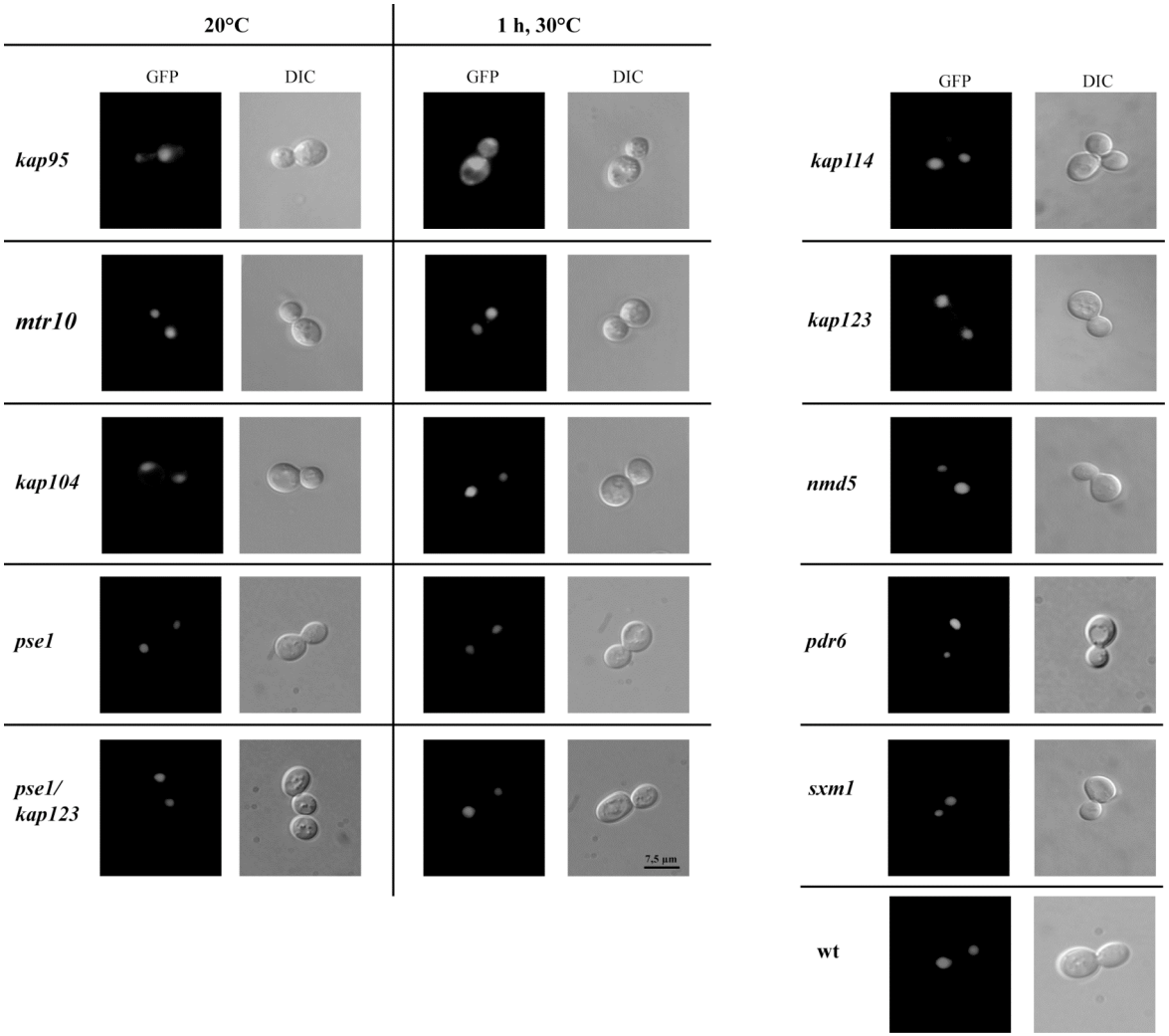


Figure 5. The mutation of the β-importin encoding *KAP95* prevent import of Pcl5p into the nucleus. Nuclear import of a functional Pcl5-GFP-fusion protein (pME2846) was analyzed in five temperature-sensitive importin mutant strains by fluorescence microscopy. Pcl5p translocation is not affected in the mutant strains *mtr10* (RH2701), *kap104* (RH2702), *pse1* (RH2703) or *pse1/kap123* (RH2706), whereas the *kap95* (RH2704) mutation impair uptake of Pcl5-GFP at the restrictive temperature of 30°C. Furthermore, five mutant strains with the non-conditional importin mutations *kap114* (RH3058), *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709), and *sxm1* (RH2710) were analyzed concerning the sub-cellular localization of Pcl5-GFP by fluorescence microscopy. Nuclear import of Pcl5p was unaffected in all five mutant strains and was indistinguishable from that in the wild-type control (RH3237).

A Pho80-Pcl5$_{aa79-178}$-Pho80p hybrid results in a Pho80p cyclin, which promotes Gcn4p specificity

We used the set of 13 truncated Pcl5-GFP versions described above to define the specificity domain for the Pho85p/Pcl5p complex, which allows promoting Gcn4p phosphorylation. Therefore we tested Pcl5p cyclin function of all Pcl5-GFP hybrids in the overexpression toxicity assay of Gcn4p described above. We found, that in the *pcl5* background the toxicity of *GCN4* moderately overexpressed from the *GAL1* promoter can be suppressed by concomitant highly amounts of Pcl5aa1-180 and 61-229, but not by that of the other truncated Pcl5-GFP hybrids (Figure 6A). The same results were observed with an induced general control by adding 10 mM 3AT to the medium. Pcl5aa 1-180 and 61-229 are able to suppress Gcn4p toxicity like FL-Pcl5p (data not shown). The functionality of the Pcl5aa1-180-GFP-fusion lacking the C-terminal part necessary for nuclear import argues for a second ancillary nuclear localization signal in the N-terminal part of Pcl5p. To analyze furthermore, whether the truncated Pcl5p versions are able to promote Gcn4p phosphorylation and degradation, we tested the effect of overexpressed *PCL5-GFP* deletions on Gcn4p degradation. Wild-type and *pcl5* mutant cells were transformed to express *GAL-myc^3-GCN4* and in addition the truncated Pcl5-GFP-fusions. Promoter-shut-off experiments of myc^3-Gcn4p revealed a rapid degradation of this protein when the additional Pcl5p amino acid stretches 1-180 or 61-229 are expressed (Figure 6B).

The central amino acid stretch of Pcl5p (aa61-180) containing the conserved cyclin box necessary for kinase interaction and cyclin activity showed no Pcl5p function by complementing the *pcl5* phenotype (Figure 6A). Furthermore, Gcn4p is strongly stabilized by simultaneous expression of the *PCL5* deletion encoding amino acids 61-180 (Figure 6B). We have shown that nuclear localization of Pcl5p is required for efficient Gcn4p degradation. This nuclear import is mediated by the 49 carboxy-terminal amino acids of the cyclin. We have substituted the N- and C-terminus of Pcl5p for that of Pho80p to create a Pho80-Pcl5-Pho80-GFP hybrid (Figure 3). Pho80p is another Pho85p kinase interacting cyclin, which, however, does not mediate Gcn4p degradation specificity (Shemer et al., 2002). This fusion protein is localized in the yeast nucleus in contrast to the Pcl5aa61-180-GFP hybrid (Figure 4). In addition, overexpression toxicity of Gcn4p is suppressed by concomitant overexpression of the Pho80-Pcl5-Pho80-hybrid (Figure 6A) and a shut-off-Western experiment showed, that this fusion protein is able to mediate Gcn4p degradation in a *pcl5* mutant strain (Figure 6B). These data, which show that a Pho80p-Pcl5p-Pho80p hybrid

protein, which includes amino acids 79-178 of Pcl5p, results in a Pho80-cyclin that promotes Gcn4p specificity, identify the central region of Pcl5p as the Gcn4p specificity region.

The substrate specificity and the nuclear localization signal of cyclins Pho80p and Pcl5p correspond to distinct domains

A fusion of either the Pho80 N- or C-terminus to the Pcl5p amino acid stretch 79-178 was constructed to clarify whether both Pho80 domains are necessary for full Pcl5p function. We made N-Pho80-Pcl5-GFP and Pcl5-Pho80-C-GFP hybrids to distinguish between these parts of Pho80p. Our results indicate, that only the Pho80-Pcl5-GFP-fusion carrying the Pho80 N-terminus is localized in the nucleus (Figure 4) and able to suppress the overexpression toxicity of Gcn4p (Figure 6A). Furthermore, Gcn4p is degraded by simultaneous expression of *PHO80-PCL5* encoding Pho80aa1-73-Pcl5aa79-178-GFP (Figure 6B). In contrast, the Pcl5-Pho80-GFP hybrid containing the Pho80 C-terminus is visualized via GFP as aggregates over the whole cell similar to the localization of the Pcl5p middle part alone (Figure 4). Furthermore, this fusion protein is not active by its ability to complement the *pcl5* phenotype (Figure 6A). Unexpectedly, we found, that in *pcl5* cells expressing $PCL5_{aa79\text{-}178}$-$PHO80_{aa170\text{-}294}$-*GFP* Gcn4p was hardly detectable, but seems to be degraded. In the corresponding wild-type cells no Gcn4p was found.

In summary, these data suggest that in contrast to Pcl5p, which depends on a C-terminal NLS, Pho80p is transported into the nucleus by a N-terminal NLS. Therefore, the substrate specificity and the nuclear localization signal of cyclins Pcl5p and Pho80p correspond to distinct domains, which can be exchanged.

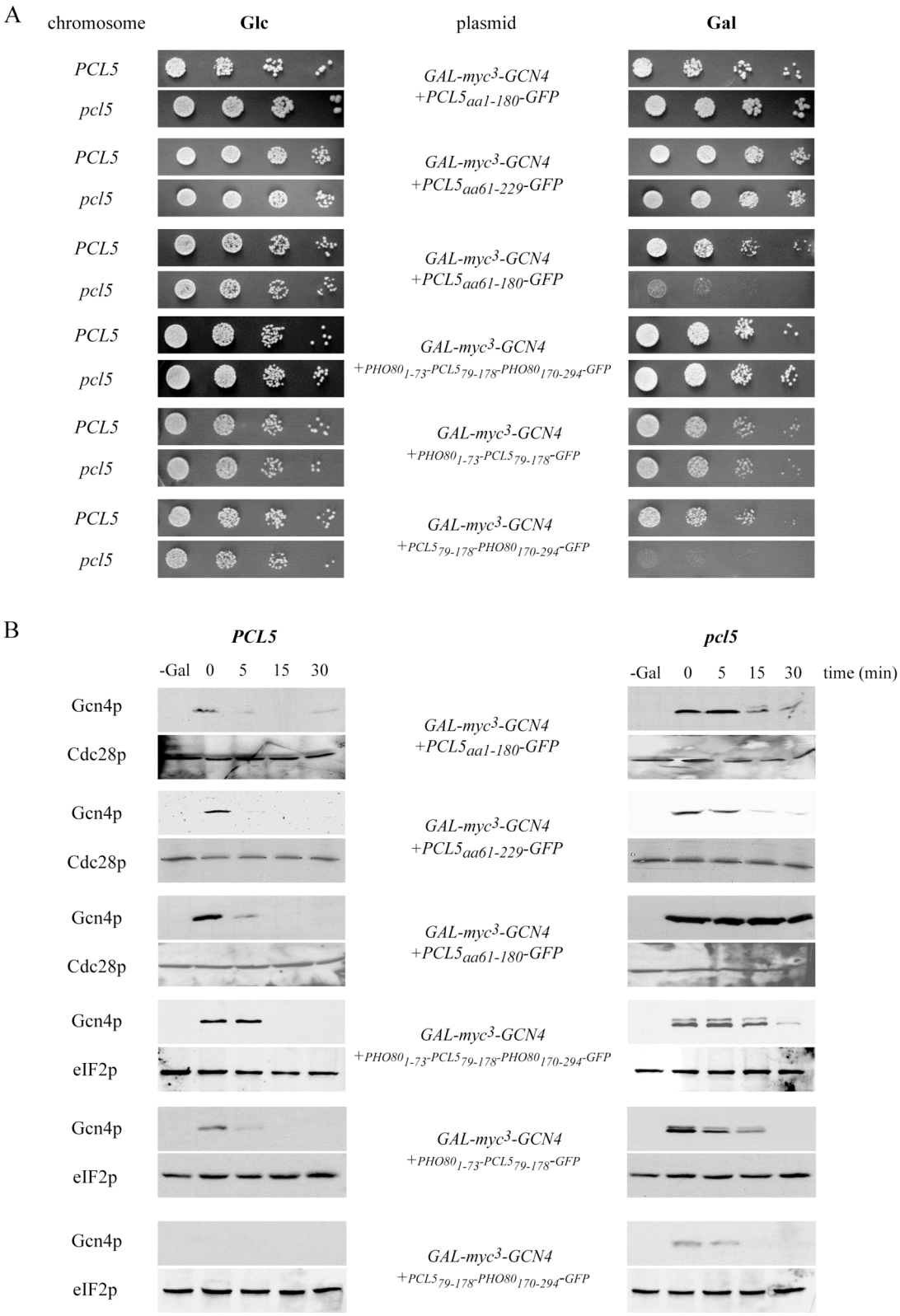


Figure 6. The Pcl5p amino acid domain 78-178 changes the substrate specificity of Pho80p in a chimera protein resulting in a Pho80p protein, which is specific to mediate Gcn4p degradation. **(A)** The Pho80-Pcl5aa79-178-Pho80 hybrid is able to suppress the overexpression toxicity of Gcn4p like full-length-Pcl5p in *pcl5* yeast cells. Wild-type cells (RH3237) and *pcl5* mutant cells (RH3238) expressing myc^3-*GCN4* from the *GAL1* promoter (pME2848) together with $PCL5_{aa1\text{-}180}$-*GFP* (pME2853), $PCL5_{aa61\text{-}229}$-*GFP* (pME2854),

