Metabolic and developmental functions of the transcription factor Gcn4p of *Saccharomyces cerevisiae*

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D7

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ABBREVIATIONS

α	Alpha
λ	Wavelength
Δ	Deletion
∅	Diameter
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
3AT	3-Amino-1,2,4-triazol
5MT	5-Methyl-tryptophan
aa	Amino acid
A, Ala	Alanine
Amp^R	Ampicillin resistance
ATP	Adenosine triphosphate
bp	Base pairs
bZIP	Basic-region leucine zipper
C, Cys	Cysteine
CAAD	Central acidic activation domain
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin-dependent kinase
CH_2Cl_2	Methylene chloride
Cm^R	Chloramphenicol resistance
CPC	Cross-pathway control
C-terminus	Carboxy terminus
DB	DNA binding domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol

ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
e.g.	For example
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
Fig.	Figure
FRE	Filamentous response element
g	Gram
G, Gly	Glycine
GAAC	General amino acid control
GCD	General control derepressed
GCN	General control non-derepressable
GCRE	Gcn4 protein recognition element
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPI	Glycosyl-phosphatidylinositol
GTP	Guanosine triphosphate
IgG	Immunoglobuline G
IPTG	Isopropyl-thio-β-D-galactoside
h	Hour
HCl	Hydrochloric acid
H_20	Water
H_20_2	Hydrogen peroxide
K	Lysine
kanMX4	Kanamycin resistance
kb	Kilobase pairs
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
l	Liter
L, Leu	Leucine
LB	Luria-Bertani
LiOAc	Lithium acetate

LZ	Leucine zipper domain
M	Molar
MAPK	Mitogen-activated protein kinase
MAT	Mating type
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minute
ml	Milliliter
mM	Millimolar
MMS	Methyl methansulfonate
MnCl	Manganese chloride
mRNA	Messenger RNA
n	Chromosome set
NaCl	Sodium choride
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
natMX4	Nourseothricin resistance
ng	Nanogram
$(NH_4)_2SO_4$	Ammonium sulfate
NLS	Nuclear localization sequence
nm	Nanometer
nM	Nanomolar
nt	Nucleotide
NTAD	N-terminal activation domain
N-terminus	Amino terminus
OD	Optical density
ORF	Open reading frame
ori	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

Pfu Polymerase	*Pyrococcus furiosus* Polymerase
pH	*Potentia hydrogenii*
PIPES	Piperazine-N, N'-bis(2-ethane sulfonic acid)
PKA	Protein kinase A
prom.	Promoter
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
S	Svedberg unit
S, Ser	Serine
SAP	Shrimp alkaline phosphatase
sec	Second
SOB	Bacteria complex medium
SOC	SOB with glucose
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SUMO	Small ubiquitin-like modifier
T, Thr	Threonine
TAE	Tris / acetate / EDTA
Taq Polymerase	*Thermus aquaticus* Polymerase
TE	Tris EDTA buffer
term.	Terminator
Tm	Tunicamycin
Tris	Tris(-hydroxymethyl)-aminomethane
t-RNA	Transfer RNA
Trp	Tryptophan
U	Unit
UAS	Upstream activation site
uORF	Upstream open reading frame
UPR	Unfolded protein response
UPRE	Unfolded protein response element
Ura	Uracil

URS	Upstream repression site
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/o	Without
WT	Wild type
w/v	Weight per volume
YEPD	Yeast extract peptone dextrose (rich medium)
YNB	Yeast nitrogen base (minimal medium)

SUMMARY

The bakers' yeast *Saccharomyces cerevisiae* executes two well established pathways, the 'General Amino Acid Control' (GAAC) and the 'Unfolded Protein Response' (UPR), which are in contrast to mammals not essential but enable yeast cells to adapt to environmental changes and different stress conditions.

The bZIP transcription factor Gcn4p represents the global key regulator of the GAAC and herein regulates transcription of numerous metabolic genes of amino acid or purine biosynthesis in response to amino acid starvation. Gcn4p is also involved in the regulation of the developmental cell-surface flocculin Flo11p, which is required for diploid pseudohyphae formation and for adhesion upon nutrient starvation. This dual function as metabolic and developmental activator could be separated by a Gcn4p variant carrying a single amino acid substitution in its leucine zipper, Gcn4p^{L267S}. This mutation abolishes *FLO11* expression and results in a reduced but sufficient transcriptional activity for the induction of amino acid biosynthetic genes. Gcn4p^{L267S} is impaired in homodimer formation and presents a significantly more stable protein compared to wild type Gcn4p. A helix breaker substitution in Leu253 results in a transcriptionally inactive, but highly stable protein variant. This is due to a feedback circuit between transcriptional activity of Gcn4p and its own stability, which depends on the Gcn4p-controlled cyclin Pcl5p. Gcn4p^{L253G} reduces the expression of Pcl5p and therefore its own degradation.

Hac1p plays an important role in the yeast UPR system and represents a bZIP transcription factor, alike Gcn4p. This work presents first evidence for a so far unknown function of Hac1p in the GAAC. Hac1p is not only able to activate Gcn4p specific target genes, but also *FLO11* expression is reduced in yeast cells deleted for *HAC1* and diploids can neither grow adhesively when starved for amino acids nor develop pseudohyhae upon nitrogen starvation. Promoter analysis of *FLO11* identified a promoter element influenced by both, Hac1p and Gcn4p, in response to amino acid starvation that was previously identified to confer regulation by amino acid starvation. Transcription factor specific stress situations result in repression of the respective antagonist. First results indicate novel evidence in Hac1p regulation, which might be of clinical interest due to the involvement of the UPR system in tumorogenesis in mammalian.

ZUSAMMENFASSUNG

In der Bäckerhefe *Saccharomyces cerevisiae* existieren zwei gut erforschte Signalwege, die 'Allgemeine Kontrolle der Aminosäurebiosynthese' (GAAC) und die 'Antwort auf ungefaltete Proteine' (UPR), die im Vergleich zu Säugetieren zwar nicht essentiell, aber für die Hefe dennoch von großer Bedeutung sind, um sich an unterschiedliche Umwelt- und Stressbedingungen anzupassen.

Der bZIP Transkriptionsfaktor Gcn4p ist der zentrale Regulator der GAAC und aktiviert unter Aminosäuremangel die Transkription vieler Gene aus Aminosäure- und Purinbiosynthesewegen. Des Weiteren ist Gcn4p an der Regulation des Zellwandproteins Flo11p beteiligt, das sowohl für das diploide Pseudohyphenwachstum, als auch für das adhäsive Wachstum unter Nährstoffmangel erforderlich ist. Diese doppelte Funktion als metabolischer und Entwicklungsaktivator konnte durch eine Gcn4p Variante getrennt werden, welche eine einzige Aminosäuresubstitution im Leucin-Zipper aufweist, Gcn4p^{L267S}. Diese Mutation führt dazu, dass die *FLO11* Expression zwar unterdrückt wird, aber die transkriptionelle Aktivität für die Induktion der Aminosäurebiosynthesegene ausreicht. Gcn4p^{L267S} beeinträchtig die Homodimerbildung und stellt, verglichen zum Wildtyp-Protein, ein stabileres Protein dar. Der Austausch von Leu253 gegen einen Helixbreaker führt zu einem inaktiven, aber sehr stabilen Transkriptionsfaktor. Dies ist auf eine Feedback-Regulation zurückzuführen, in der Gcn4p an der Regulation von Pcl5p beteiligt ist, welches wiederum für den Abbau von Gcn4p benötigt wird. Da Gcn4p^{L253G} nicht in der Lage ist, Pcl5p zu aktivieren, unterdrückt es folglich seinen eigenen Abbau.

Hac1p gehört wie Gcn4p zu der Gruppe der bZIP Transkriptionsfaktoren und nimmt eine wichtige Rolle im UPR System der Hefe ein. Die Ergebnisse dieser Arbeit weisen auf eine bisher unbekannte Rolle von Hac1p im Netzwerk der GAAC hin. Hac1p ist nicht nur in der Lage Gcn4p spezifische Zielgene zu aktivieren, sondern ist auch an der Regulation von Flo11p beteiligt. Aufgrund der reduzierten *FLO11* Expression können diploide *hac1*-Deletionsstämme weder unter Aminosäuremangel adhäsiv wachsen, noch unter Stickstoffmangel Pseudohyphen ausbilden. Analysen des *FLO11* Promotors weisen auf ein Promotorelement hin, dass von Hac1p und Gcn4p unter Aminosäuremangel beeinflusst wird. Für dieses Element wurde schon zuvor eine Aminsäuremangel-abhängige Funktion beschrieben. Spezifische Stresssituationen des jeweiligen Transkriptionsfaktor bewirken die Repression des jeweils anderen. Erste Ergebnisse deuten auf neue Erkenntnisse bzgl. der Hac1p Regulation hin, die von klinischem Interesse sein könnte, da das UPR System auch an der Tumorentwicklung in Säugern beteiligt ist.

1. INTRODUCTION

1.1 Regulation of dimorphism in *Saccharomyces cerevisiae*

Regulated dimorphism and adherence are essential virulence factors for a variety of human or plant pathogenic fungi, which threaten human health or agricultural products. Dimorphic fungi are able to attach to and penetrate into a host but also to propagate in liquid media including body fluids (San-Blas *et al.*, 2000; Sánchez-Martínez and Pérez-Martín, 2001; Klein and Tebbets, 2007). The non-pathogenic fungus *Saccharomyces cerevisiae* represents an established unicellular model system for the eukaryotic cell. In contrast to common laboratory strains, most natural yeasts are dimorphic diploids and are able to switch between a unicellular yeast and a multicellular filamentous pseudohyphal growth mode (Mortimer, 2000).

1.1.1 Life cycle of *S. cerevisiae*

Conjugation of two haploid cells (1n to 2n) or sporulation of one diploid cell (2n to 1n) enables the budding yeast *S. cerevisiae* to live either as a haploid (1n) or as a diploid (2n) organism (Fig. 1). The existence of two different mating types, called *MAT**a*** and *MATα*, is the basic prerequisite for conjugation of haploids cells, which enables them to conjugate with the respective mating partner to form diploid (*MAT**a**/α*) yeast 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 stationary phase. Haploid cells constitutively secrete small peptide pheromones into the medium, which is perceived by haploid cells with respective opposite mating type. This in turn leads to an activation of a signal cascade which induces alterations in different cellular processes including cell cycle arrest, polarity and morphology changes, and adherence. The initial contact of the '**a**' and 'α' cells is mediated amongst others by adhesion and finally results in cell and nuclear fusion (Roy *et al.*, 1991; Cappellaro *et al.*, 1994). Haploid cells show an unicellular ellipsoid cell morphology, called yeast form. During prolonged growth upon glucose limitation haploid cells penetrate into the substrate, also referred as haploid invasive growth (Cullen and Sprague, 2000).

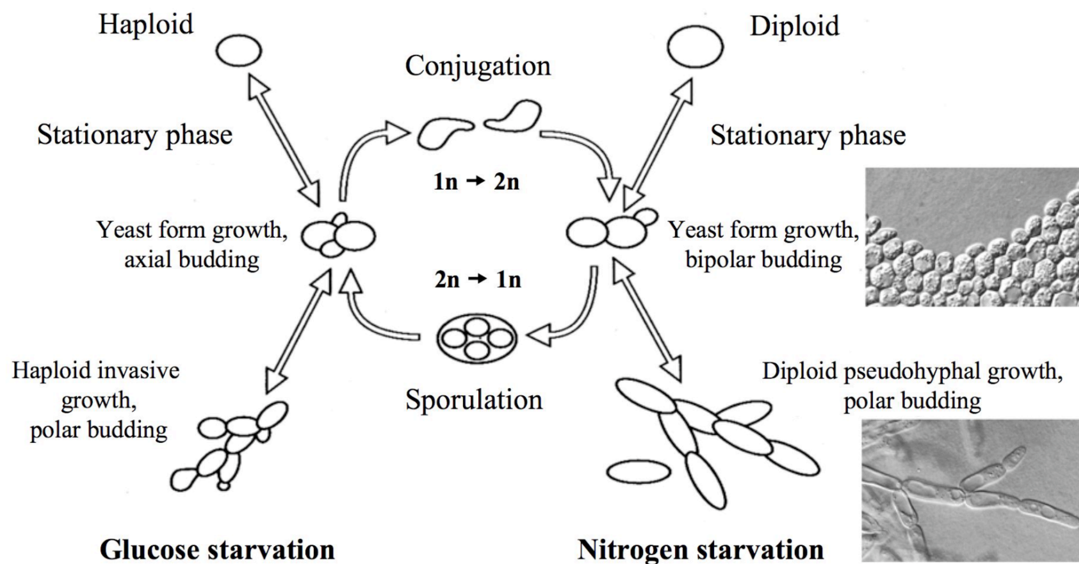


Fig. 1: Life cycle of *Saccharomyces cerevisiae* (adapted from Mösch (2000)).

Both haploid and diploid yeast cells have three growth options. They can either grow vegetatively in the yeast form or arrest growth in the stationary phase and switch to an invasive growth mode, respectively. Haploid cells (1n) are able to conjugate with respective mating partner to form diploids (2n) which in turn can sporulate to form haploids. Nitrogen starvation causes the formation of multicellular, filamtentous networks of diploids, called pseudohyphae. Haploid invasive growth occurs during glucose limitation leading to the formation of small microfilaments.

Diploid yeast cells can either grow as yeast with unicellular ellipsoid cell morphology or switch into filamentous growth upon nitrogen starvation. Diploid pseudohyphal growth of *S. cerevisiae* is characterized by chains of long, thin and elongated cells, which are attached to each other (Gimeno *et al.*, 1992; Mösch, 2000). The switch from yeast to pseudohyphae is accompanied by changes in several distinct processes. The budding pattern changes from bipolar to unipolar distal, which results in linear filamentous chains of cells. Furthermore, incomplete cell separation in pseudohyphal cells leads to formation of long multicellular chains where cells remain attached to each other.

The dimorphic switch in haploid yeast cells and diploid filamentous growth includes cell-cell adhesion and a direct substrate invasion whereas haploids show only limited changes in cell morphology (Roberts and Fink, 1994; Cullen and Sprague, 2000). The filamentous growth form of *S. cerevisiae* depends on its nutritional state and there are significant differences in triggering for haploid or diploid filaments.

1.1.2 Environmental sensing system

Cellular development of *S. cerevisiae* is tightly controlled according to the nutrient availability. For optimal growth conditions haploid or diploid yeast cells are usually cultivated in media that provide an excess of all required nutrients. On such media containing a fermentable carbon source such as glucose and abundant nitrogen, both haploid and diploid cells favor growth in the yeast form. Upon nitrogen depletion diploid cells develop pseudohyphae, even in the presence of a fermentable carbon source. This growth mode can be suppressed using standard concentrations of ammonium, arginine, glutamine or glutamate. In contrast, standard amounts of proline, histidine or uracil act permissive for the pseudohyphal growth (Gimeno *et al.*, 1992). The responsible sensor system, that differentiates between divers nitrogen components and therefore control pseudohyphal growth is not completely understood. The fungal high affinity permease Mep2p was identified to function as ammonium sensor in this system. Cells lacking Mep2p do not undergo pseudohyphal growth or exhibit any change in the activity of nitrogen metabolic enzymes (Lorenz and Heitman, 1998). In addition to Mep2p, invasive growth requires the Npr1 kinase, and elements of the PKA and the mitogen-activated protein (MAP) kinase pathway, which finally acts as downstream effector of the ammonium receptor function of Mep2p (Rutherford *et al.*, 2008).

Beside nitrogen, carbon is a further nutrient relevant for pseudohyphal differentiation. The fermentable carbon sources that promote filamentous growth are glucose, galactose, sucrose, maltose and raffinose (Gimeno *et al.*, 1992; Kron *et al.*, 1994; Lorenz *et al.*, 2000b). One sensor for different carbon sources represents Gpr1p, which encodes a cell surface G-protein coupled receptor and interacts with the heterotrimeric GTP-binding protein alpha subunit Gpa2p and with Plc1p, a phosphatidyl-inositol-specific phospholipase C (Yun *et al.*, 1998; Ansari *et al.*, 1999; Kraakman *et al.*, 1999). This trimeric complex regulates pseudohyphal growth via the cAMP pathway. Furthermore, components of the Ras/cAMP pathway are involved in glucose sensing (Broach, 1991a, b). Upon nitrogen starvation, small GTP-binding protein Ras2p is activated which in turn induces hyperfilamentous growth in diploids (Mösch *et al.*, 1996). Filament formation can also be induced by growth in the presence of low concentrations of fusel alcohols (Dickinson, 1996; Lorenz *et al.*, 2000a; Dickinson, 2008).

Haploid yeasts also change their growth phenotype upon environmental stimuli. Contrary to diploid strains, Ras2p is required for haploid invasive growth differentiation under non-starvation conditions (Mösch *et al.*, 1999). However, glucose starvation also induces haploid invasive growth and biofilm formation (Cullen and Sprague, 2000; Reynolds and Fink, 2001).

Additionally, amino acid limitation represents a further nutritional signal that triggers adhesive growth in both haploid and diploid cells. This signal even overrides the presence of the adhesion suppressors nitrogen and glucose (Braus *et al.*, 2003). Adhesive growth during amino acid starvation requires the two proteins Gcn4p and Gcn2p, key factors of a genetic network called the 'general amino acid control' (GAAC). Deletion of either *GCN2* or *GCN4* results in an adhesion deficient phenotype when cells are starved for amino acids. Furthermore, Gcn4p is also required for pseudohyphal growth upon nitrogen starvation (Braus *et al.*, 2003).