PCL5$_{aa61\text{-}180}$*-GFP* (pME2858), *PHO80*$_{aa1\text{-}73}$*-PCL5*$_{aa79\text{-}178}$*-PHO80*$_{aa170\text{-}294}$*-GFP* (pME2860), *PHO80*$_{aa1\text{-}73}$*-PCL5*$_{aa79\text{-}178}$*-GFP* (pME2948) or *PCL5*$_{aa79\text{-}178}$*-PHO80*$_{aa170\text{-}294}$*-GFP* (pME2949) were spotted in five-fold dilutions on glucose and galactose to induce expression of *GCN4* driven by the *GAL1* promoter. The plates were incubated for 2 days at 30°C. **(B)** The Pho80-Pcl5aa79-178-Pho80 hybrid is able to promote Gcn4p degradation like full-length-Pcl5p in *pcl5* yeast cells. The same transformed yeast cells as described in (A) were pre-grown to early log-phase in selective medium containing glucose as carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express *myc*^{3}*-GCN4*. After 3 h of induction the *GAL1* promoter was turned off by adding 2% glucose to the medium (0-min time point). Samples were analyzed at the indicated time points after the shift to glucose medium. Levels of myc^{3} tagged Gcn4p were determined by immunoblotting using myc-antibodies. Cdc28p and eIF2p were used as loading control.

Discussion

The cyclin Pcl5p represents the Pho85p cyclin specificity factor for the initiation of Gcn4p degradation (Shemer et al., 2002). Pcl5p is a very unstable protein with a half-life of only 2,5 minutes, both under normal growth conditions and in starved cells, and was therefore proposed to constitute a sensor of cellular biosynthetic capacity (Shemer et al., 2002). In this study we could analyze this unstable cyclin Pcl5p with the help of a stable GFP-Tag, which did not impair Pcl5p function.

The substrate specificity of Pcl5p resides in the central domain of the protein (Pcl5aa 79-178). This distinct specificity region resembles an adaptor necessary for recognition of Gcn4p by the Pho85p/Pcl5p complex. Furthermore, it was possible to create a Pho80p-like cyclin containing the specificity domain of Pcl5p that confers its substrate specificity on Gcn4p. The cyclin Pho80p has previously been identified as being specific for Pho85p-dependent phosphorylation of Pho4p, the transcription factor of the phosphate metabolism (O'Neill et al., 1996). In addition, Pho80p is not able to inhibit Gcn4p function *in vivo* and therefore to initiate Gcn4p degradation (Shemer et al., 2002). Thus in our study, the Pho4p-specific cyclin Pho80p is converted into a Pcl5p like Gcn4p adapter. The question occurs, what are the reasons for the different cyclin specificities. So far, the molecular interactions required for the direct binding between Pcl5p to Pho85p are hardly known. Amino acid substitutions of three Pcl5p residues (K140, E167 and L171), which were predicted to contact Pho85p, have no effect on Pcl5p nuclear localization or functionality and therefore, do not alter the Pcl5p specificity on Gcn4p (Figure 3). In contrast, it was previously reported that the corresponding lysine and glutamic acid of yeast G1 cyclins Cln2p and Cln3p are required for Cdc28p binding and associated kinase activity (Levine et al., 1996; Miller et al., 2005). Specific interactions between cyclin-CDK complexes and cellular proteins including inhibitors provide an additional possible mechanism for cyclin-dependent functional specificity of cyclin-CDK activity. By analogy with the co-crystal structure of cyclinA-cdk2 (Jeffrey et al., 1995), α helices 3 and 5 of cyclinA interact with the PSTAIRE helix of the CDK (Jeffrey et al., 1995). The three analyzed Pcl5p residues are also located in α helix 3 and in α helix 5 (Lee et al., 2000). The CKI Pho81p is involved in stabilization of Gcn4p in response to amino acid starvation and able to interact with Pcl5p when Gcn4p is rapidly degraded (Bömeke et al., 2006). So far it is unclear, whether the Pcl5p/Pho81p binding is a prerequisite for the interaction of the cyclin with Pho85p. The two residues R121 and E154 of cyclin Pho80p were identified to be required for Pho81p binding (Huang et al., 2001). These residues are

conserved among the Pcl cyclin family (Pcl5aa R121 and D162) and also located in the helices 3 and 5 of the cyclin box (Bazan, 1996; Huang et al., 2001). The binding between Pho81p and Pcl5p might be the first step for a strong binding and the correct assembly between Pho85p and Pcl5p. In a possible model Pho81p binds the region of R129 and D162 of Pcl5p helices 3 and 5 followed by interaction of Pho85p with Pcl5p residues K140, E167 and L171 in the same regions. Therefore, substitutions of the three analyzed Pcl5p amino acids might result in a loosening but not in a disruption of the Pho85p/Pcl5p interaction. In addition, the stability of cyclins may contribute to cyclin functional specificity. So far, there is not much known about the degradation pathway of the highly unstable cyclin Pcl5p. The half-life of this protein is determined as 2,5 minutes, whereas amino acid starvation does not influence its stability (Shemer et al., 2002). We have shown before, that the cyclin Pcl5p is able to interact with different proteins such as the kinase Pho85p and the inhibitor Pho81p, whereas both complexes dissociate in response to amino acid starvation (Bömeke et al., 2006). One possibility is that there are two or more populations of Pcl5p with different half-lives including a free, unbound form and a cyclin form associated to another protein. This might reflect that the turnover rate of Pcl5p is dependent on the assembly and disassembly of Pcl5p/protein complexes.

The distinct Pcl5p specificity domain can be combined with a N-terminal or a C-terminal NLS motif. In case of cyclin Pho80p only the N-terminus fused to the specificity domain of Pcl5p allows its nuclear localization. In contrast, Pcl5p is transported into the nucleus by a C-terminal NLS. The amino-terminal portion of Pcl5p is localized to the plasma membrane, whereas a Pcl5p deletion lacking the C-terminus, which includes a NLS, is also detected in the nucleus. Therefore the question occurs, whether there is an additional role of the amino terminal part of Pcl5p in forcing the protein into the nucleus? The N-terminal Pcl5aa 1-59 contain 8 R/K residues (in particular the RKK stretch) that resemble an ancillary non-classical nuclear localization motif. They might act in concert with the C-terminal part of the full-length protein to ensure nuclear localization. This would be similar to the substrate Gcn4p that contains two NLS motifs, from which NLS2 is the essential and specific transport signal in contrast to NLS1 playing only an auxiliary role in nuclear import (Pries et al., 2004). These data suggest that Pcl5p has multiple nuclear trafficking pathways. Possible, these pathways utilize sequences throughout the protein and the different pathways vary in their efficiency to target Pcl5p to the nucleus. The plasma membrane localization of the Pcl5p-N-terminus might reflect additional yet unknown activities of this cyclin.

In *pcl5* mutant cells expressing the Pcl5p specificity domain fused to the C-terminus of Pho80p Gcn4p is hardly detectable and seems to be efficiently degraded. In contrast, this Pcl5-Pho80-hybrid is not able to complement the *pcl5* growth phenotype and is not localized in the nucleus, the place of Pcl5p function. Since Pcl5p is required for rapid Gcn4p turnover (Shemer *et al.*, 2002), these results point to the existence of an additional Pho85p/Pcl5p-independent Gcn4p degradation. One possible explanation is that the Pcl5-Pho80-hybrid may exhibit a new additional function in the cytoplasm including binding to a protein that is normally required for stabilization of Gcn4p.

The carboxyl terminus of the protein localized to the nucleus with equivalent efficiency of the full-length protein. This Pcl5p part contains the motif PV**KR**P**R**ESD of the amino acids 207-215, that is similar to the c-myc NLS (PAA**KR**V**K**LD). This Pcl5p motif alone is not sufficient for directing the normally cytoplasmic protein Aro7p into the nucleus. However, it is not always the case that nuclear import is exclusively determined by the presence or absence of an NLS. A functional NLS of a nuclear protein might not always be sufficient to direct nuclear import of a normally non-nuclear protein. The lymphoid specific factor RAG2 is an example. This protein is essential for the diversication of antigen receptors on B and T lymphocytes and contains an analog motif (PA**KK**SFL**RR**LFD) that is necessary but not sufficient for nuclear import. A 37-aa minimal region containing this motif is necessary for nuclear targeting of RAG2 (Corneo et al., 2002). In case of Pcl5p the 49 C-terminal amino acids 181-229 are sufficient for directing GFP into the nucleus and therefore represent a non-classical NLS.

Nuclear import of Pcl5p is mediated through the action of the β-importin Kap95p as single karyopherin. Kap95p was besides the α-importin Srp1p also identified to be specifically required for nuclear import of the transcription factor Gcn4p (Pries et al., 2004). This might reflect similarities of the Pcl5p/Kap95p and Gcn4p/Kap95p folding. However, Pcl5p is being targeted into the nucleus in a Gcn4p-independent manner, because of a constitutive Pcl5p nuclear localization in the corresponding *gcn4* mutant strain. Opposite to this, a Pcl5p mislocalization into the cytoplasm results in a constitutively stabilized Gcn4p corroborating that nuclear localized Pcl5p is a prerequisite for initiating the Gcn4p degradation pathway. So far, there is no hint that mislocalization of Pcl5p into the cytoplasm result in an alteration of the function spectrum of Pcl5p as it was shown before in case of the yeast G1 cyclins Cln2p and Cln3p. If normally nuclear Cln3p is mislocalized in the cytoplasm, the place of Cln2p, this Cln3p mutant is able to assume Cln2p-like functions (Miller and Cross, 2000).

In summary, we show here that it was possible *in vitro* to transform the substrate specificity from one Pho85p cyclin to another by combining their domains. These findings implicate that it might be possible to change the activity of Cdk5, the mammalian homolog of Pho85p. Cdk5 is assumed to promote neurodegenerative processes as in e.g. Parkinson's disease (Smith et al., 2003) and down-regulation of this activity might have an important impact for patients.

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CHAPTER IV

Stabilized Gcn4p correlates to a decreased transcriptional activity in response to amino acid starvation in *Saccharomyces cerevisiae*

Abstract

The *JUN* homolog *GCN4* encodes the *Saccharomyces cerevisiae* key regulator of the 'general amino acid control', a genetic network that secures the appropriate response to starvation of amino acids. Besides this metabolic function Gcn4p is required for the amino acid starvation-induced adhesive growth phenotype that depends also on the expression of the cell-surface flocculin encoding gene *FLO11*. We report that one amino acid substitution of Gcn4p results in a separation of metabolic and developmental functions. Thus, mutational analysis of *GCN4* revealed that amino acid-dependent stability and transcriptional activity of Gcn4p are affected by the amino acid substitution Leu267Ser. Cells expressing the $GCN4^{LEU267SER}$ mutant allele are also impaired in *FLO11* expression and therefore adhesion upon amino acid limitation. Deletion of the Pho85p cyclin encoding gene *PCL5* that is specifically required for degradation of Gcn4p results in a stabilized transcription factor with an affected activity in response to amino acid starvation. Pcl5p is also required for the transcription of *FLO11* upon amino acid limitation and therefore a *PCL5* deletion causes an adhesion deficient growth phenotype for amino acid starved yeast cells. In summary, cells expressing the $GCN4^{LEU267SER}$ mutant allele as well as cells impaired in *PCL5* show a similar phenotype including a stabilized Gcn4p, which is less active in response to amino acid starvation. We therefore conclude that degradation and turnover of Gcn4p is a prerequisite for full transcriptional activity.

Introduction

A couple of fungal cells have the ability to adhere to human tissues or plastic prostheses to gain access to the bloodstream and internal organs of patients (Kojic and Darouiche, 2004). Adhesion-dependent fungal biofilms on prostheses and catheters are highly resistant against antifungal drugs and therefore a major clinical problem, especially in immuno-compromised patients (Kojic and Darouiche, 2004).

The budding yeast *S. cerevisiae* is a non-pathogenic fungus that is able for cell-cell and cell-surface adhesion in response to different environmental stimuli. The prerequisite for adhesion in *S. cerevisiae* is the expression of the cell-surface flocculin *FLO11*, which is required for haploid invasive growth (Guo et al., 2000; Roberts and Fink, 1994), diploid pseudohyphal development (Gimeno et al., 1992; Mösch and Fink, 1997) and biofilm formation (Reynolds and Fink, 2001).

In *S. cerevisiae* five adhesin *FLO* genes are known, from which four (*FLO1*, *FLO5*, *FLO9* and *FLO10*) are transcriptionally silenced (Halme et al., 2004; Verstrepen et al., 2004). Only *FLO11* is expressed in *S. cerevisiae* laboratory strains, whereas in diploid cells growing in the yeast form *FLO11* expression is almost silenced resulting in a hardly detectable mRNA (Braus et al., 2003; Gimeno et al., 1992; Fischer et al., 2006). The switch from non-adherent to adherent yeast cells, and therefore, the level of *FLO11* gene expression depend on nutrient supply and allow the cells to adapt to stress (Braus et al., 2003; Gimeno et al., 1992; Lambrechts et al., 1996). In diploid cells, nitrogen starvation causes activation of *FLO11* and subsequently leads to pseudohyphal growth to penetrate substrates in an attempt to forage for new nutrients (Gagiano et al., 2002; Liu et al., 1993; Lo and Dranginis, 1998; Robertson and Fink, 1998). In contrast, glucose starvation leads to an activation of *FLO11* expression in haploid strains of *S. cerevisiae* and therefore to invasive growth and biofilm formation (Cullen and Sprague, 2000; Reynolds and Fink, 2001).

Beside nitrogen or glucose starvation, also amino acid limitation is a nutritional signal that activates *FLO11* expression and therefore haploid adhesive growth and diploid pseudohyphal development (Braus et al., 2003). This occurs even in the presence of the adhesion suppressors nitrogen or glucose and requires besides Flo8p and Tpk2p the two proteins Gcn2p and Gcn4p, elements of a genetic network called the ‘General Amino Acid Control’ (GAAC) for activation of *FLO11* expression (Braus et al., 2003).

Besides these developmental processes, Gcn4p is required for several metabolic responses. Gcn4p is the key regulator of the general control system and therefore required for

transcriptional regulation of amino acid and purine biosynthetic genes (Hinnebusch, 1992). This regulatory network is induced by several environmental stimuli or stress circumstances including amino acid starvation, glucose limitation, UV radiation or high salinity (Hinnebusch, 1992; Yang et al., 2000; Engelberg et al., 1994; Goossens et al., 2001) and conserved from yeast to man. Activity of the *GCN4* gene product is regulated via rapid protein synthesis and degradation processes. Starvation for amino acids leads to an increase in *GCN4* mRNA translation that is mediated by phosphorylation of the general translation initiation factor eIF2 α by the kinase Gcn2p (Dever et al., 1992; Hinnebusch, 1984). Gcn4p activates its target genes by direct binding to specific Gcn4p-response elements in their promoter regions (Hope and Struhl, 1986; Oliphant et al., 1989). In addition, amino acid starvation induces the stabilization of the highly unstable Gcn4 protein (Kornitzer et al., 1994). Gcn4p degradation is initiated by the two cyclin-dependent kinases Pho85p and Srb10p, whereas Srb10p phosphorylation of Gcn4p occurs independently of the availability of amino acids. The kinase cyclin complex Pho85p/Pcl5p phosphorylates Gcn4p at the residue Thr165 in amino acid sated cells (Shemer et al., 2002), but dissociates in response to amino acid starvation leading to a stabilized Gcn4p (Bömeke et al., 2006). After phosphorylation, Gcn4p gets poly-ubiquitinated by the E2 ubiquitin-conjugating enzyme Cdc34 together with the E3 SCF^{CDC4} RING ubiquitin ligase and finally Gcn4p is degraded in the 26S proteasom (Kornitzer et al., 1994; Meimoun et al., 2000). Recently, it has been reported, that mutations in SCF^{CDC4} or inhibition of the proteasome decreased transcription of Gcn4p targets. Therefore, turnover of Gcn4p might stimulate its gene expression activity (Lipford et al., 2005).

The protein Pcl5p is one of ten different cyclins able to interact with the kinase Pho85p (Measday et al., 1997). Pcl5p has been specifically identified to be required for phoshorylation and degradation of the transcription factor Gcn4p in the W303 genetic background and therefore a *PCL5* deletion resulted in a strong stabilization of Gcn4p (Shemer et al., 2002). According to their sequence homology and functional relationship the ten Pho85p cyclins have been divided into the Pho80 and Pcl1,2 sub families (Measday et al., 1997). The Pho80 family consists of Pho80p, Pcl6p, Pcl7p, Pcl8p and Pcl10p and is functionally involved in distinct metabolic pathways (Andrews and Measday, 1998). Members of the Pcl1,2 subfamily such as Pcl1p, Pcl2p, Pcl5p, Pcl9p and Clg1p participate in association with Pho85p in cell cycle control (Measday et al., 1997; Tennyson et al., 1998) as well as in the regulation of cell wall maintenance (Andrews and Measday, 1998).

Gcn4p displays a regulator of metabolism and development, two processes that are directly connected in various multicellular organisms. The Gcn4p like ATF4 of mammalian cells is the central regulator of the general control system and also involved in processes like learning and long-term memory (Costa-Mattioli et al., 2005; Hao et al., 2005). In the filamentous fungus *Aspergillus nidulans* CPCA is the key player of this regulatory network known as cross-pathway-control (CPC) (Sachs, 1986) that can induce an arrest of cleistothecia formation in response to amino acid starvation, indicating a connection of metabolism and sexual development in filamentous fungi (Hoffmann et al., 2000). The *GCN4* homolog *cpcA* gene encodes CpcA of *Aspergillus fumigatus* that plays an important role in the virulence of this opportunist fungal pathogen. The fungal cross pathway control of amino acid biosynthesis has been shown to function as a general stress response system (Krappmann et al., 2004).

The dimorphic human pathogen *Candida albicans* is able to switch from a unicellular yeast form to a multicellular hyphal form, which causes disseminated systemic infection in immuno-compromised individuals. This infective hyphal form is also induced by different environmental stimuli, like carbon or nitrogen starvation or elevated temperatures (Ernst, 2000). Furthermore, it has been shown recently that a *C. albicans* mutant deleted for Ca*CDC4* shows constitutive filamentous growth, suggesting that ubiquitin-mediated protein degradation is involved in the dimorphic switch (Atir-Lande et al., 2005). In addition, homologous genes for Gcn4p and Pcl5p were identified in *C. albicans* which are necessary for promoting amino acids and for inducing of filamentous growth in response to amino acid starvation (Gildor et al., 2005; Tripathi et al., 2002).

However, it has not been reported so far whether the regulation function of Gcn4p in developmental and metabolic processes could be separated in *S. cerevisiae*. For the first time we present evidence that only the Gcn4p amino acid substitution Leu267Ser leads to an adhesion deficient growth phenotype and a decreased *FLO11* expression, but an intact response of the general control in amino acid starved cells. This reduced adhesion was confirmed in a *pcl5* mutant strain, where degradation of Gcn4p is decreased. Gcn4Leu267Ser and wild-type Gcn4p of *pcl5* mutant cells display a strongly stabilized protein with a decreased transcriptional activity suggesting that turnover of Gcn4p might stimulate its full activity.

Materials and Methods

S. cerevisiae strains and growth conditions

All yeast strains used in this study are listed in Table I. They are congenic to *S. cerevisiae* Σ1278b genetic background. Standard methods for genetic crosses and transformation were used as described (Guthrie and Fink, 1991).

The genetic background of the *S. cerevisiae* W303 laboratory strain differs from that of wild-type strains like Σ1278b and its derivatives. Like cells of the S288c background cells with the W303 background have lost the ability of wild-type cells for haploid adhesive growth and diploid pseudohyphal development (Liu et al., 1993). Therefore, the W303 background is not appropriate for the study of adhesion or differentiation in yeast in contrast to Σ1278b cells that adhere on plastic surfaces after amino acid starvation conditions (Kleinschmidt et al., 2005).

Yeast strains RH3279 and RH3280 (*pcl5Δ::SpHIS5*) were constructed by PCR-mediated gene replacement (Goldstein and McCusker, 1999). Primers were designed specific for amplification of *pcl5Δ::SpHIS5* with DNA of plasmid pUG27 containing the *his5*$^{+}$ gene of *Schizosaccharomyces pombe* that complements the *S. cerevisiae his3* mutation (Goldstein and McCusker, 1999). The PCR product was directly transformed into strains RH2585 and RH2586 and plated onto minimal medium without histidin. Transformants were replica-plated onto the same medium and deletions were confirmed by PCR. The haploid strains were crossed to produce the diploid *pcl5* mutant strain RH3281.

Yeast strains RH3277 and RH3282 were obtained by introducing the *FLO11-lacZ-URA*-cassette using *Apa*I linearized plasmid pME2213 (Table 2) into the *URA3* locus of yeast strains RH2817 and RH3279. The haploid *FLO11-lacZ* containing strains RH3277 and RH3282 were crossed with strain RH2818 or RH3280 to produce the diploid strain RH3278 and the *pcl5* mutant strain RH3303. Yeast strain RH3304 was obtained by introducing the *6GCRE-lacZ-URA*-cassette using *Stu*I linearized plasmid pME1112 (Table 2) into the *URA3* locus of yeast strain RH3279. The haploid *GCRE-lacZ* containing strain RH3304 was crossed with RH3280 strain to produce the diploid *pcl5* mutant strain RH3305.

The strains were grown in standard yeast extract-peptone-dextrose (YPD: 1% yeast extract, 2% peptone, 2% dextrose) and minimal yeast nitrogen base media (YNB: 1,5 g/l yeast nitrogen base lacking amino acids and ammonium sulfate, 4,5 g/l ammonium sulfate, 2% dextrose or galactose and supplemented with the appropriate amino acids).