1.1.3 Cell surface adhesion in *S. cerevisiae*

The linkage between metabolic and developmental processes is less pronounced in *S. cerevisiae*, but described for various multicellular organisms. Developmental processes in *Candida albicans* such as morgphogenesis and biofilm formation are directly connected with the response to the availability of amino acids (Tripathi *et al.*, 2002; Brega *et al.*, 2004).

A prerequisite for dimorphism of *S. cerevisiae* cells is the expression of the *FLO* gene family that encode for specific glycosyl-phosphatidylinositol (GPI)-linked cell-surface glycoproteins which include the five *FLO* (flocculation) genes *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11.* 'Flocculins' or rather 'adhesins' are characterized by a C-terminal GPI-anchor for adhesion, which is trimmed off at the plasma membrane before incorporation in the cell wall (Lipke and Ovalle, 1998; Pittet and Conzelmann, 2007). The central part is variable in length and consists of highly repeated amino acid sequences that are heavily N- and O-glycosylated (Dranginis *et al.*, 2007). These unstable repeats drive slippage and recombination reactions within and between *FLO* genes leading to a constant generation of novel *FLO* alleles and pseudogenes (Verstrepen *et al.*, 2004; Verstrepen *et al.*, 2005). The N-terminal part of the protein is required for ligand binding and thus is thought to confer the specificity of adhesion phenotypes (Kobayashi *et al.*, 1998; Zupancic *et al.*, 2008).

The *FLO* gene family evolves and diverges very quickly (Hahn *et al.*, 2005), and in contrast to the common domain structure, different families of adhesins are expressed in different yeasts. The laboratory strain S288c, for example is impaired in adhesion, biofilm formation and pseudohyphal development, as it carries a nonsense mutation in the *FLO8* regulatory gene encoding one of the main transcriptional activators of the *FLO* genes (Liu *et al.*, 1996). Repair of *FLO8* results in the expression of *FLO1* and *FLO11*, whereas the other *FLO* genes are transcriptionally silenced (Halme *et al.*, 2004; Verstrepen *et al.*, 2004; Fichtner *et al.*, 2007). In the *S. cerevisiae* laboratory strain Σ1278b the Flo8p binding site in the *FLO1* promoter has been mutated and therefore only *FLO11* can be induced during pseudohyphal growth of this yeast strain (Fichtner *et al.*, 2007).

The *FLO11* gene, which is also named *MUC1* (Lambrechts *et al.*, 1996), encodes a (GPI)-anchored cell wall adhesin, which does not only mediate diploid pseudohyphal development (Gimeno *et al.*, 1992; Mösch and Fink, 1997), but also haploid invasive growth (Roberts and Fink, 1994; Guo *et al.*, 2000) or biofilm formation (Reynolds and Fink, 2001). *FLO11* is almost silenced in diploids growing in the yeast form resulting in hardly any detectable mRNA (Braus *et al.*, 2003). The level of *FLO11* gene expression reflects differences in nutrient supply for diploids and haploids (Braus *et al.*, 2003). In diploids, nitrogen starvation causes activation of *FLO11* and subsequently leads to pseudohyphal growth enabling the penetration of substrates in order to forage for nutrients (Liu *et al.*, 1993; Lo and Dranginis, 1998; Robertson and Fink, 1998; Gagiano *et al.*, 2002). In haploids, glucose starvation leads to an activation of *FLO11* expression and causes invasive growth and biofilm formation (Cullen and Sprague, 2000; Reynolds and Fink, 2001). Amino acid limitation is a further nutritional signal that activates *FLO11* expression and therefore haploid invasive growth and diploid pseudohyphal development of *S. cerevisiae* (Braus *et al.*, 2003).

1.1.4 Signal transduction pathways for adhesion

Regulation of *FLO11* expression is relatively complex as its promoter covers a region of approximately 3 kb and therefore is one of the largest promoters to be found in *S. cerevisiae*. It integrates multiple inputs from different pathways such as the cAMP pathway, the MAPK cascade, the mating type, and nutritional signals (Rupp *et al.*, 1999). The two main signal transduction pathways required for the regulation of haploid

invasive growth and diploid pseudohyphal filamentous growth are the cAMP-dependent (PKA) pathway and the highly conserved mitogen-activated protein kinase (MAPK) cascade (Fig. 2) (Kronstad *et al.*, 1998; Mösch, 2000; Elion *et al.*, 2005; Qi and Elion, 2005; Sengupta *et al.*, 2007).

The small GTP-binding protein Ras2p possesses a prominent role in both pathways. Activated Ras2p stimulates the adenylate cyclase Cyr1p, and therefore, leads to increased intracellular cAMP levels. High levels of cAMP in turn activate the protein kinase A (PKA) by removing the inhibitory subunit Bcy1p from one of the three catalytic subunits named Tpk1p, Tpk2 and Tpk3p (Broach, 1991a). Although, all three subunits are redundant for viability, only Tpk2p is required for activating filamentous growth and pseudohyphal development (Robertson and Fink, 1998; Robertson *et al.*, 2000). The antagonistic acting transcription factors Flo8p and Sfl1p are known targets of the PKA. Phosphorylation activates Flo8p and thus *FLO11* expression (Pan and Heitman, 1999; Rupp *et al.*, 1999; Pan and Heitman, 2002) whereas Sfl1p acts as repressor (Robertson and Fink, 1998).

Activated Ras2p also affects filamentous growth via a further GTP-binding protein, Cdc42p, which plays an essential role in regulating proliferation and differentiation in all eukaryotes (Mösch *et al.*, 1996; Johnson, 1999). Cdc42p in turn stimulates the MAPK cascade consisting of the protein kinases Ste20p (MAPKKKK), Ste11p (MAPKKK), Ste7p (MAPKK) and Kss1p (MAPK). Finally, the cascade results in phosphorylation of the transcription factor Ste12p in response to environmental stimuli (Madhani and Fink, 1997). Ste12p teams up with Tec1p and together they activate target genes required for diploid pseudohyphal growth and haploid invasive growth (Bürglin, 1991; Madhani and Fink, 1997; Mösch and Fink, 1997) by binding as heterodimer to filamentous response elements (FREs) in the promoter regions, including *TEC1* itself (Madhani and Fink, 1997) and *FLO11* (Lo and Dranginis, 1998). Additionally, Tec1p alone is able to activate target genes via Tec1p binding sites (TCS) (Köhler *et al.*, 2002; Heise *et al.*, 2010). In the absence of stimuli, Kss1p remains unphosphorylated and thus binds to Ste12p and therefore prevents Ste12p-dependent activation of *FLO11* expression or other target genes (Bardwell *et al.*, 1998).

Mss11p has been described as the pivotal element underlying all of these regulatory networks controlling *FLO11* expression. Mss11p is essential for functionality of Tpk2p, Flo8p and Tec1p, respectively, and thus plays an important role in both

MAPK cascade and cAMP pathway (van Dyk *et al.*, 2005). In *Saccharomyces diastaticus* Mss11p forms a heterodimer with Flo8p, which activates expression of *STA1* harbouring a similar promoter as *FLO11* in *S. cerevisiae* (Kim *et al.*, 2004). Furthermore, Flo8p and Mss11p are the major regulators of *FLO1* expression (Fichtner *et al.*, 2007).

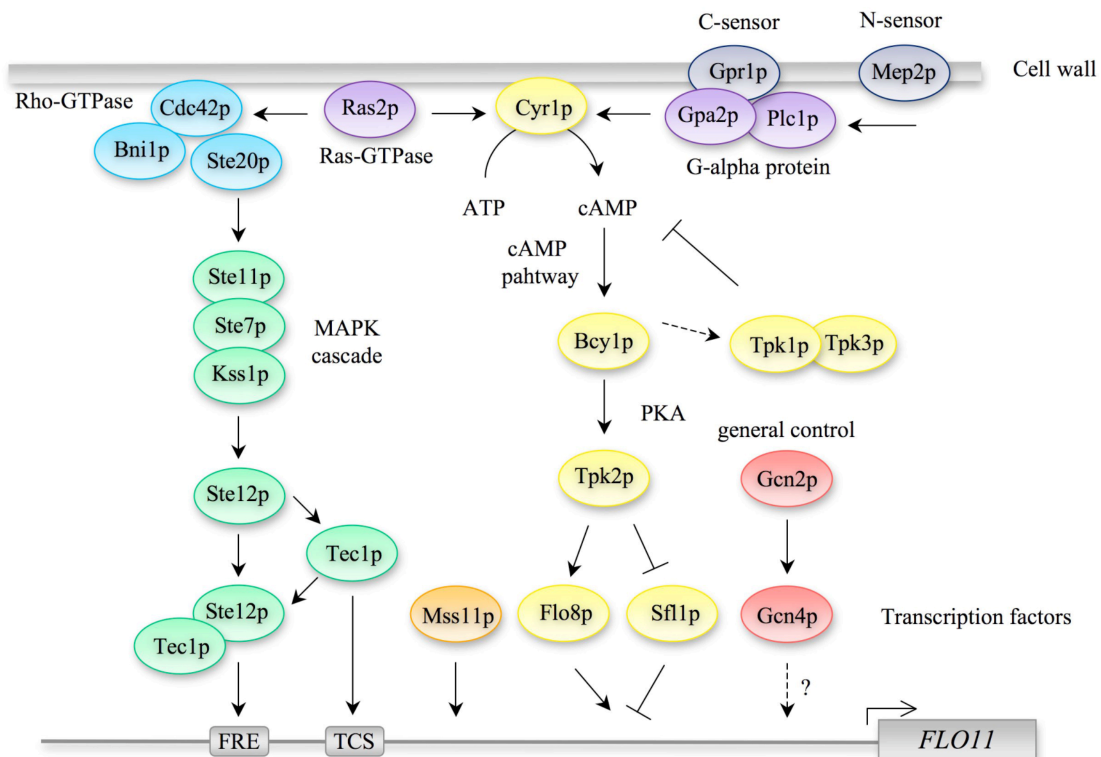


Fig. 2: Model of signalling pathways regulating adherence and pseudohyphal growth in *S. cerevisiae* (modified according to Mösch (2000) (see text for details).

In addition to the Mss11p-related networks, *FLO11* is also regulated by amino acid starvation via elements of the 'general amino acid control'. Amino acid starvation-induced adhesion is independent of the pseudohyphal MAPK cascade, but requires the proteins Tpk2p and Flo8p which represent the central elements of the cAMP pathway. Upon amino acid starvation uncharged tRNAs accumulate in the cell and activate the sensor kinase Gcn2p which in turn phosphorylates the eukaryotic translational initiation factor 2 (eIF2) and finally results in a translational derepression of *GCN4* (Hinnebusch, 1997) (described in more detail in section 1.3.3). Both Gcn2p and Gcn4p are necessary for adhesion and *FLO11* expression in response to amino acid starvation. Furthermore, Gcn4p is also required for haploid invasive growth and diploid pseudohyphal

development (Braus *et al.*, 2003). So far, no direct binding of Gcn4p to the *FLO11* promoter has been shown which suggests an indirect regulation of *FLO11* expression or in concert with other transcription factors. A connection between the 'general amino acid control' and adherence-dependent differentiation processes has also been described for the human pathogen *C. albicans* (Tripathi *et al.*, 2002; Tournu *et al.*, 2005). Herein, both Ras-cAMP pathway and MAPK cascade are involved in cellular differentiation (Lo *et al.*, 1997; Ernst, 2000; Brown *et al.*, 2007).

Additionally, the Gβ-WD40-protein Cpc2p/Asc1p was identified to be involved in regulation of *FLO11* expression and therefore deletion of *CPC2* results in non-adherent cells and pseudohyphal growth is impaired (Valerius *et al.*, 2007). These findings are in contrast to its predicted role as inhibitor of the Gα-protein Gpa2p and therefore its resulting role in the cAMP pathway (Zeller *et al.*, 2007).

1.2 Relevance of dimorphism and adhesion

Phenotypic switching from a nonpathogenic mold in the soil to a pathogenic fungus not only depends on its nutrient supply as in *S. cerevisiae,* but can also be caused by temperature changes as in *Candida* spp., *Histoplasma capsulatum* or *Coccidioides immitis*. Furthermore, alterations in pH and addition of serum can induce the switch between saprophytic and pathogenic growth forms (Sánchez-Martínez and Pérez-Martín, 2001; Klein and Tebbets, 2007). Dimorphic pathogenic fungi are responsible for primary mycoses such as histoplasmosis or coccidioidomycosis in human, and include opportunistic pathogens as *C. albicans* or *Penicillium marneffei,* which cause diseases in immuno-compromised individuals. Beside ascomycetes, some basidiomycetes also represent dimorphic pathogenic fungi. *Cryptococcus neoformans,* for example, is an encapsulated pathogenic yeast which can cause cryptococcocal disease in healthy and immuno-compromised people (San-Blas *et al.*, 2000). The basidiomycete *Ustilago maydis* in turn is a dimorphic phytopathogen which causes corn smut disease by infection through a dikaryotic mycelium (Martínez-Espinoza *et al.*, 1997; Sánchez-Martínez and Pérez-Martín, 2001).

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

Beside these developmental changes, starvation for nutrients also activates systems to ensure a satisfactory amino acid supply. Those systems sense the intra- and extracellular amino acid availability and enable yeast cells to adapt to changes by a direct amino acid uptake from the environment, recycling them through protein degradation and synthesizing all 20 amino acids *de novo*, respectively (Braus *et al.*, 2004). Such regulatory networks were first described for the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* and known as 'cross-pathway control' (CPC) (Carsiotis and Jones, 1974; Carsiotis *et al.*, 1974; Piotrowska *et al.*, 1980). The lack of one single amino acid stimulates the activation of mostly all 20 amino acid pathways in *N. crassa* (Barthelmess and Kolanus, 1990; Kolanus *et al.*, 1990). In the bakers' yeast *S. cerevisiae* this regulatory network is known as 'general amino acid control' (GAAC) and stimulates the transcription of more than 70 amino acid biosynthetic genes of 12 different pathways, pathway specific activators and genes encoding diverse aminoacyl-tRNA synthetases (Natarajan *et al.*, 2001; Hinnebusch, 2005).

1.3.1 The general amino acid control in *S. cerevisiae*

The GAAC regulatory network is not only induced by amino acid starvation or imbalances but also by other environmental stimuli including limited supply of glucose (Yang *et al.*, 2000), purines (Mösch *et al.*, 1991) and tRNA synthetases (Meussdoerffer and Fink, 1983), respectively. Furthermore, diverse stress conditions such as UV radiation (Engelberg *et al.*, 1994), high salinity (Goossens *et al.*, 2001), oxidative stress (Mascarenhas *et al.*, 2008) and treatment with rapamycin or methyl methanesulfonate (MMS) (Natarajan *et al.*, 2001; Valenzuela *et al.*, 2001) stimulate the activity of the GAAC network (Fig. 3). Under laboratory conditions, amino acid analogs such as 3-amino-1,2,4-triazole (3AT) (Klopotowski and Wiater, 1965) or 5-methyl-tryptophan (5MT) (Schürch *et al.*, 1974) were used to mimic amino acid starvation since the GAAC is not induced by growth on minimal medium. Yeast cells have a high basal expression of amino acid biosynthesis genes and therefore are able to synthesize all 20 amino acids *de novo.* Alternatively, the general amino acid control can be activated by mutations of biosynthetic enzymes resulting in auxotropic or bradytropic mutant strains (Kornitzer *et al.*, 1994).

In *S. cerevisiae* numerous *trans*-acting factors were identified required for regulation of the GAAC and subdivided into two groups. They were classified as positive regulatory *GCN* genes (general control non-derepressable) if mutations lead to a loss of transcriptional activation of the GAAC upon amino amino acid starvation. In contrast, they were described as negative regulator *GCD* genes (general control derepressed) in case of resulting in constitutive active general amino acid control (Harashima and Hinnebusch, 1986).

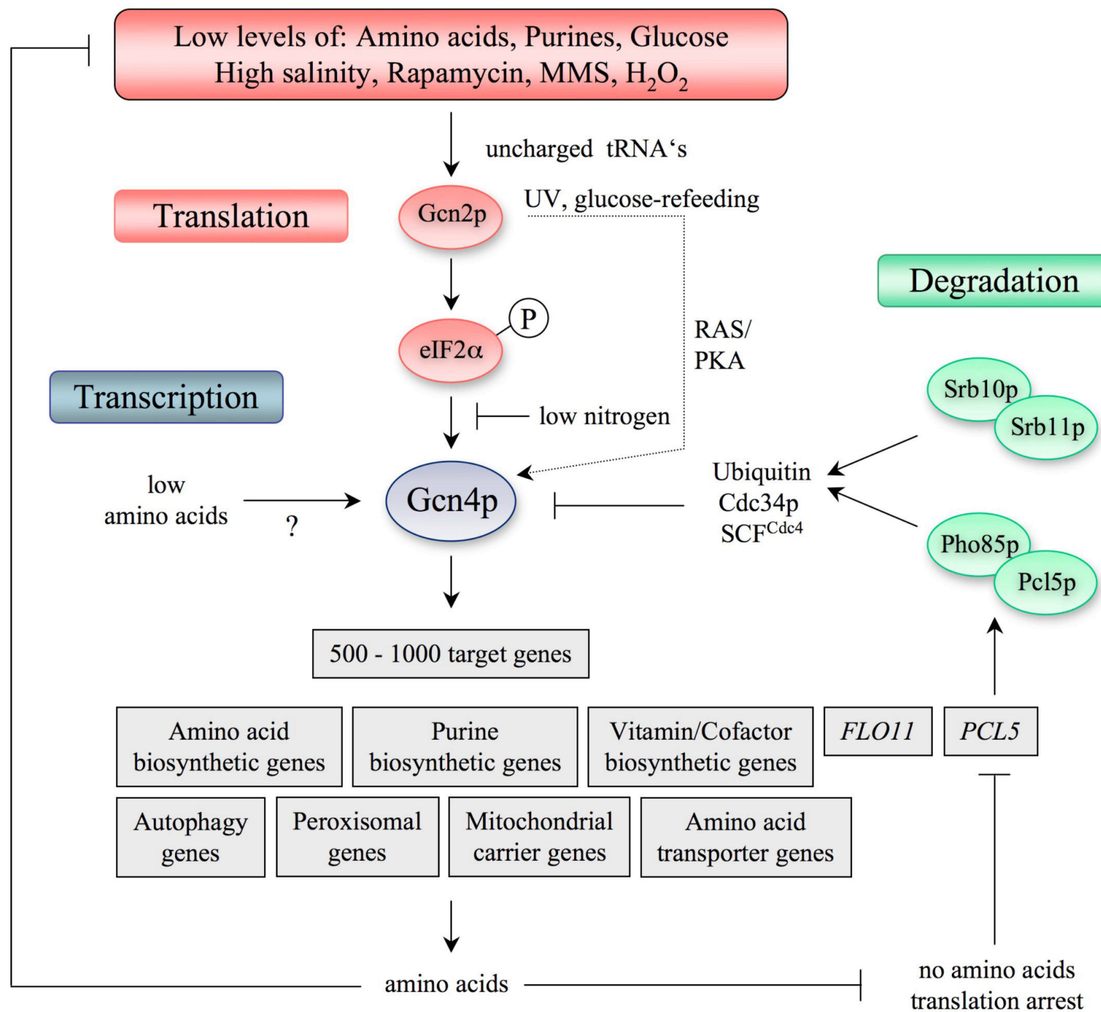


Fig. 3: Model for the major mechanisms regulating Gcn4p levels and GAAC target genes in *S. cerevisiae.*

In response to different environmental stimuli the transcriptional activator Gcn4p is regulated at levels of translation, transcription and protein degradation. This results in transcriptional regulation of more than 500 genes belonging to different biosynthetic pathways (see text for details).

Synthesis of Gcn4p, the global transcription factor of this regulatory network, is associated with the activation of the GAAC in the yeast *S. cerevisiae*. In amino acid starved cells Gcn4p function is required, directly or indirectly, for the transcription of at least 539 genes whereas its targets are not only involved in amino acid and nitrogen metabolism but also in vitamin or cofactor biosynthesis, peroxisome proliferation, autophagy, amino acid transporting, mitochondrial carrying and much more (Fig. 3) (Natarajan *et al.*, 2001). Gcn4p represents the functional homologue to CpcA in *A. nidulans* and *A. fumigatus*, CPC-1 in *N. crassa* and ATF4 in higher eukaryotes, respectively.

1.3.2 The transcription factor Gcn4p of *S. cerevisiae*

Gcn4p represents the central element of the GAAC network. This transcription factor encodes a polypeptide comprising 281 amino acids with a molecular weight of 31 kDa (Hinnebusch, 1984; Thireos *et al.*, 1984). It is the prototype of the family of alkaline leucine zipper transcription factors and structurally resembles a coiled coil (Weiss *et al.*, 1990). The sixty C-terminal amino acids of Gcn4p include the leucine zipper domain (LZ, aa 249 – 281) that mediates dimerization and the basic DNA binding domain (DB, aa 221 – 249) where interactions with DNA occur (Hope and Struhl, 1987) (Fig. 4). The leucine zipper motif is characterized by 4 or 5 leucine residues separated by 7 amino acids (Landschulz *et al.*, 1988). The highly conserved bZIP structural motif is found in many other eukaryotic transcription factors including the mammalian proteins JUN and FOS and is also present in ATF4 (Chevray and Nathans, 1992). In contrast to its mammalian homologues, yeast Gcn4p can only bind as a homodimer to a specific 9 bp palindromic nucleotide sequence (5'-ATGA(C/G)TCAT-3') (termed Gcn4 protein recognition element (GCRE)) located upstream of many genes induced by amino acid starvation (Hope and Struhl, 1986; Oliphant *et al.*, 1989). Gcn4p can also bind to naturally occurring variants of this sequence (TGATTCA, TGACTCT, TGACTGA, TGACTAT and ATGACTCT) and therefore using computer algorithm this consensus site was generalized to RRRWGASTCA (with R = purine, W = T or A, and S = G or C) (Natarajan *et al.*, 2001). Furthermore, it was shown that Gcn4p also bind to GCRE half-sites with high affinity *in vitro* (Hollenbeck and Oakley, 2000; Chan *et al.*, 2007).

Transcriptional induction of the respective target genes is mediated by an activation domain, which exists in the N-terminus of Gcn4p and spans approximately half of the protein (Drysdale *et al.,* 1995). This activation domain is subdivided into the N-terminal activation domain ((NTAD) aa 17 – 98) and the central acidic activation domain ((CAAD) aa 107 – 144). Consisting of mainly acidic and 8 hydrophobic amino acids, important for activation, both subdomains have nearly identical activation potential (Drysdale *et al.*, 1995). Furthermore, a so-called PEST region is located between these two domains, consisting of the amino acids 99 to 106. This region is named according to characteristic amino acids (Rechsteiner and Rogers, 1996) and is along with phosphorylation of threonine 165 by the cyclin-dependent kinase Pho85p responsible for the instability of Gcn4p in sated cells (Kornitzer *et al.*, 1994). The regulation of Gcn4 protein stability is described in more detail in section 1.3.4.

Finally there are two different nuclear localization sequences (NLS) to ensure a nuclear localization of Gcn4p and in turn transcriptional activity (Pries *et al.*, 2002). NLS1 consists of the amino acids 167 – 200 and acts as an ancillary motif. NLS2 is located in the DNA binding domain (aa 231 – 249) and resembles a classical bipartite NLS-motif. Nuclear import of Gcn4p requires also the presence of the karyopherins Srp1p and Kap95p (Pries *et al.*, 2004).

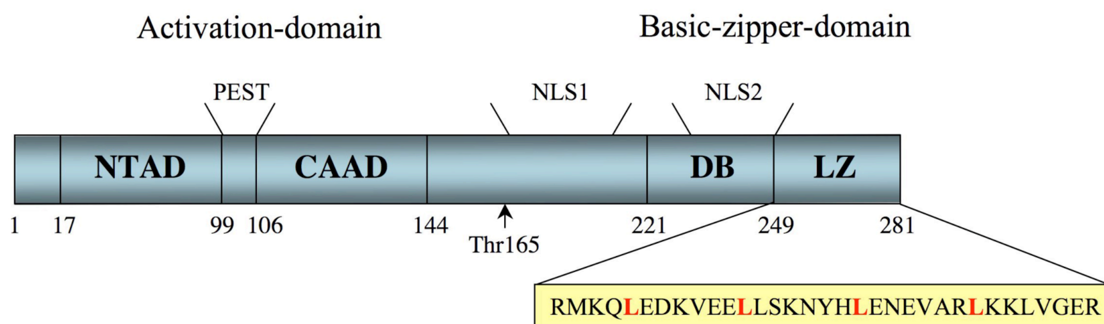


Fig. 4: Schematic structure of Gcn4p.

The transcription factor Gcn4p comprises 281 amino acids and consists of an N-terminal activation domain (NTAD, aa 17 – 98), a central acidic activation domain (CAAD, aa 107 – 144), a DNA binding domain (DB, aa 221 – 249) and a dimerization domain (LZ, leucine zipper, aa 249 – 281). The PEST region (aa 99 – 106) spans between both N-terminal domains, which is responsible for the instability of the protein. Threonine 165 represents a site of phosphorylation for the protein kinase Pho85p, which is necessary for rapid protein degradation. The nuclear localization sequences NLS1 (aa 167 – 200) and NLS2 (aa 231 – 249) are required for nuclear import of Gcn4p.

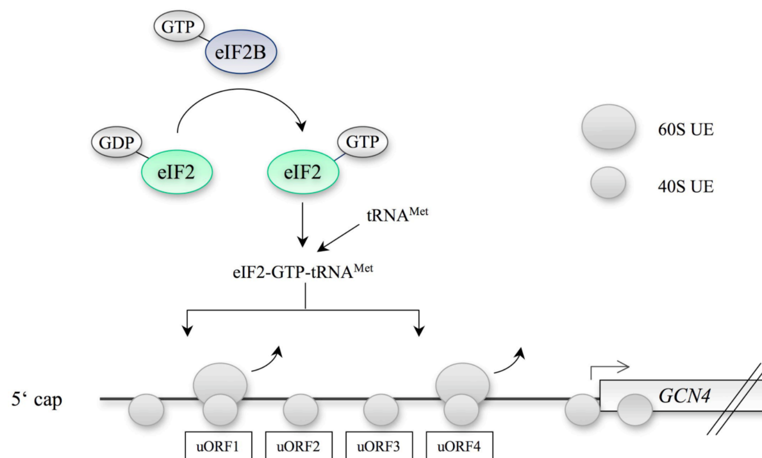
1.3.3 Translational regulation of Gcn4p

The amount of Gcn4p in the cell is controlled by multiple mechanisms and the translational control is herein an important step (Fig. 5). The regulation of *GCN4* mRNA translation in the cytoplasm is mediated by four small upstream open reading frames (uORFs) present in its 5'-untranslated region (Hinnebusch, 1984; Thireos *et al.*, 1984). When amino acids are abundant these uORFs prevent the translation of *GCN4* mRNA by limiting the flow of scanning ribosomes from the cap site to the *GCN4* initiation codon (Hinnebusch, 1997, 2005). The first and the fourth uORF are sufficient for nearly wild type translational control, whereas the second and the third uORF have only weak influence on *GCN4* translation (Mueller and Hinnebusch, 1986). Translational initiation starts with binding of the small 40S ribosomal subunit to the capped 5'-end of the *GCN4* mRNA and results in a competent 43S pre-initiation complex consisting of the small ribosomal subunit and a ternary complex (eIF2-GTP and the initiation tRNA (Met-$tRNA_i^{Met}$)). This pre-initiation complex starts downstream migration and at the AUG start codon of uORF1 teams up with the 60S ribosomal subunit to form an 80S initiation complex and in turn translation of uORF1 is initiated. During this process, the GTP bound to eIF2 is hydrolyzed resulting in an inactive eIF2-GDP binary complex. Accordingly, the 80S ribosome dissociates at the stop codon of uORF1 from the mRNA and about half of the small ribosomal subunits remain attached to the *GCN4* mRNA. The guanine nucleotide exchange factor eIF2B has to recycle eIF2-GDP to eIF2-GTP before the 40S ribosomal subunit reaches uORF4 to re-initiate translation. After translation of uORF4 the 80S dissociates again, preventing therefore translation of the real *GCN4*-ORF.

Starving or stress conditions lead to an accumulation of uncharged tRNAs in the cell which in turn activate the C-terminal histidyl-tRNA synthetase (HisRS) related domain of the sensor kinase Gcn2p (Dever *et al.*, 1992; Lanker *et al.*, 1992). For full activation of Gcn2p, the N-terminal protein kinase domain has to interact with the Gcn1p-Gcn20p protein complex (Garcia-Barrio *et al.*, 2000; Sattlegger and Hinnebusch, 2005). Activated Gcn2p phosphorylates the α-subunit of eIF2-GDP on Ser51, which inhibits the guanine nucleotide exchange factor eIF2B and thus prevents recycling of eIF2-GDP to eIF2-GTP. Consequently, the amounts of ternary complexes are reduced and as a consequence many ribosomes scan the distance between uORF1 and uORF2 without rebinding the ternary complex. The bypass of uORF4 enables the ribosomes to

re-initiate at the AUG start codon of the *GCN4*-ORF. Therefore, uORF1 is described as positive regulator for translation because translational initiation of *GCN4* is increased (Hinnebusch, 1997, 2005). In contrast, global protein synthesis is inhibited under such conditions to save nutrient resources. Cell division processes are also reduced.

A



B

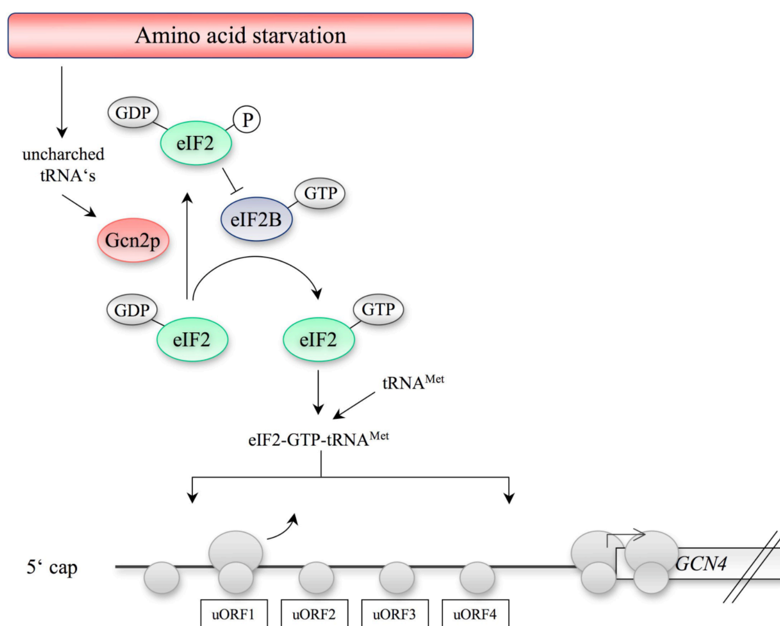


Fig. 5: Translational control of *GCN4* expression in *S. cerevisiae*.

(A) Under non-starvation conditions high levels of ternary complexes (eIF2-GTP-tRNA^{Met}) result in a re-initiation of translation at uORF4 whereas the real *GCN4*-ORF is not translated. This is due to the dissociation of the 80S ribosome, which happens after translation of uORF1 and uORF4.

(B) Amino acid starvation leads to an accumulation of uncharged tRNAs inside the cell which are in turn recognized by the sensor kinase Gcn2p. Activated Gcn2p thus inactivates the α-subunit of eIF2-GDP by phosphorlyation. This reduction leads to low levels of ternary complexes and that followed delayed re-initiation of the reassembled ribosome at uORF4. Thereupon, ribosomes bypass uORF4 and initiate *GCN4* translation.

A similar regulation was described for CpcA and ATF4, the Gcn4p homologues proteins in *Aspergilli* spp. and mammals. In response to stress stimuli phosphorylated eIF2α accumulates in the cell resulting in a bypass of uORF2 and thus uORF1 acts here also as positive regulator. Beside mGcn2p (mammalian Gcn2p) three further kinases exist in mammals, which are able to phosphorylate eIF2α upon different stress conditions such as amino acid starvation, ER stress and virus infections (Harding *et al.*, 2000; Hinnebusch, 2005).

1.3.4 Regulation of Gcn4 protein stability

The level of Gcn4p is not only regulated by a translational control in the cytoplasm but also underlies a tight protein stability control in the nucleus (Pries *et al.*, 2002). Under non-starvation conditions Gcn4p is a highly unstable protein with a half life of only a few minutes, whereas a limitation of amino acids increases the Gcn4p half life to up to 20 minutes (Kornitzer *et al.*, 1994). Its degradation is initiated by phosphorylation which is followed by the ubiquitin-proteasome system (Irniger and Braus, 2003). Two cyclin-dependent kinases (CDK), Pho85p and Srb10p, can initiate the Gcn4p degradation pathway (Meimoun *et al.*, 2000; Chi *et al.*, 2001). For activation of CDKs specific cyclin subunits are required whereby the specificity to the respective substrate is mediated (Jeffrey *et al.*, 1995; Huang *et al.*, 1998). The specific cyclins that mediate Gcn4p degradation in sated *S. cerevisiae* cells are Pcl5p and Srb11p (Shemer *et al.*, 2002). The initial step of rapid Gcn4p turnover in sated cells is the phosphorylation of Gcn4p at specific residues by the CDKs Pho85p and Srb10p in interaction with their specific cyclins. Phosphorylation marks Gcn4p for Lys48-poly-ubiquitination mediated by the SCF^{Cdc4} E3 ubiquitin ligase complex. The ubiquitin system ligates ubiquitin via an isopeptide bond to an internal lysine residue on the target protein. The specific Gcn4p E2 ubiquitin conjugating enzyme is Cdc34p which interacts with the ubiquitin ligase SCF^{Cdc4}. Finally the polyubiquitinated Gcn4p is degraded by the 26S proteasome (Kornitzer *et al.*, 1994; Meimoun *et al.*, 2000). This process is mediated by polyubiquitin receptors which bind to ubiquitinated proteins and target them to the 26S proteasome for degradation (Mayor *et al.*, 2005). Seong and co-workers identified the polyubiquitin receptor Rpn10p as receptor for ubiquitinated Gcn4p (Seong *et al.*, 2007). In addition to these specific phosphorylation and ubiquitination sites, a PEST region located between both activation domains is also responsible for the rapid turnover of

Gcn4p in sated cells. A deletion of this region results in stabilized Gcn4p (Kornitzer *et al.*, 1994).