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
RH2656	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1*	(Braus et al., 2003)
RH2817	*MATα, ura3-52, trp1::hisG, his3::hisG::HIS3*	(Fischer et al., 2006)
RH3277	*MATα, ura3-52::FLO11-lacZ-URA3, trp1::hisG, his3::hisG::HIS3*	this work
RH2818	*MATa, ura3-52, leu2::hisG, his3::hisG::HIS3*	(Fischer et al., 2006)
RH3278	*MATa/α, ura3-52/ura3-52::FLO11-lacZ-URA3, TRP1/ trp1::hisG, leu2::hisG/LEU2, his3::hisG::HIS3/his3::hisG::HIS3*	this work
RH2305	*MATa/α, ura3-52/ura3-52::GCRE-lacZ-URA3, trp1::hisG/trp1::hisG, his3::hisG/HIS3*	Grundmann, pers. comm.
RH2676	*MATa, ura3-52, trp1::hisG, leu2::hisG, gcn4::LEU2*	Grundmann, pers. comm.
RH2693	*MATα, ura3-52::FLO11-lacZ-URA3, trp1::hisG, leu2::hisG gcn4::LEU2*	Grundmann, pers. comm.
RH2694	*MATa/α, ura3-52/ura3-52, trp1::hisG/trp1::hisG, leu2::hisG/leu2::hisG, gcn4::LEU2/gcn4::LEU2*	Grundmann, pers. comm.
RH2695	*MATa/α, ura3-52/ura3-52::FLO11-lacZ-URA3, trp1::hisG/trp1::hisG, leu2::hisG/leu2::hisG, gcn4::LEU2/gcn4::LEU2*	Grundmann, pers. comm.
RH2398	*MATa/α, ura3-52/ura3-52::GCRE-lacZ-URA3, trp1::hisG/trp1::hisG, leu2::hisG/leu2::hisG, gcn4::LEU2/gcn4::LEU2, his3::hisG/HIS3*	Grundmann, pers. comm.
RH2585	*MATα, ura3-52, trp1::hisG, his3::hisG*	our collection
RH2586	*MATa, ura3-52, leu2::hisG, his3::hisG*	our collection
RH3279	*MATα, ura3-52, trp1::hisG, his3::hisG, pcl5::HIS5*	this work
RH3280	*MATa, ura3-52, leu2::hisG, his3::hisG, pcl5::HIS5*	this work
RH3281	*MATa/α, ura3-52/ura3-52, TRP1/ trp1::hisG, leu2::hisG/LEU2, his3::hisG/his3::hisG, pcl5::HIS5/pcl5::HIS5*	this work
RH3282	*MATα, ura3-52::FLO11-lacZ-URA3, trp1::hisG, his3::hisG, pcl5::HIS5*	this work
RH3304	*MATα, ura3-52::GCRE-lacZ-URA3, trp1::hisG, his3::hisG, pcl5::HIS5*	this work
RH3303	*MATa/α, ura3-52/ura3-52::FLO11-lacZ-URA3, TRP1/ trp1::hisG, leu2::hisG/LEU2, his3::hisG/his3::hisG, pcl5::HIS5/pcl5::HIS5*	this work

RH3305	*MATa/α, ura3-52/ura3-52::GCRE-lacZ-URA3, TRP1/ trp1::hisG, leu2::hisG/LEU2, his3::hisG/his3::hisG, pcl5::HIS5/pcl5::HIS5*	this work

Plasmid constructions

All plasmids used in this study are listed in Table 2. Construction of plasmids KB294 and pME2126 are described in Pries et al., 2002. pME2846 expressing *PCL5-GFP* was constructed by amplifying the *PCL5*-ORF with *Pfu*-Polymerase and cloning it as *Sma*I/*Hin*dIII-fragment into p426MET25. Afterwards a 750 bp *Bgl*II-fragment encoding the GFPuv-variant of GFP that was amplified from plasmid pBAD-GFP (Clontech, Heidelberg, Germany) was inserted behind the *PCL5*-ORF. Plasmid pME2848, expressing a triple myc epitope-tagged version of *GCN4* under the control of the *GAL1* promoter, was obtained by amplifying *GCN4* with *Pfu*-Polymerase and inserting them in p415GAL1 as blunt/*Hin*dIII-fragment. A 120 bp *Bam*HI-fragment carrying the triple myc epitope was inserted into a *Bgl*II restriction site after the fifth amino acid of Gcn4p.

Plasmid pME2911 expressing *GFPuv-GCN4*^{LEU267SER} from the *MET25* promoter was obtained by amplifying the *GCN4*^{LEU267SER} with *Pfu*-Polymerase from plasmid pME2901 and introducing it as *Nhe*I/*Cla*I-fragment into *Spe*I/*Cla*I restricted p424MET25. In front of the *GCN4* coding region the *GFPuv*-ORF from plasmid pBAD-GFP was introduced as *Bgl*II-fragment into the *Bgl*II restricted plasmid.

Plasmid pME2901, expressing *GCN4*^{LEU267SER} under the control of the *GCN4* promoter, was obtained by site-directed mutagenesis (for details see text: Library of *GCN4* mutants).

Plasmids pME2923 and pME2925, expressing a three-fold epitope-tagged version of *GCN4* or *GCN4*^{LEU267SER} under the control of the *GAL1* promoter, were obtained by amplifying *GCN4* or *GCN4*^{LEU267SER} with *Pfu*-Polymerase from plasmids pME2898 or pME2899 and inserting them as *Nhe*I/*Cla*I-fragment into *Spe*I/*Cla*I restricted p424GAL1. A 120 bp *Bgl*II-fragment carrying the three-fold myc epitope was introduced into a *Bgl*II restriction site in front of the third amino acid of Gcn4p or Gcn4Leu267Ser. *GCN4* or *GCN4*^{LEU267SER} were amplified from these plasmids and ligated as *Spe*I/*Cla*I-fragments into *Spe*I/*Cla*I restricted plasmid p426GAL1.

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pBKSII®	2.96 kb vector, *Amp*^{R} (*bla*), *lacZ*, *ori*	Stratagene (La Jolla, USA)
pRS314	*TRP, CEN, Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pRS415	*LEU, CEN, Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pRS426	*URA3*, *2µm*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
pRS424	*TRP; 2µm*, *Amp*^{R} (*bla*), *ori*	(Sikorski and Hieter, 1989)
p415GAL1	pRS415 containing *GAL1* promoter , *CYC1* terminator	(Mumberg et al., 1994)
p426GAL1	pRS426 containing *GAL1* promoter , *CYC1* terminator	(Mumberg et al., 1994)
p424GAL1	pRS424 containing *GAL1* promoter, *CYC1* terminator	(Mumberg et al., 1994)
p424MET25	pRS424 containing *MET25* promoter, *CYC1* terminator	(Mumberg et al., 1994)
p426MET25	pRS426 containing *MET25* promoter, *CYC1* terminator	(Mumberg et al., 1994)
pME1092	2,8 kb *GCN4*-fragment in pRS314	our collection
pME2126	*MET25prom-GFP-GCN4*-fusion in p426MET25	(Pries et al., 2004)
pUG27	*HIS5*-cassette from *S. pombe*	(Goldstein and McCusker, 1999)
pME2898	*GCN4wt* in pBKSII	this study
pME2899	*GCN4*^{Leu267Ser} in pBKSII	this study
pME2214	pME1092 with new restriction sites	this study
pME2901	*GCN4prom-GCN4*^{LEU267SER}*-GCN4term* in pRS314	this study
pME2911	*MET25prom-GFP-GCN4*^{LEU267SER}-fusion in p424MET25	this study
pME2923	*GAL1prom-myc*^{3}*-GCN4*-fusion in p426GAL1	this study
pME2925	*GAL1prom-myc*^{3}*-GCN4*^{LEU267SER}-fusion in p426GAL1	this study
pME2848	*GAL1prom-myc*^{3}*-GCN4*-fusion in p415GAL1	this study
pME2846	*MET25prom-PCL5-GFP*-fusion in p426MET25	this study
KB294	*GAL1-10prom-myc*^{3}*-GCN4*-fusion in *URA3*-marked *2µm* vector	(Pries et al., 2002)
pME2213	*FLO11::lacZ-URA*-cassette	our collection
pME1112	*6GCRE-lacZ-URA*-cassette	our collection

RNA-Isolation

Total RNAs from yeast were isolated following the protocol described by Cross and Tinkelenberg (Cross and Tinkelenberg, 1991). RNAs were separated on 1,4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes by electroblotting. Transcripts of *FLO11* and *ACT1* were detected by using specific ^{32}P-radiolabeled DNA-fragments with the Prime-It labelling kit from Stratagene. Hybridizing signals were visualized and quantified using a BAS-1500 phosphorimaging scanner (Fuji, Tokyo, Japan).

Protein analysis

Shut-off-Western procedure. Yeast cells were pre-grown in selective minimal medium with glucose as the carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express *myc*^{3}*-GCN4* from the *GAL1*-promoter. After 3 h, the cells were collected via centrifugation and half of these *trp*-deficient cells were starved for tryptophan by shifting them to minimal medium lacking tryptophan. 2% glucose was added to shut off the promoter after half an hour of tryptophan starvation. Samples were analyzed at the indicated time points after promoter-shut-off (0-min time point). Gcn4p protein bands were quantified using the KODAK 1D Image Analysis Software.

Whole-cell extracts of S. cerevisiae. Extracts were prepared from yeast cultures grown to exponential-phase. Cells were washed in ice-cold buffer B (100 mM Tris-HCl pH 7,5, 200 mM NaCl, 5 mM EDTA, 20% glycerol), lysed with glass beads in 200 µl of buffer B + PIM (1 mM each phenylmethylsulfonyl fluoride, tosyl-L-lysine-chloromethylketone, tosyl-L-phenylalanine-chloromethylketone, *p*-aminobenzamidine–HCl and *o*-phenanthroline) + 3% Triton X-100 + 0,8% SDS at 4°C and spun at 3500 rpm for 15 min to remove glass beads and large cell debris. Extracts (10 µl) were removed to determine total protein concentration using a protein assay kit from Bio-Rad (München, Germany). Proteins were denatured in SDS loading dye by heating at 65°C for 15 min and were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes. Myc-fusion proteins and eIF2p were detected using ECL technology (Amersham, UK). For the first incubation, monoclonal mouse anti-myc (9E10) or polyclonal rabbit anti-eIF2p antibodies were used. Peroxidase-coupled goat anti-rabbit or goat anti-mouse IgG were used as secondary antibodies (Dianova, Hamburg, Germany). Gcn4p protein bands were quantified using the KODAK 1D Image Analysis Software.

GFP fluorescence microscopy

Yeast strains harbouring the plasmid encoding GFP-Gcn4p were grown to early log-phase and analyzed under sated conditions. 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) or in case of 4',6-diamidino-2-phenylindole (DAPI) staining, a standard DAPI filter set. DAPI staining was used for visualization of nuclei. Cells were photographed using a Hamamatsu-Orca-ER digital camera and the Improvision Openlab software (Improvision, Coventry, UK).

Adhesive growth tests and photomicroscopy

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

Growth tests

Yeast strains were pre-cultured to the same optical densities (OD_{600}= 1) and spotted onto selective YNB medium supplemented with or without 100 mM 3AT or on selective medium containing galactose as carbon source. Five-fold dilution experiments started with $3x10^4$ cells per 20 ml. After 3 days of growth the spotted strains were photographed under white light.

β-galactosidase assay

Assays were performed with extracts grown in liquid medium. Specific β-galactosidase activity was normalized to the total protein (Bradford, 1976) in each extract and equalized (OD_{415} x 1,7)/(0,0045 x protein concentration x extract volume x time) (Rose and Botstein, 1983). Assays were performed for at least three independent cultures.

Library of *GCN4* mutants

GCN4 was mutagenized by PCR amplification of a 2,8 kb fragment of pME2214 containing the *GCN4*-ORF by using *Taq*-Polymerase in the presence of 0,1 mM $MnCl_2$. The resulting DNA was digested with *Nhe*I und *Aat*II and exchanged for the corresponding *Nhe*I-*Aat*II *GCN4wt*-fragment in pME2214, yielding a library of more than 24000 independent recombinants. Following identification of mutants (see below), *GCN4* alleles were sequenced using the ABI Prism Big Dye terminator sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany).

Screen for *GCN4* mutants impaired in amino acid starvation-induced adhesive growth but able to grow on 3AT

For isolation of mutants with decreased amino acid starvation-induced adhesive growth and *FLO11* expression, diploid strain RH2695 carrying a chromosomal deletion of *GCN4* and an integrated *FLO11-lacZ* reporter was transformed with the *GCN4* mutant library described above. A pool of approximately 24000 transformants was plated on solid medium containing 10 mM 3AT, from which 20000 were able to grow and therefore to complement the *gcn4* phenotype. Mutants impaired in adhesive growth were isolated by measuring expression of the *FLO11-lacZ* reporter gene by a qualitative filter assay (Breeden and Nasmyth, 1985). Initial mutant phenotypes were confirmed by isolation of the *GCN4*-containing plasmids and re-introduction into the parental strain.

Results

The *GCN4*^{LEU267SER} mutant allele

The yeast transcriptional activator Gcn4p occupies a dual function in metabolism and developmental processes. Gcn4p is the key regulator of the general amino acid control and therefore required for the appropriate response to starvation of amino acids, purines or drugs like methyl methanesulfonate (Hinnebusch, 1992; Mösch et al., 1991; Natarajan et al., 2001). Beside this metabolic response, Gcn4p has been reported to regulate adhesion and differentiation processes in *S. cerevisiae* and filamentation in *C. albicans* dependent on the availability of amino acids (Braus et al., 2003; Tripathi et al., 2002). We asked whether it is possible to separate Gcn4p regulation in metabolism and development in *S. cerevisiae*, two processes that are directly connected in various eucaryotic organisms. A screen was performed with the view to obtain a Gcn4p mutant protein not more able to activate *FLO11* expression and therefore adhesive growth in response to amino acid starvation, but still able to respond to these starvation conditions by regulating the transcripton of its target genes to secure a sufficient amino acid pool in the cell. By PCR random-mutagenesis a library of *GCN4* mutants was created that was first analyzed for mutants able to suppress amino acid limitation by growing under 3AT conditions. Diploid *gcn4* mutant cells (RH2695) carrying a chromosomally integrated *FLO11-lacZ* reporter were transformed with the *GCN4* mutant library. A pool of 24000 transformants was plated on solid medium with 10 mM 3AT, from which 20000 clones were able to grow. These transformants were analyzed concerning their ability to induce *FLO11-lacZ* expression in response to amino acid starvation using a qualitative filter assay (Breeden and Nasmyth, 1985). 207 clones were impaired in activating *FLO11* expression upon amino acid starvation, from which the *GCN4*-containing plasmids were isolated and reintroduced into the parental strain RH2695. From 83 extant clones only 8 transformants showed a significantly decrease in amino acid starvation-induced *FLO11-lacZ* expression. The diploid *gcn4* mutant strain (RH2398) carrying a chromosomally integrated *GCRE-lacZ* reporter was transformed with these *GCN4* mutant plasmids. All transformants showed a slightly reduced *GCRE-lacZ* expression and therefore Gcn4p activity upon amino acid limitation, but sufficient for an appropriate response under these conditions. Sequencing resulted in a *GCN4* mutant allele encoding a protein with the amino acid substitution Leu267Ser.

The amino acid substitution Gcn4Leu267Ser reduces adhesive growth and *FLO11* expression in haploid amino acid starved yeast cells

Cells expressing wild-type *GCN4* or the $GCN4^{LEU267SER}$ mutant allele were tested for amino acid starvation-induced adhesive growth, *FLO11* transcript levels and *FLO11-lacZ* expression. Therefore, a haploid *gcn4* yeast strain was transformed to express wild-type *GCN4* or $GCN4^{LEU267SER}$. Gcn4p is required for adhesive growth and *FLO11* expression in response to amino acid starvation independent of the availability of glucose or ammonium (Braus et al., 2003), and therefore a *gcn4* deletion strain was used as negative control. Amino acid starvation was induced by the histidin analogue 3-amino-triazole (3AT) to solid YNB medium. Figure 1C shows that under non-starvation conditions all haploid strains were non-adhesive. After starving for amino acids cells expressing wild-type *GCN4* became adhesive. In contrast, adhesive growth of haploid $GCN4^{LEU267SER}$ expressing cells is slighty reduced (Figure 1C).

We next checked whether the Gcn4p amino acid substitution Leu267Ser influences the expression of the adhesin encoding gene *FLO11* that is the key player of the tightly controlled adhesion phenotype. Therefore Northern hybridization experiments and β-galactosidase activity assays were performed. Figure 1A shows that the low basal level of *FLO11* mRNAs in haploid wild-type cells (WT) increases after 3AT treatment about a factor of eight. In contrast, investigations concerning *FLO11* transcript levels in haploid $GCN4^{LEU267SER}$ expressing cells resulted in a four-fold drop of *FLO11* mRNA in comparison to wild-type cells in response to 3AT. Similar results were obtained by β-galactosidase activity assays using a chromosomally integrated *FLO11-lacZ* as reporter (Figure 1B). A low basal β-galactosidase activity was assayed for haploid sated wild-type cells (WT) that strongly increased after 3AT treatment (1650 U). *FLO11-lacZ* expression decreased by Gcn4p amino acid substitution Leu267Ser (400 U) under 10 mM 3AT conditions showing a four-fold reduction in comparison to the expression measured in the control strain (Figure 1B).

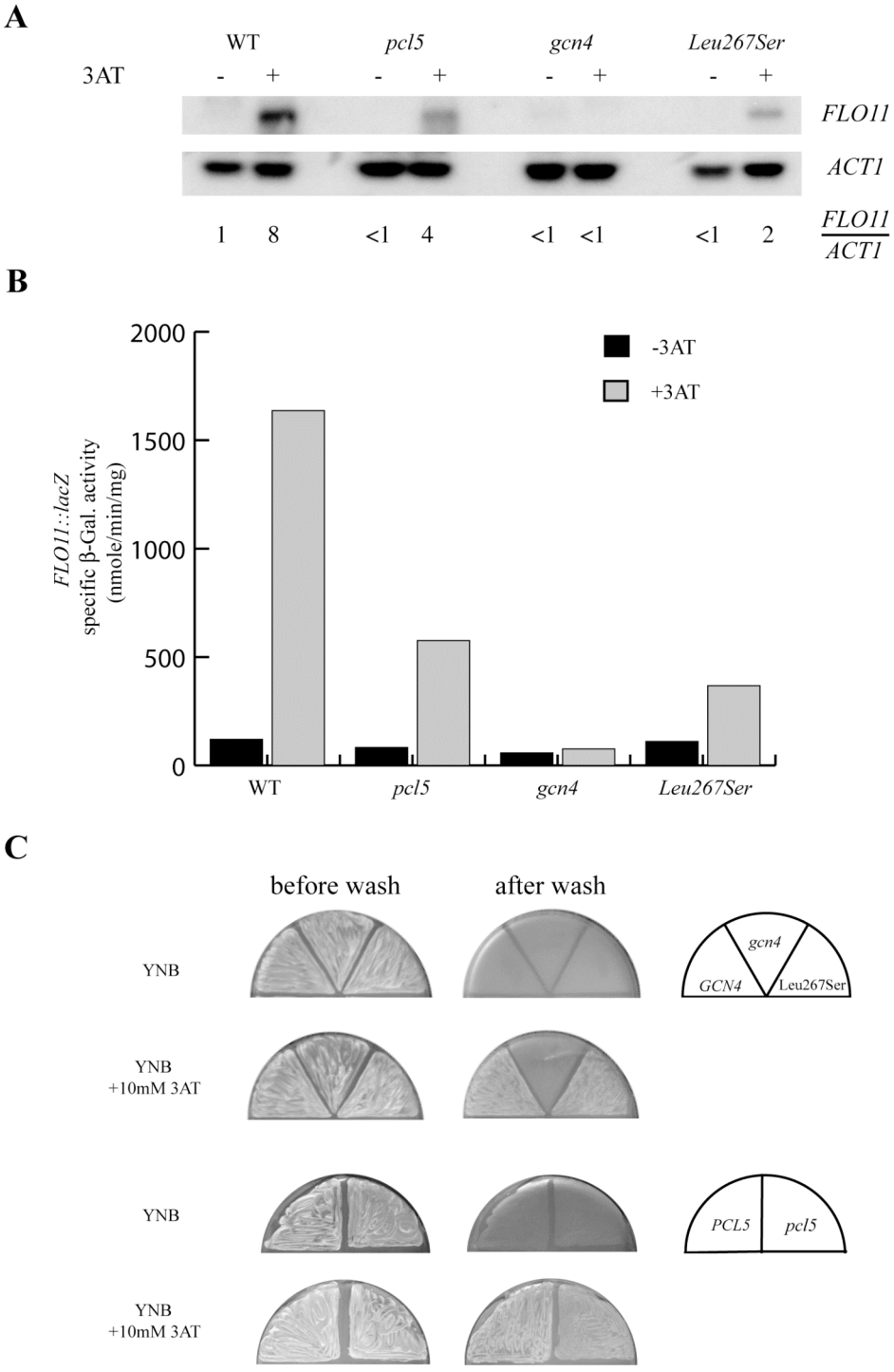

Figure 1. A Gcn4aaLeu267Ser substitution and a *pcl5* deletion reduce *FLO11* expression and impair adhesive growth in haploid Σ1278b yeast cells. **(A)** The haploid Σ1278b yeast strains RH3277 (WT), RH3282 (*pcl5*) or RH2693 (*gcn4*) transformed with pRS314 (*gcn4*) or pME2901 ($GCN4^{LEU267SER}$) were cultivated in liquid YNB medium in absence or presence of 10 mM 3AT. For Northern hybridization analysis 20 µg of total RNA were used. **(B)** The same yeast strains as described in (A) carrying a *FLO11-lacZ* reporter were grown to log-phase in YNB in absence (black bars, -3AT) or presence (grey bars, +3AT) of 10 mM 3AT before specific β-galactosidase activities were assayed. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. Bars illustration means of at least three independent measurements with a standard deviation not exceeding 20%. **(C)** The haploid Σ1278b yeast strains RH2693 (*gcn4*) transformed with pME1092 (*GCN4*), pRS314

(*gcn4*) or pME2901 (*GCN4*^{LEU267SER}), RH3277 (*PCL5*) and RH3282 (*pcl5*) were patched on solid YNB medium or solid YNB medium containing 10 mM 3AT, respectively. After incubation for two days at 30°C plates were photographed prior (total growth) and after washing (adhesive growth) by water. Non-adhesive growing cells were washed off the agar surface.

GCN4^{LEU267SER} expressing diploid cells are affected in amino acid starvation-induced adhesive growth and *FLO11* expression

In diploid yeast cells, which express *GCN4*^{LEU267SER}, adhesive growth, *FLO11* mRNA levels and *FLO11-lacZ* expression were further measured under sated and amino acid starved conditions. Sated diploid *gcn4* mutant cells expressing *GCN4*^{LEU267SER} behaved like the wild-type control with respect to their non-adhesive growth behaviour and the very low expression of *FLO11* transcripts or *FLO11-lacZ* (Figure 2). After 3AT treatment diploid wild-type cells became adhesive in contrast to *GCN4*^{LEU267SER} expressing cells that showed no adhesive growth under amino acid limitation comparable with the *gcn4* deletion strain (Figure 2C).

We next investigated the expression of *FLO11* dependent on the availability of amino acids. Figure 2A shows that the *FLO11* mRNA level in diploid wild-type cells (WT) increases significantly upon 3AT treatment. In case of the Gcn4Leu267Ser mutant protein transcript levels of *FLO11* decreased 22-fold in comparison with the control strain in response to amino acid starvation. This correlated with a decrease in expression of *FLO11-lacZ* of 5,5-fold in case of *GCN4*^{LEU267SER} expressing cells (20 U) compared to the induced levels measured in the control strain (Figure 2B).

In summary, the Gcn4p amino acid substitution Leu267Ser evokes that haploid and diploid cells are affected in cell surface adhesion and *FLO11* expression under amino acid starvation conditions.