Srb10p-dependent phosphorylation is constitutive and occurs independently of the availability of amino acids whereas phosphorylation by Pho85p-Pcl5p depends on the availability of amino acids (Meimoun *et al.*, 2000; Chi *et al.*, 2001). When amino acids are limited, Gcn4p gets stabilized with an increased half life of up to 20 minutes (Kornitzer *et al.*, 1994). The dissociation of the Pho85p-Pcl5p complex was identified as initial step leading to a stabilization of Gcn4p in amino acid starved cells (Bömeke *et al.*, 2006; Streckfuss-Bömeke *et al.*, 2009). In contrast, other studies predict that metabolic instability of Pcl5p is important for the proper response to amino acid starvation conditions (Shemer *et al.*, 2002). Furthermore, they suggest an autophosporylation-induced degradation of Pcl5p, which ensures that activity of the Pho85p-Pcl5p complex is self-limiting *in vivo* (Aviram *et al.*, 2008).

1.4 The 'Unfolded Protein Response'

The endoplasmic reticulum (ER) represents the first compartment of the secretory pathway and most of all cellular secretory and transmembrane proteins are folded and modified in the ER. Molecular chaperones and folding catalyzes which are resident in the ER lumen ensure the correct folding and oligomerziation (Gething and Sambrook, 1992; Ellgaard and Helenius, 2003). A variety of physiological or environmental stress conditions such as calcium depletion, glucose deprivation, hypoxia or misfolded proteins lead to an accumulation of misfolded or unfolded proteins in the ER lumen, which results in the induction of the 'unfolded protein response' (UPR) (Kozutsumi *et al.*, 1988; Patil and Walter, 2001; Ron, 2002; Schröder and Kaufman, 2005). Misfolded proteins are characterized by exoteric hydrophobic amino acids, which are recognized by the molecular Hsp70-chaperone BiP/Grp78 (binding protein/glucose regulated protein 78-Hsc3, in *Drosophila*; homologues to Kar2p in *S. cerevisiae*). At non-stress conditions Bip/Grp78 associates to different receptor proteins present in the ER lumen (Hendershot, 2004). In case of an accumulation of misfolded proteins in response to ER stress BiP preferably binds to the hydrophobic regions of unfolded or incompletely folded proteins to assist their refolding and preventing interactions of these proteins with surrounding molecules (Munro and Pelham, 1986; Bertolotti *et al.*, 2000; Rasheva and Domingos, 2009). As a consequence,

free receptor proteins are activated and this in turn triggers the UPR leading to a reduction in global protein synthesis and a specific up-regulation of stress-response proteins involved in divers processes including protein translocation, glycosylation, folding and degradation, lipid/inositol metabolism, vesicular trafficking, vacuolar protein sorting and cell wall biogenesis (Prostko *et al.*, 1993; Bertolotti *et al.*, 2000; Travers *et al.*, 2000; Kaufman, 2004). Finally, the UPR represents a signal transduction pathway that communicates between the ER and the nucleus and is conserved from yeast to mammals (Patil and Walter, 2001).

1.4.1 The UPR system in *S. cerevisiae*

Conserved from yeast to mammals is the sensing and response pathway that is transduced by Ire1p leading to an up-regulation of transcription levels of approximately 400 genes, i.e., 7% to 8% of the yeast genome (Cox *et al.*, 1993; Mori *et al.*, 1993; Travers *et al.*, 2000; van Anken and Braakman, 2005) (Fig. 6).

In *S. cerevisiae*, Ire1p senses the stress and mediates a signalling cascade to up-regulate responsive genes through an unusual *HAC1* mRNA splicing. Ire1p encodes a bifunctional transmembrane kinase/endoribonuclease consisting of an unfolded protein sensor domain in the ER lumen, a transmembrane domain and a cytosolic effector domain which contains an intrinsic serine/threonine kinase as well as an endoribonuclease in its C-terminus (Nikawa and Yamashita, 1992; Cox *et al.*, 1993; Mori *et al.*, 1993; Sidrauski and Walter, 1997; Liu *et al.*, 2002). Under basal conditions the chaperone BiP/Kar2p associates with the luminal domain of Ire1p thereby preventing homodimerization of Ire1p. Upon ER stress BiP/Kar2p preferably binds to unfolded proteins and thus the BiP/Kar2p-Ire1p complex dissociates and BiP/Kar2p is titrated away (Bertolotti *et al.*, 2000; Kimata *et al.*, 2003). Thereupon, Ire1p is able to oligomerize and to *trans*-autophosphorylate (Shamu and Walter, 1996; Tirasophon *et al.*, 1998) resulting in an activated cytosolic endonuclease effector domain (Sidrauski and Walter, 1997; Kohno, 2007). Though, activation of Ire1p is not dependent on BiP/Kar2p dissociation *per se*, because Ire1p lacking the region specific for BiP/Kar2p binding can still sense and signal ER stress (Kimata *et al.*, 2004; Oikawa *et al.*, 2007). In fact, there are indications for activation of Ire1p by direct binding to unfolded proteins (Credle *et al.*, 2005; Bernales *et al.*, 2006; Ron and Walter, 2007).

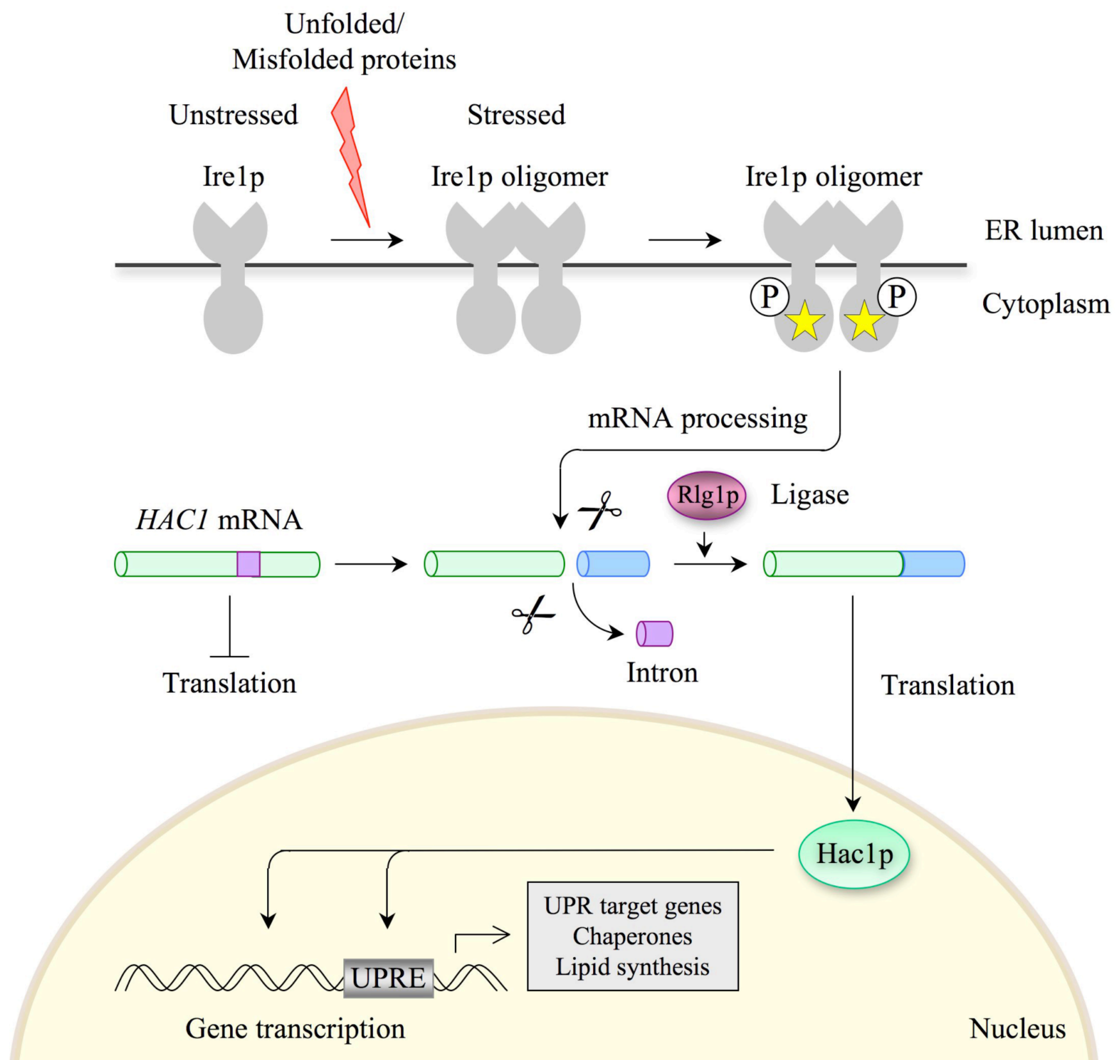


Fig. 6: Schematic illustration of the UPR system in *S. cerevisiae*.

The transmembrane endonuclease Ire1p is activated by ER stress, which in turn oligomerizes and *trans*-autophosphorylates. The endonuclease effector domain of Ire1p splices the *HAC1* mRNA in a non-canonical fashion. The tRNA ligase Rlg1p religates the *HAC1* transcript, which is then efficiently translated to the bZIP transcription factor Hac1p. Hac1p travels into the nucleus and increases the transcription of its target genes.

However, the only known substrate of Ire1p endoribonuclease activity is the *HAC1* mRNA (Niwa *et al.*, 2005) which encodes for a basic leucine zipper (bZIP) transcription factor that *trans*-activates target genes of the UPR (Cox and Walter, 1996; Mori *et al.*, 1996; Patil *et al.*, 2004). *HAC1* mRNA is constitutively synthesized as a precursor bearing a 252-nucleotide intron that blocks translation as the result of base pairing with a sequence in the 5'-untranslated region of the mRNA (Chapman and Walter, 1997; Kawahara *et al.*, 1997; Ruegsegger *et al.*, 2001). Ire1p recognizes two 'loop' structures in the *HAC1* mRNA and the endonuclease effector domain of Ire1p

splices the *HAC1* mRNA. Subsequently, the tRNA ligase Rlg1p religates causing exons to produce the mature, efficiently translated *HAC1* mRNA (Kawahara *et al.*, 1997; Sidrauski and Walter, 1997). As the level of Hac1p rises in the cell, the genes that harbor unfolded protein response elements (UPREs) within their promoters are induced at the transcriptional level (Cox and Walter, 1996). In yeast, there are approximately 381 transcriptional targets of the UPR that encode functions ranging from protein folding, protein translocation, and protein transport, to protein degradation within the secretory pathway (Travers *et al.*, 2000). UPRE was first identified as a 22-bp sequence that is necessary und sufficient for the induction of the UPR target gene Kar2p upon ER stress (Mori *et al.*, 1992). This consensus sequence was abbreviated to a partial palindrome with a spacer of one nucleotide (nt: CAGCGTG) that is essential for its function (Mori *et al.*, 1996). An additional transcriptional induction of *HAC1* can be achieved by increasing temperature parallel to UPR stress conditions (Leber *et al.*, 2004). This so-called super-UPR (S-UPR) response results in three- to four-fold increased amounts of *HAC1* mRNA and is independent of Ire1p.

The synthesis of Hac1p in response to ER stress is not only regulated on its translational level but also by mechanisms that regulate the rate of turnover of Hac1p, similar as described for the bZIP transcription factor Gcn4p (see section 1.3.4). Like Gcn4p, Hac1p is ubiquitinated by the SCF^{Cdc4} E3 ligase complex resulting in degradation by the 26S proteasome. Furthermore, phosphorylation by the CDK Srb10p marks Hac1p for ubiquitination, similar to Gcn4p, whereas phosphorylation by Pho85p was not observed so far. Hac1p also contains a PEST region, which is typically for rapid turnover transcription factors (Pal *et al.*, 2007).

In contrast to *S. cerevisiae*, the *HAC1* mRNA of *C. albicans* and higher eukaryotes only contains a 19 to 26 nucleotide long intron, which is also removed by non-canonical splicing in response to ER stress (Cox and Walter, 1996; Mulder *et al.*, 2004; Wimalasena *et al.*, 2008).

1.4.2 Role of Gcn4p in the UPR system of *S. cerevisiae*

At least 381 UPR target genes were identified in yeast whereas the predicted UPRE-1 consensus sequence (CAGNGTG) was absent in most of them (Travers *et al.*, 2000). Thereupon, Patil and co-workers identified two further UPR elements, which are recognized by Hac1p (UPRE-2, consensus sequence TACGTG; UPRE-3, consensus

sequence AGGAACAAC) (Patil *et al.*, 2004). Apart from its role as transcriptional activator of the GAAC, Gcn4p and its activator Gcn2p are required for induction of a majority of UPR target genes upon ER stress. A direct binding of Gcn4p could be demonstrated for UPRE-1 and UPRE-2 whereupon binding to UPRE-1 was Hac1p-dependent. In contrast, Gcn4p is not necessary for the regulation of genes without a recognizable UPRE, which represent half of all UPR targets. Both Hac1p and Gcn4p are bZIP transcription factors. Although a binding to DNA as heterodimer was speculated but it has not been proven yet (Patil *et al.*, 2004). A heterodimer consisting of ATF4 and XBP1, the mammalian homologous of Gcn4p and Hac1p, was also not detected so far.

Furthermore, Gcn4p levels rise soon after induction of ER stress whereas *GCN4* translation remains unaffected. Therefore, a stabilization of Gcn4p by forming a heterodimer with Hac1p is proposed. Due to the fact that eIF2α is phosphorylated under ER stress conditions (Cherkasova and Hinnebusch, 2003) an indirect activation of Gcn2p in response to ER stress is suggested (van Anken and Braakman, 2005). Although, Gcn4p levels increase in response to ER stress, it is underdosed to activate the GAAC nevertheless it is supposed that Gcn4p has a kind of 'fine-tuning' function in the UPR system (Patil *et al.*, 2004).

1.4.3 The UPR system of higher eukaryotes

The activation of the UPR in mammals is mediated by three distinct ER stress sensors: inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK). All of them are integral membrane proteins carrying a sensor domain in the ER lumen and a cytosolic effector domain (Rasheva and Domingos, 2009). The most conserved branch of the UPR is the sensing and response pathway, which is regulated by IRE1. In response to ER stress, IRE1 is activated in the same way as described for yeast and by unconventional splicing a 26 nucleotide long intron is removed from the XBP1-mRNA (X-box binding protein) which is the functional homolog to Hac1p in *S. cerevisiae*. The removal of the intron introduces a frame-shift and results in the active transcription factor XBP1(S) (Yoshida *et al.*, 1998; Shen *et al.*, 2001; Calfon *et al.*, 2002). Enzyme(s), which are responsible for religation of the exons have not been identified in higher eukaryotes. In contrast to yeast, where unspliced *HAC1* mRNA represses translation, both the precursor and

spliced form of XBP1 are translated in higher eukaryotes whereas the unspliced form (XBP1(U)) is not active as transcription factor. In fact, the unspliced form functions as negative feedback regulator of IRE1 signalling by binding and targeting XBP1(S) for degradation (Calfon *et al.*, 2002; Yoshida *et al.*, 2006). While in yeast Ire1p and Hac1p act in a linear pathway this is not the case for higher eukaryotes. Beside XBP1, IRE1 binds to other activators such as Traf2 (tumor necrosis factor receptor-associated factor 2), an upstream activator of the c-JUN N-terminal kinase (JNK) signalling pathway (Urano *et al.*, 2000). In addition, in *Drosophila* IRE1 promotes the degradation of specific mRNAs encoding ER target proteins which are located to the ER after ER stress to reduce protein synthesis (Hollien and Weissman, 2006). Both processes are independent of XBP1. Recent studies identified 13 novel mRNAs as targets of IRE1 (Oikawa *et al.*, 2010). Furthermore, analysis of the cleavage sites revealed a consensus sequence (CUGCAG) accompanied by a stem-loop structure, which is also present in known cleavage targets, such as XBP1.

Activation of ATF6 (activating transcription factor 6), a founding member of a novel class of metazoan specific ER stress transducers, represents a more rapid response to ER stress. The transmembrane precursor protein ATF6 consists of a cytosolic domain containing a bZIP motif, a transcription activation domain and an ER luminal domain that binds to BiP (Haze *et al.*, 1999; Wang *et al.*, 2000; Yoshida *et al.*, 2000; Shen *et al.*, 2005). Under ER stress, ATF6 is transported from the ER to the Golgi where it is successively cleaved by Golgi resident proteases (Ye *et al.*, 2000). The released cytosolic DNA-binding domain of ATF6 moves to the nucleus and activates the transcription of its target genes (Yoshida *et al.*, 1998; Haze *et al.*, 1999; Kokame *et al.*, 2001).

PERK (PKR-like ER kinase) represents the third ER stress transducer and resembles IRE1. Both PERK and IRE1 are transmembrane proteins consisting of a luminal stress sensing domain and a cytosolic domain with kinase activity. Similar to IRE1, PERK oligomerizes and *trans*-autophosphorylates upon ER stress, however, unlike IRE1, PERK phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) (Shi *et al.*, 1998; Shi *et al.*, 1999). As consequence, the global translational rate of newly synthesized proteins is reduced to protect the already stressed ER lumen (Harding *et al.*, 1999). Similar to yeast, phosphorylation of eIF2α leads to the translational up-regulation of specific mRNAs, such as the transcription factor ATF4

(activating transcription factor 4), the mammalian homologue of Gcn4p (for more details, see section 1.3.3) (Lu *et al.*, 2004; Vattem and Wek, 2004). Although there are many parallel translational controls upon eIF2α phosphorylation in all eukaryotes but in contrast, Gcn2p is not localized to the ER in yeast. Admittedly, it is shown that activation of the UPR is incomplete in *S. cerevisiae* cells deleted for *GCN2* (Patil *et al.*, 2004).