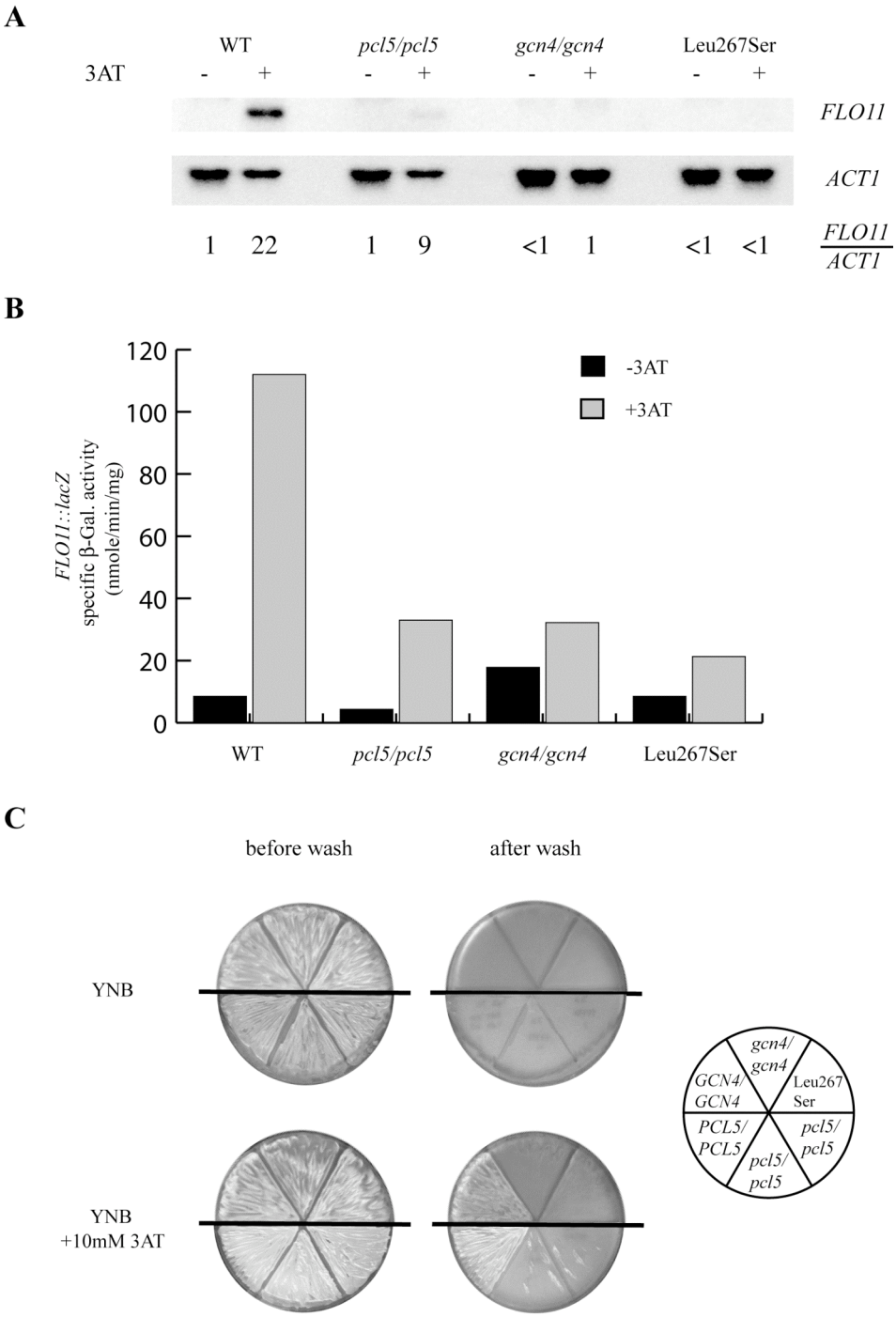

Figure 2. Gcn4aaLeu267 and *PCL5* are required for amino acid starvation-induced adhesive growth and expression of *FLO11* in diploid Σ1278b yeast cells. **(A)** The diploid Σ1278b yeast strains RH3278 (WT) and RH3303 (*pcl5/pcl5*) or RH2695 (*gcn4/gcn4*) transformed with pRS314 (*gcn4*) or pME2901 ($GCN4^{LEU267SER}$) were cultivated in liquid YNB medium in absence or presence of 10 mM 3AT. For Northern hybridization analysis 20 µg of total RNA were used. **(B)** The same yeast strains as described in (A) carrying a *FLO11-lacZ* reporter were grown to log-phase in YNB in absence (black bars, -3AT) or presence (grey bars, +3AT) of 10 mM 3AT before specific β-galactosidase activities were assayed. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. Bars illustration means of at least three independent measurements with a standard deviation not

exceeding 20%. (**C**) The diploid Σ1278b yeast strains RH2695 (*gcn4/gcn4*) transformed with pME1092 (*GCN4*), pRS314 (*gcn4*) or pME2901 ($GCN4^{LEU267SER}$), RH3278 (*PCL5/PCL5*) and RH3303 (*pcl5/pcl5*) were patched on solid YNB medium or solid YNB medium containing 10 mM 3AT, respectively. After incubation for two days at 30°C plates were photographed prior (total growth) and after washing (adhesive growth) by water. Non-adhesive growing cells were washed off the agar surface.

The Gcn4p amino acid substitution Leu267Ser affects basal and amino acid starvation-induced Gcn4p activity

Gcn4p regulates adherence in yeast by inducing *FLO11* expression in amino acid starved cells (Braus et al., 2003). Since we have shown that the amino acid substitution Leu267Ser causes an impaired *FLO11* expression and therefore an adhesion-deficient growth phenotype in response to amino acid starvation, we asked whether the transcriptional activity of Gcn4p is influenced in cells expressing this *GCN4* mutant allele. β-galactosidase activity assays were performed using a *GCRE-lacZ* as reporter to determine Gcn4Leu267Ser activity in sated and amino acid starved diploid yeast cells. Gcn4p binds specifically as a homodimer to a 9 bp nucleotide sequence termed Gcn4-protein responsive elements (GCRE) (Hope and Struhl, 1986; Oliphant et al., 1989). Under sated conditions expression of *GCRE-lacZ* increased 1,6-fold in diploid cells expressing $GCN4^{LEU267SER}$ compared to the wild-type. In contrast, upon 10 mM 3AT treatment *GCRE-lacZ* expression is down-regulated to 70% in comparison to the expression measured in case of wild-type Gcn4p (Figure 3A). These results indicate, that the Gcn4p amino acid Leu267 is required for its full transcriptional activity.

Furthermore, the Gcn4Leu267Ser mutant protein is still able to complement a *gcn4* phenotype by mediating resistance to 10 mM 3AT-induced amino acid starvation (data not shown). Figure 3B shows that $GCN4^{LEU267SER}$ expressing cells grow better on 100 mM 3AT than wild-type cells suggesting that the Gcn4Leu267Ser mutant protein mediates better resistance to high amounts of 3AT than wild-type Gcn4p.

So far our data indicate, that the complete transcriptional activation of Gcn4p seems to be necessary for an intact adhesion phenotype and differentiation processes in response to amino acid starvation. In contrast, the down-regulation of Gcn4p activity to 70% does not influence regulation of metabolic processes including an intact ‘general control’ upon amino acid starvation.

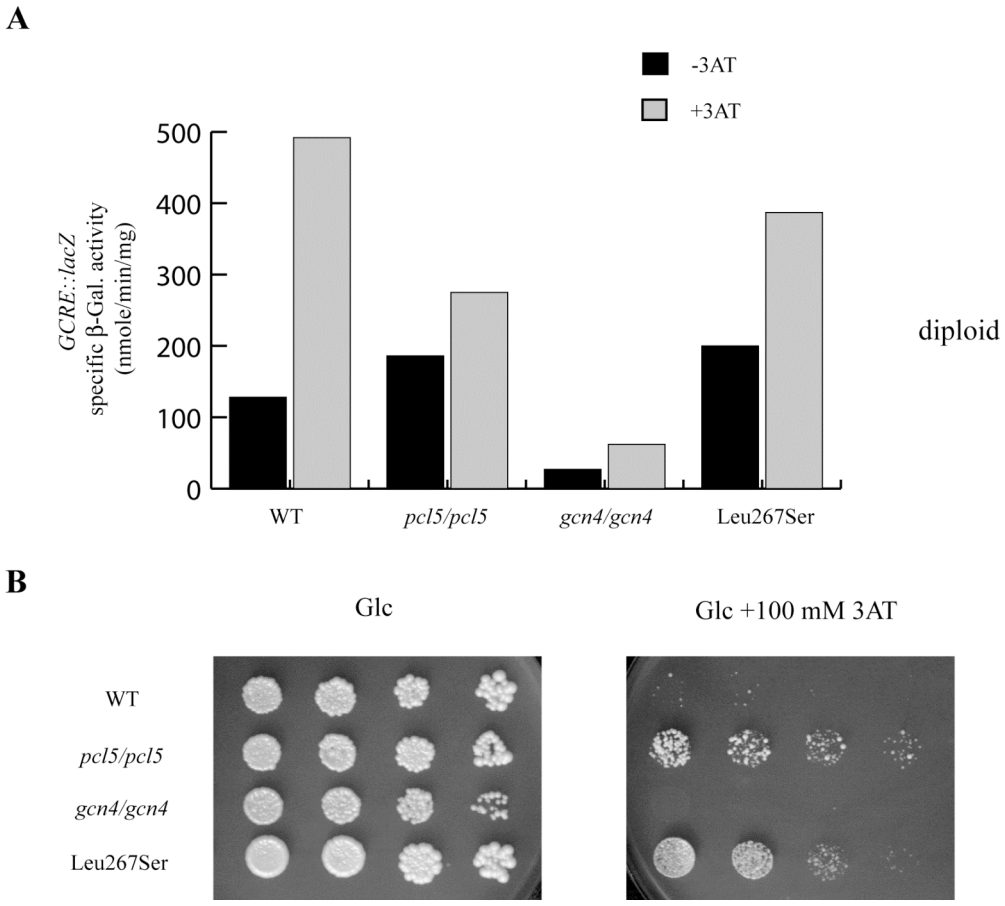

Figure 3. Basal and amino acid starvation-induced Gcn4p activity depend on Gcn4aaLeu267 and *PCL5* in diploid Σ1278b yeast cells. (**A)** The diploid yeast strains RH2305 (WT), RH3305 (*pcl5/pcl5*) and RH2398 (*gcn4/gcn4*) transformed with plasmid pRS314 (*gcn4*) or pME2901 ($GCN4^{LEU267SER}$) each carrying a *6GCRE-lacZ* reporter were grown to log-phase in YNB in absence (black bars, -3AT) or presence (grey bars, +3AT) of 10 mM 3AT before specific β-galactosidase activities were assayed. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. Bars illustration means of at least three independent measurements with a standard deviation not exceeding 20%. **(B)** The diploid yeast strains RH2656 (WT), RH3281 (*pcl5/pcl5*) and RH2398 (*gcn4/gcn4*) transformed with plasmid pRS314 (*gcn4*) or pME2901 ($GCN4^{LEU267SER}$) were spotted in five-fold dilutions on solid glucose medium and solid glucose medium containing 100 mM 3AT. After incubation for three days at 30°C plates were photographed.

Amino acid-dependent stability of the nuclear Gcn4Leu267Ser mutant is increased in diploid yeast cells

$GCN4^{LEU267SER}$ expressing haploid and primarily diploid yeast cells are impaired in amino acid starvation-induced adhesive growth and *FLO11* expression. Both, Gcn4p and its specific degradation factor Pcl5p are constitutively predominantly nuclear localized proteins independent of the availability of amino acids. Since the regulation of Gcn4p degradation occurs exclusively in the yeast nucleus (Pries et al., 2002), we investigated the sub-cellular localization of the Gcn4Leu267Ser mutant as putative tool for the limited amino acid-

dependent adhesion phenotype. Therefore, cells expressing wild-type *GFP-GCN4* or the *GFP-GCN4*^{LEU267SER} mutant allele were monitored in living yeast cells. Figure 4A illustrates that both proteins, Gcn4p and Gcn4Leu267Ser are enriched in the nucleus excluding a regulation on the level of sub-cellular localization.

Under non-starvation conditions Gcn4p is an unstable protein with a half-life of only a few minutes that increases up to 20 minutes when cells are starved for amino acids. We asked whether the stability of Gcn4Leu267Ser is regulated like wild-type Gcn4p dependent on the availability of amino acids. Therefore, we analyzed the stability regulation of Gcn4p and Gcn4Leu267Ser in sated and amino acid starved cells expressing *myc*^{3}*-GCN4* or *myc*^{3}*-GCN4*^{LEU267SER} from the inducible *GAL1* promoter. Promoter-shut-off experiments were carried out indicating that both wild-type Gcn4p and the mutant protein Gcn4Leu267Ser are rapidly degraded in sated cells (Figure 4B). In response to amino acid starvation wild-type Gcn4p is normally stabilized resulting in an increased half-life. In contrast, the amino acid substitution Leu267Ser in Gcn4p leads to a stronger stabilization of this transcription factor under starved conditions similar to that observed in sated *pcl5* deletion cells (Figure 4B and Figure 5A).

In summary, our data indicate that cells expressing the *GCN4*^{LEU267SER} mutant allele show an increased stability of the transcription factor Gcn4p in amino acid starved cells with a less transcriptional activity. This suggests that degradation of Gcn4p is a prerequisite for its full activity.