The protective mechanisms activated by the UPR are essential for viability of higher eukaryotes and is also involved in differentiation processes. In the case when misfolded proteins are not degraded they can aggregate into insoluble high order structures, which are associated with numerous neurodegenerative human diseases, such as Alzheimer's disease (Koo *et al.*, 1999; Petkova *et al.*, 2002). In contrast, activation of these mechanisms frequently correlates with tumorogenesis. For instance, several UPR downstream targets have been reported to be overexpressed in a variety of human tumors (Lee, 2001; Ma and Hendershot, 2004). Recent studies identified IRE1 as key regulator of angiogenesis and invasion in malignant glioma (Auf *et al.*, 2010). Both XBP1 and PERK are also involved in tumorogenesis (Romero-Ramirez *et al.*, 2004; Bi *et al.*, 2005; Koong *et al.*, 2006). In contrast, ATF4 mediates ER stress-induced cell death of neuroectodermal tumor cells in response to hemotherapeutic agents (Armstrong *et al.*, 2010).

In lower eukaryotes, such as *S. cerevisiae* or *C. albicans*, Hac1p/XBP1 is not essential for viability *per se*, but for *C. albicans* plays an important role in regulating the morphology and the expression of genes encoding cell surface proteins during ER stress factors that are important for virulence of this fungal pathogen (Wimalasena *et al.*, 2008). Furthermore, HacA is required for virulence of *A. fumigatus* (Richie *et al.*, 2009). In contrast, Hac1p/XBP1 represses differentiation processes in *S. cerevisiae* (Schröder *et al.*, 2000).

1.5 Aim of this work

In response to nutrient starvation, the bZIP transcription factor Gcn4p not only has a metabolic function as activator of the 'general amino acid control' but also is involved in adhesive growth and pseudohyphal development. Both developmental processes are regulated by the cell-surface flocculin Flo11p. One aim of this work was to separate these two functions. Therefore, specific leucines of the leucine zipper of Gcn4p were substituted and their functionality was determined using *GCRE6*- and *FLO11::lacZ* reporter. Developmental consequences were also documented by pseudohyphal development or adhesive growth tests. The stability of the Gcn4 mutant proteins was analyzed by promoter shut-off experiments.

In addition, the interplay of Gcn4p and Hac1p and therefore the connection between the 'general amino acid control' and the 'unfolded protein response' should be investigated. It is demonstrated that Gcn4p is required for Hac1p target gene expression under ER stress. Therefore, the role of Hac1p for the control of Gcn4p specific target gene expression in response to amino acid starvation was analyzed using *lacZ* reporter assays. The influence of Hac1p on the expression of *FLO11*, an unspecific target gene of Gcn4p, was also determined by reporter gene expression experiments and phenotypical tests, respectively, and a Hac1p-dependet UAS (upstream activation site) in the promoter of *FLO11* was defined. The rapid turnover of Hac1p bears a resemblance to the degradation of Gcn4p. Therefore so far not documented aspects were examined by protein synthesis shut-off assays.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 *Saccharomyces cerevisiae* strains

All yeast strains used in this study are listed in Table I. They are derivates of the *S. cerevisiae* Σ1278b genetic background (Gimeno *et al.*, 1992) unless otherwise stated. The *hac1* and *pho85* deletion strains RH3351, RH3352, RH3402, RH3403 and RH3426 were obtained by amplification of the kanamycin resistance cassette from the Euroscarf strain collection (Brachmann *et al.*, 1998) containing sequence homologous to the up- and downstream regions of the relevant gene and integration of deletion cassettes by homologous recombination in yeast strains RH2676, RH2816, RH2819 and RH3401. Positive transformants could be selected on YEPD medium supplemented with 200 µg/ml Geneticin G418 sulfate (CARL ROTH GmbH, Karlsruhe, Germany). For genetic crosses the kanamycin resistance cassette of RH3352 and RH3403 was replaced by a nourseothricin resistance cassette, which was amplified from plasmid pAG25 and transformants (RH3404 and RH3405) could be selected on YEPD medium containing 100 µg/ml Nourseothricin (clonNAT, WERNER BIOAGENTS, Jena, Germany). Homo- and heterozygous diploid strains RH3412 – RH3416 were obtained by crossing RH2676, RH3351 or RH3402 with strain RH2819, RH3404 or RH3405. Haploid yeast strains RH3360, RH3406, RH3407 and RH3408 were obtained by introducing the *FLO11::lacZ::URA3*-cassette, using *Apa*I linearized plasmid pME2213, into the *URA3* locus of yeast strains RH2676, RH2816, RH3351 and RH3402. The *GCRE6::lacZ::URA3* reporter gene cassette was introduced in the same four haploid yeast strains by transformation with *Stu*I linearized *GCRE6::lacZ* reporter construct pME1112 to obtain yeast strains RH3363, RH3409, RH3410 and RH3411. The haploid *FLO11*- and *GCRE6::lacZ* containing *MAT**a*** strains were crossed with *MAT*α strains RH2819, RH3352, RH3404 or RH3405 to produce diploid strains RH3349, RH3350, RH3362 and RH3417 – RH3425. All gene deletions, integrations, or replacements were confirmed by PCR and Southern blot analysis (Southern, 1975).

Table I. *Saccharomyces cerevisiae* strains used in this study.

Strain	**Genotype**	**Source**
BY4741	*MAT**a**, his3::hisG, leu2::hisG, met15::hisG, ura3::hisG* (S288c-background)	(Brachmann *et al.*, 1998)
Y00249	*MAT**a**, his3::hisG, leu2::hisG, met15::hisG, ura3::hisG, Δgcn4::kanMX4* (S288c-background)	(Brachmann *et al.*, 1998)
Y02797	*MAT**a**, his3::hisG, leu2::hisG, met15::hisG, ura3::hisG, Δpho85::kanMX4* (S288c-background)	(Brachmann *et al.*, 1998)
Y05650	*MAT**a**, his3::hisG, leu2::hisG, met15::hisG, ura3::hisG, Δhac1::kanMX4* (S288c-background)	(Brachmann *et al.*, 1998)
RH2398	*MAT**a**/α, ura3-52/ura3-52::GCRE6::lacZ::URA3, his3::hisG/HIS3, leu2::hisG/leu2::hisG, trp1::hisG/trp1::hisG, Δgcn4::LEU2/Δgcn4::LEU2*	(Grundmann *et al.*, 2001)
RH2520	*MAT**a**, ura3-52* (S288c-background)	(Grundmann *et al.*, 2001)
RH2656	*MAT**a**/α, ura3-52/ura3-52, trp1::hisG/TRP1*	(Braus *et al.*, 2003)
RH2658	*MAT**a**/α, ura3-52/ura3-52, leu2::hisG/leu2::hisG, trp1::hisG/TRP1, Δgcn4::LEU2/Δgcn4::LEU2*	(Braus *et al.*, 2003)
RH2661	*MAT**a**/α, ura3-52/ura3-52, trp1::hisG/TRP1, Δflo11::kanMX4/Δflo11::kanMX4*	(Braus *et al.*, 2003)
RH2676	*MAT**a**, ura3-52, leu2::hisG, trp1::hisG, Δgcn4::LEU2*	Grundmann, pers. comm.
RH2681	*MAT**a**, ura3-52, trp1::hisG, Δflo11::kanMX4*	Grundmann, pers. comm.
RH2693	*MATα, ura3-52::FLO11::lacZ::URA3, leu2::hisG, trp1::hisG, Δgcn4::LEU2*	Grundmann, pers. comm.
RH2694	*MAT**a**/α, ura3-52/ura3-52, leu2::hisG/leu2::hisG, trp1::hisG/trp1::hisG, Δgcn4::LEU2/Δgcn4::LEU2*	Grundmann, pers. comm.
RH2695	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, leu2::hisG/leu2::hisG, trp1::hisG/trp1::hisG, Δgcn4::LEU2/Δgcn4::LEU2*	Gundmann, pers. comm.
RH2697	*MAT**a**, ura3-52::GCRE6::lacZ::URA3, leu2::hisG, trp1::hisG, Δgcn4::LEU2*	Grundmann, pers. comm.
RH2816	*MAT**a**, ura3-52, his3::hisG::HIS3, trp1::hisG*	(Strittmatter *et al.*, 2006)
RH2819	*MATα, ura3-52, his3::hisG::HIS3, leu2::hisG*	(Strittmatter *et al.*, 2006)

Table I. *Saccharomyces cerevisiae* strains used in this study, continued.

Strain	Genotype	Source
RH3278	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, his3::hisG::HIS3/his3::hisG::HIS3, leu2::hisG/LEU2, trp1::hisG/TRP1,*	(Bömeke, 2006)
RH3279	*MATα, ura3-52, his3::hisG, trp1::hisG, Δpcl5::HIS5*	(Bömeke, 2006)
RH3349	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, leu2::hisG/leu2::hisG, trp1::hisG/trp1::hisG, Δgcn4::LEU2/Δgcn4::LEU2, Δhac1::kanMX4/Δhac1::kanMX4::natMX4*	This study
RH3350	*MAT**a**/α, ura3-52/ura3-52::GCRE6::lacZ::URA3, leu2::hisG/leu2::hisG, trp1::hisG/trp1::hisG, Δgcn4::LEU2/Δgcn4::LEU2, Δhac1::kanMX4/Δhac1::kanMX4::natMX4*	This study
RH3351	*MAT**a**, ura3-52, trp1::hisG, Δhac1::kanMX4*	This study
RH3352	*MATα, ura3-52, leu2::hisG, Δhac1::kanMX4*	This study
RH3360	*MAT**a**, ura3-52::FLO11::lacZ::URA3, trp1::hisG, Δhac1::kanMX4*	This study
RH3362	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, leu2::hisG/LEU2, trp1::hisG/TRP1, Δhac1::kanMX4/Δhac1::kanMX4*	This study
RH3363	*MAT**a**, ura3-52::GCRE6::lacZ::URA3, trp1::hisG, Δhac1::kanMX4*	This study
RH3401	*MATα, ura3-52, leu2::hisG, trp1::hisG, Δgcn4::LEU2*	Grundmann, pers. comm.
RH3402	*MAT**a**, ura3-52, leu2::hisG, trp1::hisG, Δgcn4::LEU2, Δhac1::kanMX4*	This study
RH3403	*MATα, ura3-52, leu2::hisG, trp1::hisG, Δgcn4::LEU2, Δhac1::kanMX4*	This study
RH3404	*MATα, ura3-52, leu2::hisG, Δhac1::kanMX4::natMX4*	This study
RH3405	*MATα, ura3-52, leu2::hisG, trp1::hisG, Δgcn4::LEU2, Δhac1::kanMX4::natMX4*	This study
RH3406	*MAT**a**, ura3-52::FLO11::lacZ::URA3, trp1::hisG*	This study
RH3407	*MAT**a**, ura3-52::FLO11::lacZ::URA3, leu2::hisG, trp1::hisG, Δgcn4::LEU2*	This study

Table I. *Saccharomyces cerevisiae* strains used in this study, continued.

Strain	Genotype	Source
RH3408	*MAT**a**, ura3-52::FLO11::lacZ::URA3, leu2::hisG, trp1::hisG, Δgcn4::LEU2, Δhac1::kanMX4*	This study
RH3409	*MAT**a**, ura3-52::GCRE6::lacZ::URA3, trp1::hisG*	This study
RH3410	*MAT**a**, ura3-52::GCRE6::lacZ::URA3, leu2::hisG, trp1::hisG, Δgcn4::LEU2*	This study
RH3411	*MAT**a**, ura3-52::GCRE6::lacZ::URA3, leu2::hisG, trp1::hisG, Δgcn4::LEU2, Δhac1::kanMX4*	This study
RH3412	*MAT**a**/α, ura3-52/ura3-52, leu2::hisG/LEU2, trp1::hisG/TRP1, Δhac1::kanMX4/Δhac1::kanMX4::natMX4*	This study
RH3413	*MAT**a**/α, ura3-52/ura3-52, leu2::hisG/LEU2, trp1::hisG/TRP1, Δhac1::kanMX4/HAC1*	This study
RH3414	*MAT**a**/α, ura3-52/ura3-52, leu2::hisG/leu2::hisG, trp1::hisG/TRP1, Δgcn4::LEU2/GCN4*	This study
RH3415	*MAT**a**/α, ura3-52/ura3-52, leu2::hisG/leu2::hisG, trp1::hisG/trp1::hisG, Δgcn4::LEU2/Δgcn4::LEU2, Δhac1::kanMX4/Δhac1::kanMX4::natMX4*	This study
RH3416	*MAT**a**/α, ura3-52/ura3-52, leu2::hisG/leu2::hisG, trp1::hisG/TRP1, Δgcn4::LEU2/GCN4, Δhac1::kanMX4/HAC1*	This study
RH3417	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, leu2::hisG/LEU2, trp1::hisG/TRP1*	This study
RH3418	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, leu2::hisG/LEU2, trp1::hisG/TRP1, Δhac1::kanMX4/HAC1*	This study
RH3419	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, leu2::hisG/leu2::hisG, trp1::hisG/TRP1, Δgcn4::LEU2/GCN4*	This study
RH3420	*MAT**a**/α, ura3-52/ura3-52::FLO11::lacZ::URA3, leu2::hisG/leu2::hisG, trp1::hisG/TRP1, Δgcn4::LEU2/GCN4, Δhac1::kanMX4/HAC1*	This study
RH3421	*MAT**a**/α, ura3-52/ura3-52::GCRE6::lacZ::URA3, leu2::hisG/LEU2, trp1::hisG/TRP1*	This study

Table I. *Saccharomyces cerevisiae* strains used in this study, continued.

Strain	Genotype	Source
RH3422	*MAT**a**/α, ura3-52/ura3-52::GCRE6::lacZ::URA3, leu2::hisG/LEU2, trp1::hisG/TRP1, Δhac1::kanMX4/Δhac1::kanMX4::natMX4*	This study
RH3423	*MAT**a**/α, ura3-52/ura3-52::GCRE6::lacZ::URA3, leu2::hisG/LEU2, trp1::hisG/TRP1, Δhac1::kanMX4/HAC1*	This study
RH3424	*MAT**a**/α, ura3-52/ura3-52::GCRE6::lacZ::URA3, leu2::hisG/leu2::hisG, trp1::hisG/TRP1, Δgcn4::LEU2/GCN4*	This study
RH3425	*MAT**a**/α, ura3-52/ura3-52::GCRE6::lacZ::URA3, leu2::hisG/leu2::hisG, trp1::hisG/TRP1, Δgcn4::LEU2/GCN4, Δhac1::kanMX4/HAC1*	This study
RH3426	*MAT**a**, ura3-52, trp1::hisG, Δpho85::kanMX4*	This study
S001	W303-1A wild type strain *MAT**a**, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11, 15, ura3, GAL*	(Dieckhoff *et al.*, 2004)
S099	*MATα, ubc9::TRP1, ubc9-2/ΛEY2* (W303-background)	(Dieckhoff *et al.*, 2004)
S542	*MATα, smt3-331* (W303-background)	(Dieckhoff *et al.*, 2004)

2.1.2 Bacterial strains

The *Escherichia coli* strain DH5α was used for preparation of plasmid DNA [F', *φ80dlacZΔM15, Δ(lacZYA-argF), U169*, *deo*R, *rec*A1, *end*A1, *hsd*R17, (r_K^-, m_K^+), *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1] (Woodcock *et al.*, 1989). *E. coli* strain FHK12 was employed for dimerization studies using the ToxR system [F', *lac*I^q *lac*ZΔM15, *pro*A^+B^+, *ara*, Δ(*lac-pro*AB), *rps*L, (ϕ80d*lac*ZΔM15, *att*B::(*ctx*::*lac*Z), (*amp*^{r})] (Kolmar *et al.*, 1995b).

2.1.3 Plasmids

All plasmids used in this study are listed in Table II. Plasmid pME2898 expressing wild type *GCN4* was constructed by amplifying the *GCN4*-ORF with *Pfu* polymerase from plasmid pME2214, and introducing it as *Nhe*I/*Aat*II-fragment into

equally digested pBluescript vector. The mutagenesis of plasmids pME2899, and pME3373 – pME3377 was arranged by using the 'QuickChange® site-directed mutagenesis Kit' (STRATAGENE, La Jolla, USA). Instead of *PfuUltra* HF DNA Polymerase (PROMEGA, Mannheim, Germany) the *KOD* Hifi DNA Polymerase (NOVAGEN, Darmstadt, Germany) was used to insert the substitutions. Plasmid pME2898 was amplified with two oligonucleotide primers (listed in Table III), which are complementary to the reverse strand of the vector and contain the favored substitutions. Plasmids pME2901 and pME3378 – pME3382, expressing different substituted forms of *GCN4* under the control of the *GCN4* promoter, were obtained by digestion of the corresponding pBluescript plasmids with *Nhe*I and *Aat*II and replacing the respective *GCN4* fragments in pME2214. Plasmids pME2923, pME2925, pME3383, pME3384, and pME3386 expressing a three-fold myc epitope tagged version of the *GCN4* alleles under the control of the *GAL1* promoter were obtained by amplifying the different *GCN4* alleles with *Pfu* polymerase from plasmids pME2214, pME2901, pME3378, pME3379, and pME3381 and inserting them as *Nhe*I/*Cla*I-fragments into *Spe*I/*Cla*I restricted p426GAL1. A 120 bp *Bgl*II-fragment carrying the three-fold myc epitope was introduced into a *Bgl*II restriction site directly after the ATG of Gcn4p, Gcn4p^{L267S}, Gcn4p^{L253G}, Gcn4p^{L267G}, and Gcn4p^{L267A}, respectively. Wild type *GCN4* and *GCN4*^{L253G} were amplified from pME2923 or pME3383 and ligated as *Spe*I/*Cla*I-fragment into equally restricted plasmid p424GAL1 to get pME2918 or pME3388. Plasmid pME3389 was constructed by substitution of leucine 267 with serine in the leucine zipper domain of pHKToxR'MalE/ZIP.