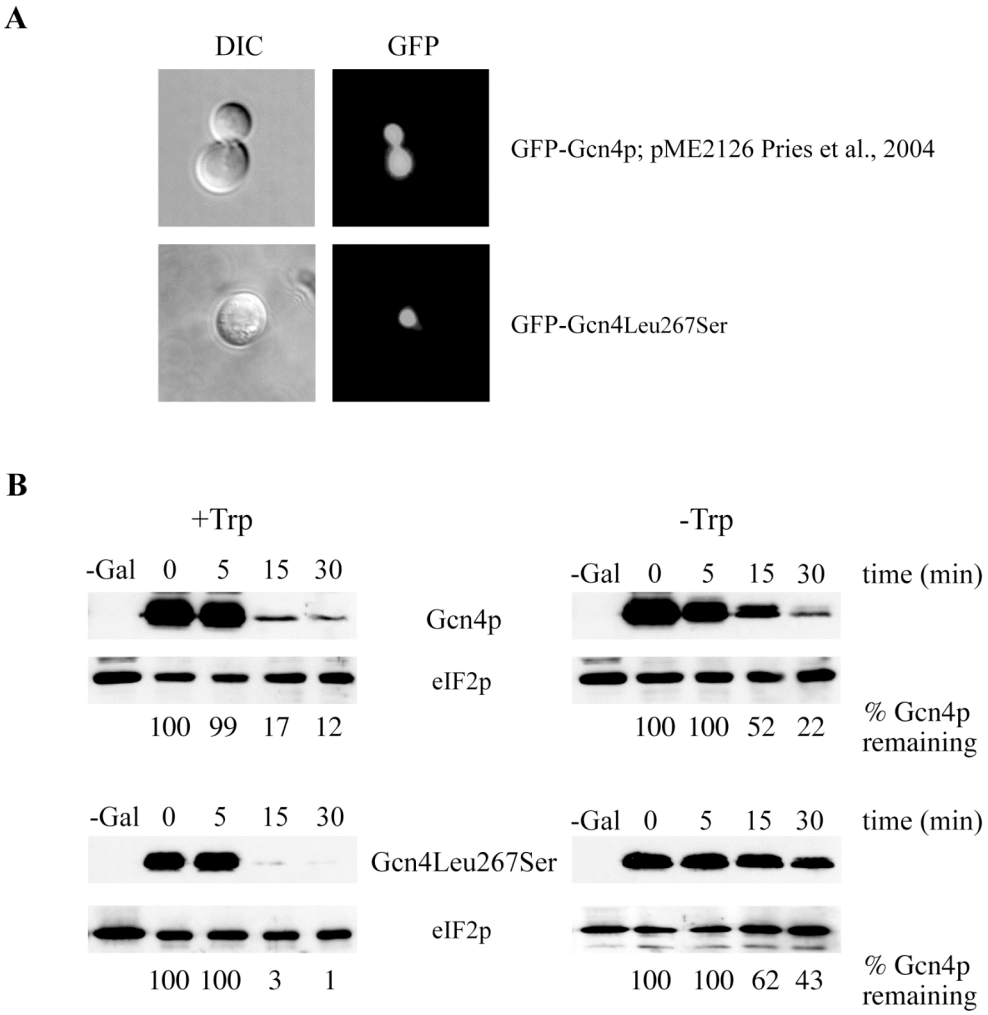

Figure 4. Amino acid-dependent stability of the nuclear Gcn4Leu267Ser mutant is increased in yeast. **(A)** Nuclear localization of GFP-Gcn4Leu267Ser mutant. *S. cerevisiae gcn4* mutant cells (RH2694) expressing *GFP-GCN4* (pME2126) or *GFP-GCN4*^{LEU27SER} (pME2911) from the *MET25* promoter were grown to early log-phase at 30°C and analyzed by DIC (differential interference contrast) microscopy (left) and fluorescence microscopy (right). **(B)** A Gcn4Leu267Ser mutation leads to an increased Gcn4p stability under amino acid starvation in yeast. The *gcn4 S. cerevisiae* strain RH2694 was transformed to express the *GAL1*-driven *myc*^{3}*-GCN4* (pME2923) or *GCN4*^{LEU27SER} (pME2925) on a high-copy-number plasmid. Cells were pre-grown to early log-phase in selective minimal medium at 30°C containing glucose as carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express *myc*^{3}*-GCN4* or *myc*^{3}*-GCN4*^{LEU267SER}. After 3 h of induction, half of these *trp1*-deficient cells were starved for tryptophan (-Trp). After half an hour the *GAL1* promoter was turned off by adding 2% glucose to the medium and samples were analyzed at the indicated time points after the shift to glucose medium (0-min time point). Protein levels of myc^3-tagged Gcn4p and myc^3-Gcn4Leu267Ser were determined by immunoblotting using myc-antibodies. The translation factor eIF2p was used as loading control. Numbers given below indicate remaining Gcn4p-percentages when compared to eIF2p as internal standard quantified by image station of the gel shown.

A *pcl5* deletion impairs adhesive growth and reduces *FLO11* expression in amino acid starved haploid Σ1278b yeast cells

In the model fungus *S. cerevisiae* rapid decay of the transcriptional activator Gcn4p is initiated in sated cells by phosphorylation of Thr165 by the kinase/cyclin complex Pho85p/Pcl5p (Shemer et al., 2002) whereas in response to a reduced amino acid supply this kinase/cyclin complex dissociates resulting in a stabilized Gcn4p (Bömeke et al., 2006). So far our results indicate, that full transcriptional Gcn4p activity seems to be required for amino acid starvation-induced adhesion and differentiation, but not for metabolic processes such as an intact general amino acid control. Furthermore, degradation of Gcn4p might be a prerequisite for its function in transcriptional activity. To confirm these hypotheses a Gcn4p degradation mutant was created by deletion of the Gcn4p specific cyclin encoding gene *PCL5* and the effect of stabilized Gcn4p was analyzed concerning developmental functions and transcriptional activity.

So far, Gcn4p stability was only analyzed in adhesion-deficient *S. cerevisiae* laboratory strains. Σ1278b yeast cells are able to adhere on plastic surfaces after amino acid starvation conditions (Kleinschmidt et al., 2005) and therefore haploid and diploid *pcl5* Σ1278b mutants were created (see Materials and Methods). First we checked the effect of a *pcl5* deletion on degradation of Gcn4p in sated cells. Diploid wild-type and *pcl5* Σ1278b yeast cells were transformed to express *myc*^{3}*-GCN4*. A promoter-shut-off experiment of the fusion gene revealed a strong stabilization of Gcn4p in the *pcl5* mutant compared to the *PCL5* wild-type strain (Figure 5A).

In *S. cerevisiae* adhesion-deficient cells a *pcl5* deletion causes hypersensitivity to moderate overexpression of Gcn4p (Shemer et al., 2002). We next checked the sensitivity of Σ1278b wild-type and *pcl5* mutant cells to Gcn4p overexpressed from the inducible *GAL1* promoter. As shown in Figure 5B the *pcl5* mutant is hypersensitive to high amounts of Gcn4p driven from the *GAL1* promoter. Additional expressed *PCL5-GFP* is able to suppress overexpression toxicity of Gcn4p and therefore able to complement the *pcl5* mutant phenotype in Σ1278b cells.

Expression of *FLO11* is required for haploid invasive growth in response to glucose starvation, diploid pseudohyphal development under nitrogen starvation conditions (Lo and Dranginis, 1998) and amino acid starvation-induced adhesive growth (Braus et al., 2003). Investigations concerning a putative impact of *PCL5* on these phenotypes resulted in a slightly reduced invasive growth upon glucose limitation compared to the control strain. In response to nitrogen starvation, diploid *pcl5* mutant cells are able to develop pseudohyphae

with a reduced length in comparison to cells with an intact *PCL5* (data not shown). Thereupon, haploid wild-type and *pcl5* Σ1278b yeast strains were tested for amino acid starvation-induced adhesive growth. Amino acid starvation was induced as described before. Figure 1C shows that both tested strains were non-adhesive under non-starvation conditions. After starvation for amino acids, wild-type cells with an intact *PCL5* allele became adhesive. In contrast, haploid *pcl5* deletion cells show a decrease in adhesive growth under amino acid limitation.

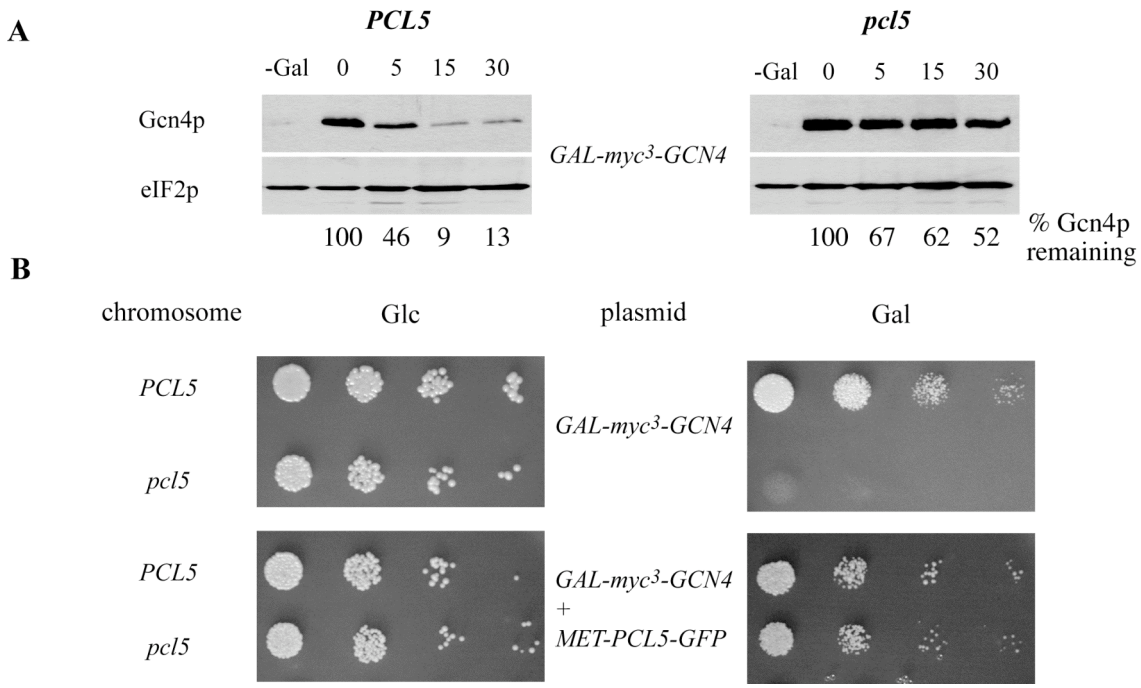

Figure 5. Overexpression of a stabilized Gcn4p results in toxicity in *pcl5 S. cerevisiae* Σ1278b cells. (**A)** A *pcl5* mutation leads to a stabilized Gcn4p. The isogenic yeast strains Σ1278b RH2656 (*PCL5/PCL5*) and RH3281 (*pcl5/pcl5*) were transformed to express the *GAL1*-driven *myc*^{3}*-GCN4* on a high-copy-number plasmid (KB294). Cells were pre-grown to early log-phase in selective minimal medium at 30°C containing glucose as carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express *myc*^{3}*-GCN4*. After 4 h of induction the *GAL1* promoter was turned off by adding 2% glucose to the medium. Samples were analyzed at the indicated time points after the shift to glucose medium (0-min time point). Levels of myc^3-tagged Gcn4p were determined by immunoblotting using myc-antibodies. eIF2p was used as loading control. Numbers given below indicate remaining Gcn4p-percentages when compared to eIF2p as internal standard quantified by image station of the gel shown. (**B)** In *pcl5* Σ1278b yeast cells the overexpression toxicity of Gcn4p is suppressed by functional *PCL5*. Wild-type cells (RH2818) and *pcl5* mutant cells (RH3280) expressing *myc*^{3}*-GCN4* from plasmid pME2848 alone or together with *PCL5-GFP* (pME2846) were spotted in five-fold dilutions on glucose and galactose to induce expression of *GCN4* driven by the *GAL1* promoter. After three days of incubation at 30°C cells were photographed.

We are further interested, whether *PCL5* influences the expression of *FLO11*. Northern hybridization experiments were performed to determine *FLO11* transcript levels under non-starvation and amino acid starvation conditions. The *FLO11* mRNA level in haploid wild-type cells (*PCL5*) is increased eight-fold upon 3AT treatment. In contrast, in haploid *pcl5* deletion cells no *FLO11* transcript was detectable in the absence of 3AT. After 3AT treatment the *FLO11* transcript level decreased two-fold in the absence of *PCL5* in comparison with the control strain. These results were confirmed by β-galactosidase activity assays with a *FLO11-lacZ* as reporter (Figure 1B). The low β-galactosidase activity in haploid sated wild-type cells (*PCL5*) increases in response to 3AT (1650 U). Contradictory, expression of *FLO11-lacZ* decreased by deletion of *PCL5* (550 U) upon 3AT treatment reflecting a three-fold reduction in comparison to the expression measured in the control strain.