Table II. Plasmids used in this study.

Plasmid	Description	Source
B3782	3 kb-*FLO11::lacZ* in YEp355	(Rupp *et al.*, 1999)
pBKSII®	2.96 kb vector, *Amp^R, lacZ, ori*	STRATAGENE (La Jolla, USA)
pRS314	*TRP, CEN, Amp^R, ori*	(Sikorski and Hieter, 1989)
pRS424	*TRP, 2µm, Amp^R, ori*	(Sikorski and Hieter, 1989)

Table II. Plasmids used in this study, continued.

Plasmid	Description	Source
pRS426	*URA3, 2µm, Amp^R, ori*	(Sikorski and Hieter, 1989)
p424GAL1	pRS424 containing *GAL1* promoter, *CYC1* terminator	(Mumberg *et al.*, 1994)
p426GAL1	pRS426 containing *GAL1* promoter, *CYC1* terminator	(Mumberg *et al.*, 1994)
p426MET25	pRS426 containing *MET25* promoter, *CYC1* terminator	(Mumberg *et al.*, 1994)
p180	*GCN4::lacZ* reporter construct on centromere vector (*URA3)*	(Hinnebusch, 1985)
pAG25	natMX4-cassettte in pFA6	(Goldstein and McCusker, 1999)
pLG669Z	*lacZ* shuttle vector	(Guarente and Ptashne, 1981)
p*FLO11-2/1* to p*FLO11-15/14*	440 bp sequence elements cloned into pLG669Z from -1 to -420 bp, -180 to -620 bp, -380 to -1020 bp until -2580 to -2980 bp	(Rupp *et al.*, 1999)
pHK vector series	Expression vector for β-galactosidase assays; *P/O_{toxR}, F1 ori, ColE1 ori, Cm^R*	(Kolmar *et al.*, 1994)
pHKToxR'MalE	t*oxR'malE* fusion gene in pHK, *Cm^R*	(Kolmar *et al.*, 1995b)
pHKToxR'MalE/ZIP	*toxR'malE/ZIP* fusion gene in pHK, *Cm^R*	(Kolmar *et al.*, 1995b)
pHKToxR'REI-T39K	t*oxR'rei-T39K* fusion gene in pHK, *Cm^R*	(Kolmar *et al.*, 1995a)
pME1092	2.8 kb *GCN4* fragment in pRS314	(Albrecht *et al.*, 1998)
pME1112	Integrative *GCRE6::lacZ* reporter construct	(Albrecht *et al.*, 1998)
pME2212	pLG669Z without UAS	(Braus *et al.*, 2003)
pME2213	Integrative *FLO11::lacZ* reporter construct	Grundmann, pers. comm.
pME2214	pME1092 with new restriction sites	Grundmann, pers. comm.
pME2846	*MET25prom-PCL5-GFP*-fusion in p426MET25	(Streckfuss-Bömeke *et al.*, 2009)
pME2898	*GCN4* wt fragment in pBKSII	This study

Table II. Plasmids used in this study, continued.

Plasmid	Description	Source
pME2899	*GCN4*^{L267S} fragment in pBKSII	This study
pME2901	*GCN4prom-GCN4*^{L267S}*-GCN4term* in pRS314	This study
pME2918	*GAL1prom-myc*^{3}*-GCN4*-fusion in p424GAL1	This study
pME2923	*GAL1prom-myc*^{3}*-GCN4*-fusion in p426GAL1	This study
pME2925	*GAL1prom-myc*^{3}*-GCN4*^{L267S}-fusion in p426GAL1	This study
pME3373	*GCN4*^{L253G} fragment in pBKSII	This study
pME3374	*GCN4*^{L267G} fragment in pBKSII	This study
pME3375	*GCN4*^{L267T} fragment in pBKSII	This study
pME3376	*GCN4*^{L267A} fragment in pBKSII	This study
pME3377	*GCN4*^{L267C} fragment in pBKSII	This study
pME3378	*GCN4prom-GCN4*^{L253G}*-GCN4term* in pRS314	This study
pME3379	*GCN4prom-GCN4*^{L267G}*-GCN4term* in pRS314	This study
pME3380	*GCN4prom-GCN4*^{L267T}*-GCN4term* in pRS314	This study
pME3381	*GCN4prom-GCN4*^{L267A}*-GCN4term* in pRS314	This study
pME3382	*GCN4prom-GCN4*^{L267C}*-GCN4term* in pRS314	This study
pME3383	*GAL1prom-myc*^{3}*-GCN4*^{L253G}-fusion in p426GAL1	This study
pME3384	*GAL1prom-myc*^{3}*-GCN4*^{L267G}-fusion in p426GAL1	This study
pME3386	*GAL1prom-myc*^{3}*-GCN4*^{L267A}-fusion in p426GAL1	This study
pME3388	*GAL1prom-myc*^{3}*-GCN4*^{L253G}-fusion in p424GAL1	This study
pME3389	pHKToxR'MalE/ZIP containing amino acid substitution L267S	This study

2.1.4 Oligonucleotides

The oligonucleotides used in this study are listed in Table III.

Table III. List of oligonucleotides used in this study.

Oligo	Size	Sequence (5' – 3')	Application
BH3	37mer	GCT TTC GAA AAA TTA TCA CAC GGA AAA TGA GGT TGC C	Insertion of Gcn4p amino acid substitution L267T
BH4	37mer	GGC AAC CTC ATT TTC CGT GTG ATA ATT TTT CGA AAG C	
BH5	37mer	GCT TTC GAA AAA TTA TCA CTG CGA AAA TGA GGT TGC C	Insertion of Gcn4p amino acid substitution L267C
BH6	37mer	GGC AAC CTC ATT TTC GCA GTG ATA ATT TTT CGA AAG C	
BH7	37mer	GCT TTC GAA AAA TTA TCA CGC GGA AAA TGA GGT TGC C	Insertion of Gcn4p amino acid substitution L267A
BH8	37mer	GGC AAC CTC ATT TTC CGC GTG ATA ATT TTT CGA AAG C	
BH9	37mer	GCT TTC GAA AAA TTA TCA CGG GGA AAA TGA GGT TGC C	Insertion of Gcn4p amino acid substitution L267G
BH10	37mer	GGC AAC CTC ATT TTC CCC GTG ATA ATT TTT CGA AAG C	
BH11	29mer	GCA AAG AAT GAA ACA AGG TGA AGA CAA GG	Insertion of Gcn4p amino acid substitution L253G
BH12	29mer	CCT TGT CTT CAC CTT GTT TCA TTC TTT GC	
BH22	21mer	CGG CAG ACA ATG CAG AAG TTG	Amplification of Δ*hac1::kanMX4* cassette from Y05650
BH23	22mer	GGA CTA CAG AGA GCC GTG AGA G	
BH86	20mer	AGA CAA TCG CAA GAG GGT AT	*HAC1* probe for Southern verification
BH87	19mer	CGG CGT TAG CAT CAA GTC T	
BH117	20mer	GTC ATT GAC ACA GTC TGT GA	*URA3* probe for Southern verification
BH118	20mer	GTG GAT GAT GTG GTC TCT AC	
BH119	19mer	GTG TCA AGT GAG AGA GCA G	*PHO85* probe for Southern verification
BH120	19mer	CGT TGA CGT GGT AGA GGT C	

Table III. List of oligonucleotides used in this study, continued.

Oligo	Size	Sequence (5' – 3')	Application
KB47	37mer	GCT TTC GAA AAA TTA TCA CTC GGA AAA TGA GGT TGC C	Insertion of Gcn4p amino acid substitution L267S
KB48	37mer	GGC AAC CTC ATT TTC CGA GTG ATA ATT TTT CGA AAG C	
KB49 (*ClaI*)	32mer	CCA TCG ATG GTC AGC GTT CGC CAA CTA ATT TC	Amplification of the *GCN4*-ORFs from the yeast vector
KB60 (*SpeI*)	31mer	GGA CTA GTC CAT GAG ATC CTC TAG AGG TGA A	
PFH30	23mer	CAA ATT ATC CAG GTT TAC TCG CC	
KB56	29mer	GGG ACC TAG ACT TCA GGT TGT CTA ACT CC	Sequencing of *GCN4* and Controlling right clones via colony PCR
PFH32	23mer	GAG CAG CAG GAT CAC TGG ATT CG	
RP36 (*ClaI*)	30mer	TAA ATC GAT CTT TCC AAC ATG ATG TGA CTT	
RP71 (*ClaI*)	32mer	CCA TCG ATA TGG CTA GCA AAG GAG AAG AAC TT	
RP87	27mer	GTT AAT ATT TCC TCT ATA CTT TAA CGT	
RP89 (*BglII*)	51mer	TAA ATG TCC GAA TAT GGA AGA TCT TCC CAG CCA AGT TTA TTT GCT TTA AAT	
T3	20mer	AAT TAA CCC TCA CTA AAG GG	
T7	22mer	GTA ATA CGA CTC ACT ATA GGG C	
KB84	24mer	GAG AAA GGA GAA GTA TCA CAA GAT	Amplification of Δ*pho85::kanMX4* cassette from Y02797
KB85	24mer	GCT TGC TTT GTT CTG ATC AAG ACC	
PR78	20mer	CCT TGA CAG TCT TGA CGT GC	Amplification of *natMX4* cassette from pAG25
PR79	20mer	CGC ACT TAA CTT CGC ATC TG	

2.2 Cultivation of microorganisms

2.2.1 *Escherichia coli*

Cells were grown in Luria-Bertani medium (LB: 1% tryptone, 0.5% yeast extract, 1% NaCl) at 37ºC. Isopropyl-thio-β-D-galactoside (IPTG) was added to liquid media at a final concentration of 0.4 mM. Ampicillin and chloramphenicol were used at a final concentration of 100 or 25 µg/ml, respectively. Solid medium contained 2% agar. SOB and SOC media for preparation of competent *E. coli* were prepared as described by Inoue *et al.* (1990).

2.2.2 *Saccharomyces cerevisiae*

The yeast strains were routinely cultivated in standard yeast extract-peptone-dextrose (YEPD: 1% yeast extract, 2% peptone, 2% glucose) or in minimal yeast nitrogen base media (YNB: 1,5 g/l yeast nitrogen base lacking amino acids and ammonium sulfate, 5 g/l ammonium sulfate, 2% glucose or galactose, supplemented with the appropriate amino acids) at 30°C. Temperature-sensitive strains were cultivated at permissive temperature of 23°C before shifting to restrictive temperature of 37°C, respectively. Solid media were prepared by using 2% agar.

3-Amino-1,2,4-triazole (3AT) (SIGMA-ALDRICH, Steinheim, Germany) was added to a final concentration of 5 to 100 mM to induce amino acid starvation. Solid media contained 5 or 10 mM 3AT and for promoter shut-off experiments a final concentration of 100 mM 3AT was used to mimic amino acid starvation. Tunicamycin (Tm) (CALBIOCHEM/MERCK KGaA, Darmstadt, Germany) was used in final concentrations of 0.5 µg/ml to 5 µg/ml to induce ER stress. Medium with a low concentration of ammonium (SLAD; YNB medium containing 50 µM ammonium sulfate) was used to observe pseudohypal formation.

Yeast growth was followed by measuring the optical density at 600 nm against the corresponding medium. The $OD_{600} = 1$ is equivalent to a cell density of 1.5×10^7 cells/ml (Sherman *et al.*, 1986). Growth times or inoculation volumes were calculated according to the following formula using a growth rate (µ) of 0.29/h for YNB or 0.45/h for YEPD, respectively:

$$V_i = \frac{V_c \text{ x } OD_t \text{ x } e^{-\mu \text{ x } \Delta t}}{OD_s}$$

V_i = volume of inoculum [ml]
V_c = volume of culture [ml]
OD_t = desired OD at the time t
OD_s = OD of starter culture
μ = growth rate
Δt = growth time [h].

2.3 Isolation of nucleic acids

2.3.1 Plasmid DNA purification

Plasmid DNA was purified using the 'QIAPrep Spin Miniprep Kit' (QIAGEN, Hilden, Germany). A single colony of *E. coli* was inoculated into 5 ml LB medium supplemented with ampicillin to a final concentration of 100 µg/ml and incubated overnight at 37°C while shaking. The cells were harvested by centrifugation at 13,000 rpm and remaining steps were based on the instruction's manual provided. The isolated plasmids were stored at -20°C.

2.3.2 Isolation of DNA from yeast cells by 'Smash & Grab'

'Smash & Grab' was performed for rapid DNA isolation (Hoffman and Winston, 1987). Yeast cells of an overnight culture were collected by centrifugation (3,000 rpm, 4 min) and the pellet was resuspended in 0.5 ml H_2O. After 5 sec centrifugation at 13,000 rpm supernatant was discarded and the pellet was resuspended in the residual liquid. 0.2 ml of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) and 0.2 ml of phenol equilibrated with methylene chloride and isoamyl alcohol (25:24:1) were added. Furthermore, 0.3 g of glass beads (∅ 0.25 – 0.50, CARL ROTH GmbH, Karlsruhe, Germany) were added to disrupt the cells by vortexing for 4 min at 4°C. After subsequent centrifugation for 5 min at 13,000 rpm the DNA containing aqueous phase could be removed and transferred into a new reaction tube. For higher purity, extraction could be repeated, optionally. DNA was precipitated by addition of 1 ml ice-cold absolute ethanol and subsequently the precipitated DNA was collected by centrifugation for 5 min at 13,000 rpm. The supernatant was discarded and precipitated DNA was resuspended in 0.4 ml H_2O to which 3 µl of RNase A

solution (10 mg/ml) was added. After incubation for 5 min at 37°C 10 µl of 4 M ammonium acetate was added and DNA was precipitated again by addition of 1 ml ice-cold 100% ethanol. DNA was collected by centrifugation for 2 min at 13,000 and dried at room temperature or at medium speed in a vacuum dryer (Speed Vac RC3B plus, SAVANT). Finally, DNA was dissolved in 100 µl H_2O and used for analytical purposes like PCR. The dissolved DNA was stored at -20°C.

2.3.3 Pure DNA isolation from yeast cells via glass capillary

For higher DNA purification a further method was used. An overnight culture of the respective yeast strain in YEPD was centrifuged and after washing the cells with H_2O the pellet was resuspended in 500 µl SPM buffer (0.9 M sorbitol, 50 mM sodium phosphate pH 7.5, 140 mM β-mercaptoethanol). 5 µl zymolyase (10 mg/ml) was added and incubated at 37°C. After 30 min 50 µl proteinase K (10 mg/ml), 50 µl EDTA (0.5 M) and 150 µl SDS (10%) were added and incubated for further 30 min at 37°C. The solution was once extracted with an equal volume of phenol (pH 8.0). After vortexing for 10 sec and following centrifugation for 5 min at 13,000 rpm the DNA containing aqueous phase including the interphase could be removed and transferred into a new reaction tube. Extraction was repeated with one volume of phenol equilibrated with methylene chloride and isoamyl alcohol (25:24:1) and after centrifugation aqueous phase was removed without the interphase. Last extraction step was performed with one volume of CH_2Cl_2/isoamyl alcohol (24:1). The aqueous phase was transferred into a new reaction tube and DNA was precipitated for 5 min on ice by adding 1.5 volumes of ethanol. Afterwards the precipitated DNA was spun on a glass capillary and resuspended in 200 µl H_2O. Precipitation was repeated by adding another 1.5 volume of ethanol and DNA was resuspended in 100 µl H_2O. The DNA thus obtained was used for analytical purposes like PCR. The dissolved DNA was stored at -20°C.

2.4 Cloning techniques

2.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) (Saiki *et al.*, 1985) was performed for rapid amplification of DNA for various purposes. Depending on the application, different thermostable polymerases were used as recommended in the manufacturers's protocols.

For cloning, either *KOD* HiFi DNA Polymerase (NOVAGEN, Darmstadt, Germany) or *Pfu* polymerase (PROMEGA, Mannheim, Germany) was used. For amplification of DNA for site-specific homologous recombination *Phusion* High-Fidelity Polymerase (FINNZYMES, Espoo, Finland) was applied. For analytical purposes the self-made *Taq* polymerase was used. Therefore, the following temperature profile was applied after an initial denaturation at 94°C for 1 min: 1 min denaturation at 94°C, 40 sec hybridisation at a specific annealing temperature and finally elongation at 72°C, which lasted according to the fragment size (1 min/kb). After 25 or 30 cycles a final extension at 72°C for 5 min followed. In general, primer oligonucleotides were used in a concentration of 5 – 50 nmol. Reaction buffer, polymerase and dNTPs were added according to manufacturers's recommendations.

2.4.2 Restriction digestion of DNA

For cloning and analytical confirmation, approximately 1 µg DNA was digested in a total volume of 20 µl using restriction enzymes (FERMENTAS, St. Leon-Rot, Germany), whereas preparative amounts ranged from about 5 to 10 µg DNA. Restriction buffers were used according to the manufacturers's instruction and the restrictions were performed at 37°C for at least 2 h.

2.4.3 Agarose gel electrophoresis

Digested or amplified DNA was mixed with 0.1 volumes of loading dye (25% w/v Ficoll 400, 0.25% bromphenol blue sodium salt, 0.25% w/v xylene cyanol, 200 mM EDTA, pH 8.0) before fragments were separated in 1% agarose gels containing about 0.5 µg/ml EtBr in TAE buffer (40 mM Tris-acetate, 20 mM Na-acetate, 2 mM EDTA, pH 8.3). Bands were visualized with an UV transilluminator (λ = 254 nm). The 'GeneRuler 1kb DNA Ladder' (FERMENTAS, St. Leon-Rot, Germany) was used for size determination of fragments.

2.4.4 Isolation of DNA fragments from agarose gels

After separating digested or amplified DNA on a standard agarose gel, the desired bands were excised under UV light (λ = 366 nm). Isolated DNA was purified using the 'QIAquick Gel Extraction Kit' (QIAGEN, Hilden, Germany). Purification was

performed according to the instruction manual provided. The dissolved DNA was stored at -20°C.

2.4.5 Dephosphorylation of DNA

For cloning, the digested vector DNA was dephosphorylated in order to remove 5’ phosphates to avoid unnecessary religation. Dephosphorylation was performed using Shrimp Alkaline Phosphatase (SAP) from FERMENTAS (St. Leon-Rot, Germany) and the respective buffer. The reaction mixture was incubated at 37°C for 1 h. The digested and dephosphorylated vector DNA was then purified as described in 2.4.4.

2.4.6 Ligation of DNA fragments

Linear DNA fragments with cohesive or blunt ends were incubated overnight at 16°C or for two hours at room temperature in a 20 µl mixture consisting of 2 µl T4 DNA ligase (FERMENTAS, St. Leon-Rot, Germany) and 2 µl 10x ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8). The ratio between digested vector DNA and digested insert DNA was 1:5 or 1:10. DNA ligase was inactivated by heating the reaction mix at 65°C for approximately 10 min before transformed in *E. coli*.

2.4.7 Site directed mutagenesis

Site directed mutagenesis was used to substitute codons encoding leucines of the leucine zipper of Gcn4p. The mutagenesis was carried out by applying the ‘QuickChange® Site-Directed Mutagenesis Kit’ (STRATAGENE, La Jolla, CA, USA). A thermocycler and *KOD* Polymerase instead of *PfuUltra* HF DNA Polymerase were used to establish the favored substitution whereas 10 ng of the pBluescript vector containing a *GCN4* wild type open reading frame (*GCN4*-wt-ORF) (pME2898) served as template DNA. Two primers containing the desired mutations were designed spanning the target region in opposing directions (Table III). With a PCR thermocycler 16 cycles were executed: 30 sec denaturation at 95°C, 1 min annealing at 55°C and 5 min elongation at 68°C. After amplification 2 µl T4 ligase and appropriate volume of buffer were added and ligation was incubated at room temperature for 2.5 h. The reaction was stopped through heat inactivation (5 – 10 min, 65°C) and subsequently 1 µl of *Dpn*I restriction enzyme was added to each reaction and incubated at 37°C for 1 h. The *Dpn*I enzyme is

specific to methylated and hemi-methylated DNA and therefore only digests the parental template DNA. The enzyme reaction was stopped by heat inactivation and afterwards 1 µl of undigested DNA was used to transform *E. coli* DH5α or FHK12, respectively. Plasmids were isolated from the resulting colonies and the successful substitution was verified by sequencing with specific primers (Table III).

2.4.8 DNA sequencing

Crucial cloning steps and mutagenesis were validated by sequencing on an ABI Prism 310 capillary sequencer (APPALERA DEUTSCHLAND GmbH, Darmstadt, Germany) at the Göttingen Genomics Laboratory. DNA sequences were verified using the 4Peaks software (available at http://mekentosj.com/4peaks/).

2.5 Methods of transformation

2.5.1 Preparation of chemically competent *E. coli* cells

Preparation was performed according to Inoue *et al.* (1990). About 10 colonies of *E. coli* DH5α were inoculated in 250 ml SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). The culture was incubated at 20°C for approximately 24 h until OD_{600} = 0.6 and cooled on ice for 10 min before centrifuging at 2,500 rpm for 10 min at 4°C. The pellet was resuspended in 80 ml TB buffer (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, adjustment of pH 6.7 with KOH, 55 mM $MnCl_2$) and kept on ice. After 10 min incubation, cell suspension was centrifuged and the obtained pellet was resuspended gently in 20 ml TB buffer followed by the addition of DMSO with swirling to obtain a final concentration of 7%. This cell suspension was incubated for further 10 min on ice and dispended in 500 and 700 µl aliquots which were first subjected to shock freezing in liquid nitrogen before storing at -80°C.

2.5.2 Transformation of *E. coli* DH5α

An aliquot of competent cells was carefully thawn on ice. 20 µl of a ligation reaction was added to 200 µl of competent *E. coli* DH5α and incubated for 30 min on ice. The cells were then subjected to heat shock at 42°C for 90 sec and subsequently placed on ice again for further 3 min. The samples were mixed with 800 µl LB medium

followed by recovery at 37°C under constant shaking for 1 h. Afterwards cells were harvested by centrifugation at 5,000 rpm for 2 min, resuspended in the remaining supernatant and plated on LB agar supplemented with ampicillin to a final concentration of 100 µg/ml. Plates were incubated overnight at 37°C.

2.5.3 Preparation of $CaCl_2$-competent *E. coli* FHK12 cells

A single colony of *E. coli* FHK12 was inoculated into 5 ml LB medium supplemented with ampicillin to a final concentration of 100 µg/ml, and incubated overnight at 30°C on a rotary shaker. 100 ml LB medium supplemented with ampicillin were inoculated with 1 ml of this pre-culture and incubated at 30°C until OD_{600} = 0.6 – 0.8. Cells were harvested by centrifugation at 4,000 rpm for 20 min at 4°C and resuspended in 20 ml sterile ice-cold 0.1 M $CaCl_2$ solution. After 24 h incubation on ice cells were harvested again and resuspended in 3 ml sterile ice-cold 0.1 M $CaCl_2$ solution. Cell suspension was dispensed in 200 µl aliquots, which were incubated for further 30 min on ice. Subsequently 7 µl DMSO was added to each aliquot and cells could be used for direct transformation or stored at -80°C.

2.5.4 Transformation of *E. coli* FHK12

An aliquot of competent *E. coli* FHK12 cells was carefully thawn on ice. 1 µg DNA (in maximal 10 µl) was added to 200 µl of the competent cells and incubated for 30 min on ice. Cells were subjected to heat shock at 37°C for 3 min before 800 µl LB medium were added to the mixture followed by recovery at 37°C under constant shaking for 45 min. 50, 100 and 150 µl of this cell suspension were plated on solid LB medium supplemented with ampicillin and chloramphenicol. Incubation was overnight at 37°C.

2.5.5 Transformation of *S. cerevisiae*

Transformation was performed according to the lithium acetate method as described by Ito *et al.* (1983). 10 ml cultures of *S. cerevisiae* were pre-grown in YEPD medium at 30°C overnight. 10 ml YEPD medium was inoculated with 400 µl of this pre-culture and incubated at 30°C for further 5 – 6 h. Cells were harvested by centrifugation at 3,000 rpm for 3 min and resuspended in 4 ml 1x LiOAc/TE (0.1 M LiOAc, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After short whirling, cells were

harvested by centrifugation. For chromosomal integration of linear DNA fragments this whirling step was extended up to 20 – 30 min. After centrifugation cells were resuspended in 400 µl 1x LiOAc/TE prior dividing it into two samples of 200 µl, respectively. Approximately 20 µl of pre-warmed (65°C) carrier DNA (salmon sperm DNA) (10 mg/ml) was added to each tube with 200 µl competent yeast cells. For transformation of plasmid DNA 5 – 8 µl high copy or 15 µl low copy plasmid DNA was added to one of the tubes. The other one served as negative control. For transformation of linear DNA 20 – 200 µl of purified PCR product were used. After addition of 800 µl 50% PEG 4000 samples were vortexed and incubated at 30°C for 30 min. The cells were then subjected to heat shock at 42°C for 20 – 25 min and subsequently centrifuged at 7,000 rpm for 30 sec. The supernatant was discarded and the cell pellets were resuspended in 1 ml YEPD and incubated at 30°C for 1 h while shaking. For chromosomal integration this step was extended up to 2 – 3 h. Finally, cells were pelletized by centrifugation at 4,000 rpm for 10 – 20 sec and the resuspended cells were plated on selective media and incubated at 30°C for about 3 days until colonies were visible. Transformation of yeast with linear DNA was plated on solid YEPD medium containing the appropriate drug for selection and incubated at 30°C for 4 days.

2.6 Protein analysis

2.6.1 Shut-Off-Western procedure

Yeast cells were pre-grown in selective minimal medium with glucose as carbon source to OD_{600} = 0.6. Cells were collected by centrifugation (3,000 rpm, 4 min, 4°C) and incubated in minimal medium containing 2% galactose and 0.5% raffinose to express *myc^{3}-GCN4* from the *GAL1* promoter. After 3.5 hours, cells were collected via centrifugation and half of these tryptophan-auxotrophic cells were starved for tryptophan by shifting them to minimal medium lacking tryptophan. Alternatively, 100 mM 3AT was used to induce amino acid starvation. 3% glucose was added to shut-off the promoter after half an hour of starvation. Samples (50 ml) were analyzed at the indicated time points after promoter-shut-off (0-min time point).

2.6.2 Protein Synthesis Shut-Off Assay

Yeast cells were cultivated in 150 ml liquid synthetic minimal medium (YNB) or rich medium (YEPD) to OD_{600} = 0.6 at 30°C. Temperature-sensitive yeast strains were cultivated at 25°C (permissive temperature). To examine the turnover of Hac1p, tunicamycin (Tm) was added to a final concentration of 5 µg/ml in order to induce the UPR. After 90 min post Tm-induction, cycloheximide (APPLICHEM GmbH, Darmstadt, Germany) was added to a final concentration of 1 mg/ml and samples (15 ml) were analyzed at the indicated time points after halt of protein synthesis (0-min time point). Temperature-sensitive strains were shifted from 25°C to restrictive temperature of 37°C for 30 min before cycloheximide was added.

2.6.3 Whole-cell extracts of *S. cerevisiae*

Cell extracts were prepared from yeast cultures grown to exponential phase. Cells were washed in 2.5 ml ice cold buffer B (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 20% glycerine), lysed with glass beads (∅ 0.25 – 0.50 mm, CARL ROTH GmbH, Karlsruhe, Germany) in 500 µl of B-buffer^{+} containing protease inhibitors (Complete, EDTA-free, ROCHE DIAGNOSTICS GmbH, Mannheim, Germany) and 14.3 mM β-mercaptoethanol through vigorous shaking on a Ika-Vibrax-Mixer at 4°C. To remove glass beads and large cell debris samples were spun at 13,000 rpm for 12 min (4°C). For preparation of extracts from the protein synthesis shut-off assay 150 µl of B-buffer^{+} were used. Part of the supernatant (10 µl) was used for Bradford (1976) assay to determine the protein concentration while the remaining supernatant was denatured at the ratio 3:1 in 3x SDS loading dye (0.25 M Tris-HCl pH 6.8, 15% β-mercaptoethanol, 30% glycerine, 7% SDS, 0.3% bromphenol blue) by heating at 65°C for 15 min.

2.6.4 SDS-Polyacrylamide Gel Electrophoresis (Laemmli, 1970)

Yeast cell extracts were prepared for electrophoretic separation as described in section 2.6.3. Extracts containing equal amounts of protein from each sample were separated by 12% SDS-PAGE consisting of two different gel types (running gel: 375 mM Tris pH 6.8, 3% v/v acrylamide, 0.08% v/v bisacrylamide, 0.1% SDS; stacking gel: 375 mM Tris pH 8.8, 12% v/v acrylamide, 0.4% v/v bisacrylamide, 0.1% SDS). Running of the gel was carried out at 200 V in electrophoresis buffer (25 mM Tris Base,

25 M glycine, 1% w/v SDS, 0.34% w/v EDTA). The 'PageRuler™ Prestained Protein Ladder' (FERMENTAS GmbH, St. Leon-Rot, Germany) was used as marker.

2.6.5 Protein immunoblotting

For Western hybridization experiments, separated proteins were transferred onto a nitrocellulose membrane (SCHLEICHER & SCHUELL BIOSCIENCES GmbH, Dassel, Germany) by electrophoretic blotting in a 'Mini-Trans-Blot-Electrophoretic-Cell' from BIO-RAD (BIO-RAD LABORATORIES GmbH, Munich, Germany) in transfer buffer containing 30 mM Tris-base, 200 mM glycine, 0.002% SDS and 20% methanol for 1.5 h at 100 V or overnight at 35 V.

After protein transfer, free binding sites on the nitrocellulose membrane were blocked by treatment with 4 – 5% milk powder in PBS (140 mM NaCl, 10 mM sodium phosphate, pH 7.5) for one hour. Afterwards the membrane was incubated for 1 – 2 h at RT or overnight at 4°C with the respective primary antibody diluted in a suspension of 4 – 5% milk powder in PBS. The used primary antibodies were monoclonal mouse anti-GFP (CLONTECH, Heidelberg, Germany), monoclonal mouse anti-myc (9E10) (SANTA CRUZ BIOTECHNOLOGY, Heidelberg, Germany), polyclonal rabbit anti-Cdc42p (SANTA CRUZ BIOTECHNOLOGY, Heidelberg, Germany), polyclonal rabbit anti-Hac1p (gift from Kazutoshi Mori, Kyoto University, Japan), polyclonal rabbit anti-eIF2αp [pS52] (#44728G, INVITROGEN, Darmstadt, Germany) or rabbit anti-eIF2αp (gift from Thomas Dever, MD, USA). Residual antibody was washed away by shaking the membrane 3x 10 min in PBS. Peroxidase-coupled goat anti-rabbit IgG (#G21234, MOBITEC, Göttingen, Germany) or goat anti-mouse IgG (INVITROGEN GmbH, Karlsruhe, Germany) were used as secondary antibodies. The membrane was incubated with the secondary antibodies for 60 min and afterwards washed as described for treatment with primary antibodies. The respective proteins were detected using 'Enhanced Chemiluminescence' (ECL) technology (Tesfaigzi *et al.*, 1994). The ECL solution 1 (100 µl of 2.5 mM Luminol and 44 µl of 40 µM Paracumaric acid in a total volume of 10 ml of 10 mM Tris, pH 8.5) was mixed with ECL solution 2 (6.15 µl of 30% H_2O_2 in 10 ml of 10 mM Tris, pH 8.5) just prior for use. The membrane was incubated in this mixture for 1 min and subsequently exposed to the Hyperfilm™-ECL™ (AMERSHAM BIOSCIENCES, Munich, Germany). Quantification of detected bands was performed

using the KODAK MI 4.05 software (EASTMAN KODAK COMPANY, Rochester, NY, USA).

2.7 Southern analysis

Genetic integrations or manipulations of strains were confirmed by Southern analysis according to Southern (1975). Non-radioactive labeling of probes and detection was performed using the 'Gene Images™ Random-Prime DNA labeling kit' and the 'Gene Images™ CDP-Star™ Detection Kit (GE HEALTHCARE LIFE SCIENCES, Munich, Germany). The chemiluminescent signals were exposed to the Hyperfilm™-ECL™ (AMERSHAM BIOSCIENCES, Munich, Germany).

2.8 β-galactosidase assays

2.8.1 β-galactosidase assay in *S. cerevisiae*

Strains carrying either a chromosomally integrated *lacZ* reporter or expressing a *lacZ* reporter from a plasmid were cultivated in liquid synthetic minimal medium (YNB) overnight at 30°C, diluted into fresh medium, and cultivated for 6 h before assaying enzymatic activities. For amino acid starvation, 3AT (SIGMA-ALDRICH, Steinheim, Germany) was added to fresh diluted cultures to a final concentration of 10 mM, and cells were incubated for 8 h before measuring the β-galactosidase activity. For nitrogen starvation, cells grown to logarithmic phase were washed twice with 2% glucose and incubated for 24 h in liquid YNB medium containing 50 µM ammonium sulfate (instead of 50 mM) as the sole nitrogen source. Tunicamycin (Tm) (CALBIOCHEM/MERCK KGaA, Darmstadt, Germany) was added to fresh diluted cultures to a final concentration of 1 µg/ml and cultures were incubated for 6 h to induce ER stress. Additionally, cultures grown to log phase (6 h YNB) were treated with 1 µg/ml Tm for 15, 30, 60 and 90 min.

Extracts were prepared and assayed for specific β-galactosidase activity as described previously (Rose and Botstein, 1983) and normalized to the total protein concentration (Bradford, 1976), resulting in the specific enzyme activity (OD_{420} x 0.35)/(0.0045 x protein concentration x extract volume x time). Assays were performed at least three times from independent cultures. The standard errors of the mean values were below 15%.