In summary, the yeast Pho85p cyclin encoding gene *PCL5* is necessary for expression of *FLO11* in haploid Σ1278b yeast cells and therefore for efficient amino acid starvation-induced adhesive growth. These data might reflect, that stability of Gcn4p is connected to its regulation in amino acid-dependent developmental functions.

Amino acid starvation-induced adhesive growth and expression of *FLO11* requires *PCL5* in diploid Σ1278b yeast cells

We further analyzed adhesive growth, *FLO11* transcript levels and *FLO11-lacZ* expression in diploid wild-type and *pcl5* mutant cells. Under sated conditions, the diploid wild-type strain is non-adhesive and shows a very low expression of *FLO11* mRNA or *FLO11-lacZ* similar to the data observed for the diploid *pcl5/pcl5* mutant strain (Figure 2). Upon 3AT treatment a *PCL5* deletion reduces the adhesive growth compared to the adhesively growing wild-type strain. These results correlated well with those obtained from Northern hybridization experiments or β-galactosidase activity assays. The amino acid starvation-induced transcript levels of *FLO11* decreased 2,4-fold in the absence of *PCL5* in comparison with the control strain (Figure 2A). This finding was confirmed with a decrease in expression of *FLO11-lacZ* of 3,3-fold in the *pcl5/pcl5* strain (35 U) in comparison to the activated levels measured in the control strain (Figure 2B). The data obtained in haploid yeast cells were therefore intensified in diploid cells by showing that amino acid starvation-induced adhesive growth and expression of *FLO11* require *PCL5*.

Basal and amino acid starvation-induced Gcn4p activity depend on *PCL5*

The *pcl5* deletion phenotype suggests that Gcn4p degradation is also required for amino acid starvation-dependent adhesive growth and *FLO11* expression. Therefore, we asked whether the activity of Gcn4p is influenced in cells impaired in *PCL5*. By β-galactosidase activity assays with *GCRE-lacZ* as reporter Gcn4p activity was measured in sated and amino acid starved diploid wild-type and *pcl5* mutant cells. Under non-starvation conditions expression of *GCRE-lacZ* increased from 125 U in a control strain to 195 U in the *pcl5* mutant strain (Figure 3A). An increase of Gcn4p activity in *pcl5* cells in comparison to wild-type cells has been observed previously (Shemer et al., 2002). This indicates that Pcl5p reduces Gcn4p activity. After 3AT treatment to induce amino acid starvation expression of *GCRE-lacZ* enhanced up to 500 U in the wild-type strain. Surprisingly, expression of *GCRE-lacZ* decreased by a deletion of *PCL5* under 10 mM 3AT conditions, corresponding to a two-fold drop in comparison to the expression measured in the control strain. These data suggest that Pcl5p is required for full Gcn4p activity in response to amino acid starvation.

Overexpression of *GCN4* inhibits cellular growth (Tavernarakis and Thireos, 1995). This Gcn4p overexpression toxicity can be measured by hypersensitivity of *pcl5*-deficient yeast cells (Shemer et al., 2002). The question occurs, whether a decreased induction of Gcn4p activity in *pcl5* amino acid starved cells, can be monitored by measuring the growth behaviour in response to strong amino acid starvation. We analyzed the sensitivity of wild-type and *pcl5* mutant cells to different Gcn4p activities induced by 100 mM 3AT. As shown in Figure 3B, growth of *pcl5* yeast cells is increased on 100 mM 3AT compared to wt cells suggesting that wild-type cells are more sensitive to high amounts of 3AT than *pcl5* mutant cells.

In summary, our results suggest that the stabilization of Gcn4p in *pcl5* mutant cells (Figure 5A) correlates to a decreased transcriptional activity and therefore might be the reason for a reduced toxicity of this transcription factor under amino acid starvation conditions (Figure 3A).

Discussion

The *JUN* homolog *GCN4* of *S. cerevisiae* encodes a global transcriptional factor of a genetic system that secures the appropriate amount of amino acids in the cell (Hinnebusch, 2005). Beside this metabolic function Gcn4p regulates various developmental processes including adhesive growth and pseudohyphal development that depend also on the cell surface flocculin Flo11p (Braus et al., 2003). Metabolism and development are two processes that are directly connected in various multicellular organisms (Costa-Mattioli et al., 2005; Gildor et al., 2005; Hoffmann et al., 2000; Krappmann et al., 2004). We report, that it was possible to separate the regulatory functions of Gcn4p in metabolic and developmental processes by substitution of one Gcn4p amino acid.

Cells expressing the mutant allele $GCN4^{LEU267SER}$ are affected in *FLO11* expression and adhesive growth in response to amino acid starvation but able to suppress these limitations by inducing the general control system. A slightly decreased activity of Gcn4Leu267Ser points to the suggestion that complete transcriptional Gcn4p activity is required for amino acid starvation-induced adhesion, but not for metabolic processes such as an intact general amino acid control. The second hypothesis that turnover of Gcn4p as a prerequisite for its complete function in transcriptional activation is based on the stabilized Gcn4Leu267Ser with a down-regulated activity to 70% compared to wild-type Gcn4p. The importance of activator turnover in gene expression was recently shown by Lipford et al. (2005). Thus, the degradation by the ubiquitin-proteasome system of transcriptional activators, like Gcn4p, Gal4p and Ino2/4 can stimulate their transcriptional activity. This is based on their results, where the inhibition of the proteasome and furthermore mutations in the Gcn4p ubiquitin ligase SCF^{CDC4} impaired the induction of Gcn4p target gene expression. Investigations concerning a stabilized unphosphorylated Gcn4p mutant-protein (Chi et al., 2001) revealed that despite an increase of Gcn4p amount, stability and target promoter association its specific activity is decreased (Lipford et al., 2005). Therefore, a model is proposed from this group in which degradation is necessary to remove used Gcn4 activator proteins for resetting the promoter. This mechanism is called ‘activation by destruction’.

The increased amino acid starvation-induced stability of Gcn4Leu267Ser leads to the questions, whether the amino acid residue Ser267 of this mutant protein resembles a putative phosphorylation site. Besides degradation initiation phosphorylation of transcription factors can also result in a stabilization of these proteins as it was shown before in case of the human transcriptional activator JUN (Naumann et al., 1999). Amino acid substitutions of Leu267

against threonine, an additional putative phosphorylation site, or alanine would clarify this possibility. The amino acid leucin 267 represents the third of four repeats of leucine residues of a classical leucine zipper domain, which are separated by seven amino acids (Landschulz et al., 1988). This motif is described to be necessary for dimerization of yeast Gcn4p that can only bind as a homodimer in the promoter regions of its target genes (Paluh and Yanofsky, 1991). One possibility for the reduced activity of Gcn4Leu267Ser in response to amino acid starvation is a decreased dimerization and therefore a limited level of Gcn4p homodimers associated to target gene promoters. Chromatin immunoprecipitation (Chip) assays of Gcn4Leu267Ser-myc with a typical target gene promoter, like the *HIS4* promoter, would elucidate the association efficiency of putative less dimerized Gcn4Leu267Ser. Furthermore, it might be possible, that dimerization per se influences protein stability leading to a stabilized Gcn4p monomer in contrast to a faster degraded Gcn4p dimer.

The hypothesis that degradation of Gcn4p is required for its transcriptional activity was corroborated in a Gcn4p degradation mutant with a deleted *PCL5* gene that encodes the cyclin specifically required for Gcn4p phosphorylation and degradation (Shemer et al., 2002). Deletion of *PCL5* resulted in a stabilized Gcn4p with an affected transcriptional activity in sated and amino acid starved cells and a defect in developmental processes. According to this, Pcl5p has to be involved in these developmental steps in a Gcn4p-dependent manner, in which the Gcn4p activity is regulated about its stability. To verify the link between the Gcn4p activity and stability the endogenous Gcn4p amounts in wild-type and *pcl5* deletion cells dependent on the availability of amino acids have to be analyzed. Furthermore, the question occurs whether a disruption of the kinase Pho85p results also in a reduced Gcn4p activity and an adhesion-deficient growth phenotype for amino acid starved Σ1278b cells. A Pho85p-requirement for differentiation processes would argue for a Gcn4p-dependent involvement of Pcl5p. Unexpectedly, it was shown before by Lipford et al., (2005) that single *pho85* or *srb10* mutants display a similar Gcn4p activity in sated cells as the wild-type and only the disruption of both kinases leads to an increased transcriptional activity and a suppression of the sensitivity of Gcn4p activity to a defect ubiquitin pathway.

Another possibility to explain the contribution of Pcl5p to the adhesion response under amino acid starvation conditions is that this cyclin plays a positive role in this response and therefore acts in a Gcn4p-independent manner. According to this facility, the lack of amino acid starvation-induced adhesive growth of the *gcn4* mutant may only reflect the positive role of Gcn4p in Pcl5p expression. This Gcn4p-independent way of Pcl5p function in developmental processes might also include the stability regulation of other proteins, namely Tpk2p and

Flo8p, required for amino acid starvation-induced adhesion and *FLO11* expression (Braus et al., 2003). We do not favour this possibilty, because investigations concerning glucose starvation-induced adhesive growth of *pcl5* mutant cells on YEPD resulted in only little difference between the wild-type and the mutant strain (data not shown). In addition, nitrogen-induced pseudohyphal development on SLAD medium showed a slightly reduced ability for pseudohyphal development of a *pcl5* deletion compared to wild-type cells (data not shown). These data indicate that neither glucose starvation nor nitrogen limitation is a signal for the induction of a Pcl5p-dependent adhesion or *FLO11* expression. Alternatively, Pcl5p could regulate other transcriptional factors that bind in combination with Gcn4p directly to yet unknown DNA sequence elements in the *FLO11* promoter. In this case, a Gcn4p-dependent Pcl5p involvement in adhesion would take place, but independent of the stability of Gcn4p.

High levels of Gcn4p caused by an increased translation and stability in parallel are toxic for the cell and result in inhibited cellular growth (Tavernarakis and Thireos, 1995). This toxic Gcn4p overexpression effect is based on multiple interactions resulting in transcriptional activation of different pathways. This is for example the case, when Gcn4p is moderately overexpressed from the *GAL1* promoter in a *pcl5* mutant strain compared to wild-type (Figure 5B). Here we have shown that cells expressing the $GCN4^{LEU267SER}$ mutant allele or cells with a deleted *PCL5* gene are less sensitive to amino acid starvation than wild-type cells (Figure 4B). This effect is pronounced in case of an overexpression of $GCN4^{LEU267SER}$ compared to wild-type cells (data not shown). This points to a reduced toxicity of Gcn4Leu267Ser or Gcn4p in *pcl5* mutant cells in response to amino acid starvation that could be explained by a decreased Gcn4p transcriptional activity of other pathways (Figure 3A). All together this suggests that it is not Gcn4p activity per se, but rather adequate modulation of Gcn4p activity that is required to fulfill its function.

Comparison to *C. albicans*

The infective hyphal form of *C. albicans* is induced by different environmental stimuli including carbon or nitrogen starvation, elevated temperatures or amino acid limitation (Ernst, 2000; Tripathi et al., 2002). High concentrations of specific amino acids or amino acid mixtures induce filamentation in wild-type cells, in contrast to Ca*gcn4* and Ca*pcl5* deletion strains with a reduced hyphae formation (Gildor et al., 2005). In contrast, a *C. albicans* mutant deleted for the F-box protein encoding gene Ca*CDC4*, the homolog of the *S. cerevisiae CDC4*, shows constitutive filamentous growth (Atir-Lande et al., 2005). This

suggests that the dimorphic switch of *C. albicans* depend on CaGcn4, the protein stability of CaGcn4 itself and SCF^{CDC4} ubiquitin ligase-mediated protein degradation in general. It has been recently shown that mutations in *S. cerevisiae* SCF^{CDC4} result in a stabilized Gcn4p but in a reduced transcription of its target genes suggesting that Gcn4p degradation is required for its transcriptional activity (Lipford et al., 2005). These findings conform to our favoured model, in which regulated Gcn4p turnover is necessary for its complete activity that, in turn, seems to be required for the switch from the yeast to the hyphal form.

In summary, we show here that it was possible to separate metabolism and development in *S. cerevisiae*, two processes that are regulated by the transcriptional activator Gcn4p. These data are fundamental applications for various multicellular organisms where development and metabolism are directly connected and required for, e.g. long-term memory and learning (Hao et al., 2005; Costa-Mattioli et al., 2005).

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