2.8.2 β-galactosidase assay in *E. coli*

For β-galactosidase assays of chimeric protein activity *E. coli* strain FHK12 transformed with the respective pHKToxR' derivate was pre-grown for 4 hours at 37°C in LB in the presence of chloramphenicol (25 µg/ml) and ampicillin (100 µg/ml). 5 µl of each pre-culture was used for at least three main cultures, which were grown overnight at 30°C in LB in the presence of chloramphenicol, ampicillin and 0.4 mM IPTG. 15 µl of each culture were transferred into a microtiter well and 100 µl chloroform-saturated Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) was added. Afterwards, OD_{620} was determined using an ELISA plate reader. Further steps were performed as reported by Kolmar *et al.* (1995b). Assays were performed for at least three independent cultures.

2.9 Growth tests

For spot dilution assays, yeast strains were pre-cultured to the same optical density (OD_{600} = 0.6) and then diluted five- or ten-fold, starting with 3 x 10^4 cells per 20 µl. For each dilution 10 µl (YEPD) or 20 µl (YNB) were spotted onto solid YNB or YEPD medium with or without 0.5 µg/ml tunicamycin for ER stress survival assays, and on selective YNB medium with or without 5 mM 3AT for resistance upon amino acid starvation. After incubation for 3 days at 30°C (temperature sensitive mutants were also incubated at 25 and 37°C) plates were photographed under white light.

2.10 Adhesive growth

Amino acid starvation induced adhesive growth tests on solid YNB medium were performed as described previously (Roberts and Fink, 1994; Braus *et al.*, 2003). Strains were pre-grown at 30°C for 20 hours on solid YNB medium containing the respective supplements. Cells were streaked on YNB medium containing fresh supplements and 10 mM 3AT to induce amino acid starvation. After incubation for three days at 30°C, plates were photographed to visualize total growth and then carefully washed under a stream of water. Afterwards, the plates were photographed again to document adhesive cell growth. For visualization of biofilms in wells of polystyrene plates, assays were performed as described in Reynolds and Fink (2001). Cells were pre-grown in YNB at 30°C overnight and used for inoculation of main

cultures followed by an incubation at 30°C. At an OD_{600} = 0.6, 300 µl of each culture were transferred into a microtiter plate and incubated with or without 5 – 10 mM 3AT at 30°C for 1 – 2 days. Then, 100 – 150 µl of crystal violet was added to the cells and incubated for approximately 15 min. Finally, the plate was carefully washed under a gentle stream of water to remove all non-adhesive cells, dried and ultimately photographed.

2.11 Pseudohyphal growth

For qualitative diploid pseudohyphal development assays cells were grown for five days on solid SLAD medium containing 50 µM $(NH_4)_2SO_4$ as nitrogen source (Gimeno *et al.*, 1992). 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).

3. RESULTS

3.1 A feedback circuit between transcriptional activation and self-destruction of Gcn4p separates its metabolic and morphogenic function in diploid yeasts

Various transcription factors, which regulate the expression of genes involved in differentiation processes, are targets for polymorphisms. Single nucleotide polymorphisms (SNPs) provoke changes in their function and activity whereby expression in different yeast strains can result in varying adaptations and phenotypes (Gerke *et al*., 2009; Dowell *et al*., 2010). We aimed to dissect the developmental from the metabolic function of the transcription factor Gcn4p in diploid yeast cells.

3.1.1 The *GCN4*^{L267S} mutant allele separates the metabolic and the developmental function of a transcription factor in diploids

The initial step to separate the metabolic and the developmental function of Gcn4p was the generation of a *GCN4* mutant library by random mutagenesis via PCR. Diploid mutants which still have an intact metabolic function and provide sufficient amounts of translational precursors were screened for growth under amino acid starvation conditions mediated by the drug 3-amino-1,2,4-triazole (3AT) (Klopotowski and Wiater, 1965). A *gcn4* deletion strain is unable to grow on media containing 3AT, because it causes histidine starvation acting as false feedback-inhibitor of the corresponding amino acid biosynthetic pathway nevertheless could grew significantly reduced compared to wild type cells when Δ*gcn4* cells were thickly streaked out (Fig. 7A and 12B). The developmental function of Gcn4p was monitored using an additional reporter. The *GCN4* mutant library was transformed into diploid Δ*gcn4* mutant cells (RH2695) that carry a chromosomally integrated *FLO11::lacZ* reporter. Diploids were only able to adhere and form pseudohyphae when Gcn4p was active and in turn the *FLO11* gene was expressed which can be visualized and quantified in this strain by β-galactosidase activity assays.

A pool of 24,000 (100%) transformants was plated on solid YNB medium containing 10 mM 3AT. 20,000 clones (83%) were able to grow in the presence of 3AT suggesting that an intact metabolic function of Gcn4p provided sufficient amounts of

amino acids for growth. *FLO11::lacZ* expression was determined using a qualitative filter assay (Breeden and Nasmyth, 1987). 207 clones of primarily 24,000 (0.86%) showed an impairment in the activation of *FLO11* expression upon amino acid starvation scored by less intense colored the filters. The corresponding *GCN4*-containing plasmids were isolated and re-introduced into the parental strain (RH2695) as secondary screen for a separation of the dual function of Gcn4p. This second screen resulted in 83 transformants (0.58%) and eight clones (0.03%) featured a significant decrease in amino acid starvation-induced *FLO11::lacZ* expression. DNA sequencing of the isolated *GCN4* mutant alleles revealed a shared codon exchange resulting in a Leu267Ser amino acid substitution. This amino acid represents the third of four conserved leucines of the leucine zipper region, which is located in the C-terminal region of the Gcn4 protein.

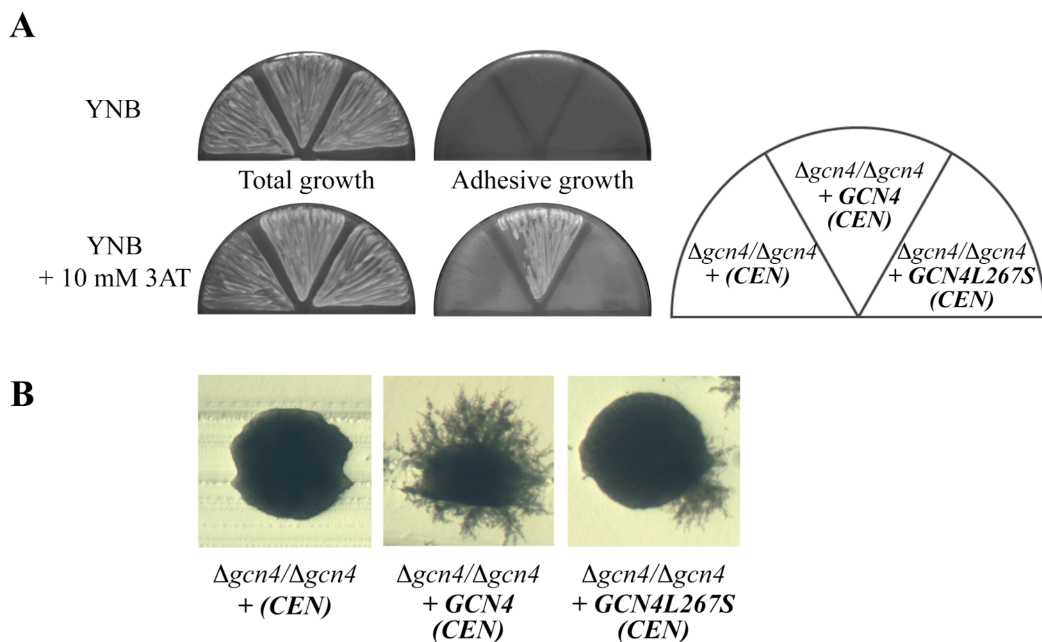


Fig. 7: Diploid *GCN4*^{L267S} yeasts are able to grow under amino acid starvation, but do not adhere to agar.

(A) Diploid yeast Σ1278b strain RH2695 (Δ*gcn4*/Δ*gcn4*) carrying low copy *CEN* plasmids with wild type *GCN4* (pME1092), *GCN4*^{L267S} (pME2901) or as control (Δ*gcn4*) the empty vector (pRS314) was streaked out on solid YNB medium (non-starved cells) and with 10 mM 3AT (histidine starved cells), respectively. After incubation for three days at 30°C adhesive growth was determined. Plates were photographed prior (total growth) and after washing under a stream of water (adhesive growth) to document remaining cells on the agar surface.

(B) Strains were streaked out on solid nitrogen starvation medium (Natarajan *et al.*, 2001) containing only 50 µM $(NH_4)_2SO_4$ as nitrogen source to induce pseudohyphal growth. After five days incubation at 30°C pseudohyphal colonies were visualized with an Axiovert microscope and photographed.

The developmental phenotype was analyzed in more detail. Under non-starvation conditions all diploid strains carrying an intact *FLO11* gene and an additional *FLO11::lacZ* reporter were growing (Fig. 7A). The growth mode was non-adhesive, since washing detached all strains from the agar surface. In contrast, diploid cells expressing wild type *GCN4* grew adhesively when treated with 3AT whereas cells containing the $GCN4^{L267S}$ mutant allele were unable to adhere similar to the control strain, which expressed an empty vector and therefore mimiced a Δ*gcn4*/Δ*gcn4* mutant strain. Pseudohyphae formation of diploid Δ*gcn4* yeast strains expressing wild type *GCN4* or the $GCN4^{L267S}$ mutant allele were analyzed by streaking them on solid nitrogen starvation medium containing 50 µM $(NH_4)_2SO_4$ as nitrogen source. This reduced amount of ammonium sulfate triggered pseudohyphal growth of diploid wild type cells (*GCN4*). In contrast, cells expressing $GCN4^{L267S}$ showed an impaired ability in pseudohyphae formation (Fig. 7B).

We conclude that a separation of the dual function of Gcn4p is possible when the amino acid starvation regulated metabolic function is still intact but the developmental function is abolished.

3.1.2 Transcriptional activity of Gcn4p^{L267S} in starved diploids is significantly lower for *FLO11::lacZ* in comparison to Gcn4p target gene expression

Our screen revealed only a single mutation in the C-terminus of the transcription factor, which allowed the separation of the metabolic and developmental function. The reason why corresponding diploid *GCN4* mutant cells were unable to differentiate was investigated by measuring *FLO11* expression representing the key player for adhesion and pseudohyphal growth. First, β-galactosidase activity assays were performed using the chromosomally integrated *FLO11::lacZ* reporter.

Amino acid starvation led to a significant induction of β-galactosidase activity in diploid wild type cells carrying intact Gcn4p (*GCN4*) (66 U) (Fig. 8). In contrast, cells containing the substitution ($GCN4^{L267S}$) showed only a partial induction corresponding to 25% of the wild type *FLO11::lacZ* expression (16 U) when treated with 3AT. These data suggest that the amino acid substitution Leu267Ser of Gcn4p causes an impaired ability to induce *FLO11* expression and thereby an adhesion-deficient growth phenotype in response to amino acid starvation. The $GCN4^{L267S}$ mutant strains are still able to grow in the presence of 3AT starvation conditions. To analyze the

transcriptional activity of the mutant Gcn4p on the upstream elements of metabolic genes (Gcn4 protein recognition elements (GCRE)) (Hope and Struhl, 1986; Oliphant *et al.*, 1989) β-galactosidase assays of *GCRE6::lacZ* reporter were performed. This reporter carries six copies of the 9 base pairs nucleotide GCRE sequence representing a specific binding site for the Gcn4p homodimer. Activity of wild type and mutant Gcn4p was monitored in sated and amino acid starved diploid yeast cells. Under sated conditions expression of *GCRE6::lacZ* was low in both diploid cells expressing *GCN4*^{L267S} and wild type *GCN4*. Upon 10 mM 3AT treatment *GCRE6::lacZ* induction in the Gcn4p^{L267S} mutant displayed approximately 50% (394 U) of the expression measured in cells expressing wild type Gcn4p (783 U) (Fig. 8).

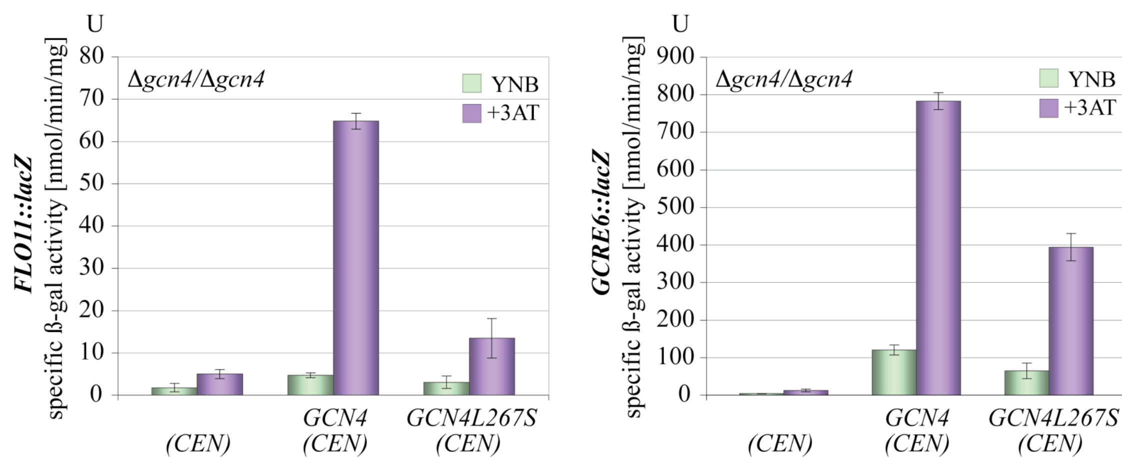


Fig. 8: ***FLO11* and Gcn4p-dependent reporter gene expression in diploid *GCN4*^{L267S} yeasts.**

The diploid yeast strain RH2695 carrying a chromosomally *FLO11::lacZ* reporter transformed with plasmids expressing wild type *GCN4* (pME1092), *GCN4*^{L267S} (pME2901) or as control (Δ*gcn4*) the empty vector (pRS314) was grown to log-phase in YNB in absence (green bars, YNB) or presence of 10 mM 3AT (purple bars, +3AT) before specific β-galactosidase activities were measured. Additionally, galactosidase activity was determined in diploid Σ1278b strain RH2398 (Δ*gcn4*/Δ*gcn4*) carrying a *GCRE6::lacZ* reporter expressing the same *GCN4* plasmids. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. The bars represent the mean value of at least three independent measurements.

Taken together, the mutant protein Gcn4^{L267S} has a significantly reduced activity in diploids compared to wild type Gcn4p. Quantitative differences of Gcn4p^{L267S} on metabolic or developmental gene expression resulted in distinct phenotypes. The reduced activation of metabolic genes by Gcn4p^{L267S} in comparison to wild type Gcn4p is apparently sufficient to permit growth during amino acid starvation conditions. This

suggests that there is a decent buffer in the basal Gcn4p independent metabolic activity for GCRE controlled genes of the yeast cell. In contrast, the activity of Gcn4p^{L267S} on *FLO11* expression is significantly more impaired when compared to wild type and seems to be below a threshold, which is required for adhesive and pseudohyphal growth. Therefore, only full Gcn4p activity is able to provide sufficient *FLO11* expression for development. This might be reflect by the fact that there is hardly any basal activity of *FLO11*, because this is a decision between two growth mode alternatives that are mutually exclusive and result in either yeast or pseudohyphal development.

3.1.3 The separation of the dual function of Gcn4p is diploid-specific

Haploid cells of *S. cerevisiae* have the ability to grow adhesively when starved for either amino acids or glucose (Cullen and Sprague, 2000; Braus *et al.*, 2003). We investigated whether the Gcn4p amino acid substitution Leu267Ser leads to a similar separation of the metabolic and developmental function of Gcn4p in starved haploids as observed in diploids. A haploid Δ*gcn4* Σ1278b strain (RH2693) carrying a *FLO11::lacZ* reporter was transformed to express wild type *GCN4* or the *GCN4^{L267S}* mutant allele and tested for amino acid starvation-induced adhesive growth. In contrast to diploids, cells expressing either wild type *GCN4* or *GCN4^{L267S}* became adhesive upon starvation for amino acids (Fig. 9A). Consistently, *FLO11::lacZ* expression during amino acid starvation resulted in a significantly increased *FLO11* derived β-galactosidase activity in the presence of wild type Gcn4p or mutant Gcn4p^{L267S}. A direct comparison showed that the Gcn4p derived expression of *FLO11::lacZ* (464 U) displayed half of the expression monitored in strains expressing wild type *GCN4* (1172 U) (Fig. 9B). The partial transcriptional activation of *FLO11* provided by the mutant is sufficient to induce haploid adhesive growth after amino acid starvation. The *GCRE6::lacZ* reporter activity was measured to compare the effect of the mutated Gcn4p on metabolic genes in haploids and diploids. The transcriptional activity of Gcn4p^{L267S} was slightly reduced to 82% (1323 U) upon amino acid starvation compared to the expression mediated by wild type Gcn4p (1619 U) (Fig. 9B).

These data suggest that the impact of the Gcn4p amino acid substitution Leu267Ser on haploids points to a similar direction as the impact on diploids, but the reduction in transcriptional activation capacity is less severe. Therefore the separation

of the metabolic and the developmental function of Gcn4p by the Leu267Ser exchange is specific for diploids and reveals subtle ploidy dependent differences for the same SNPs.

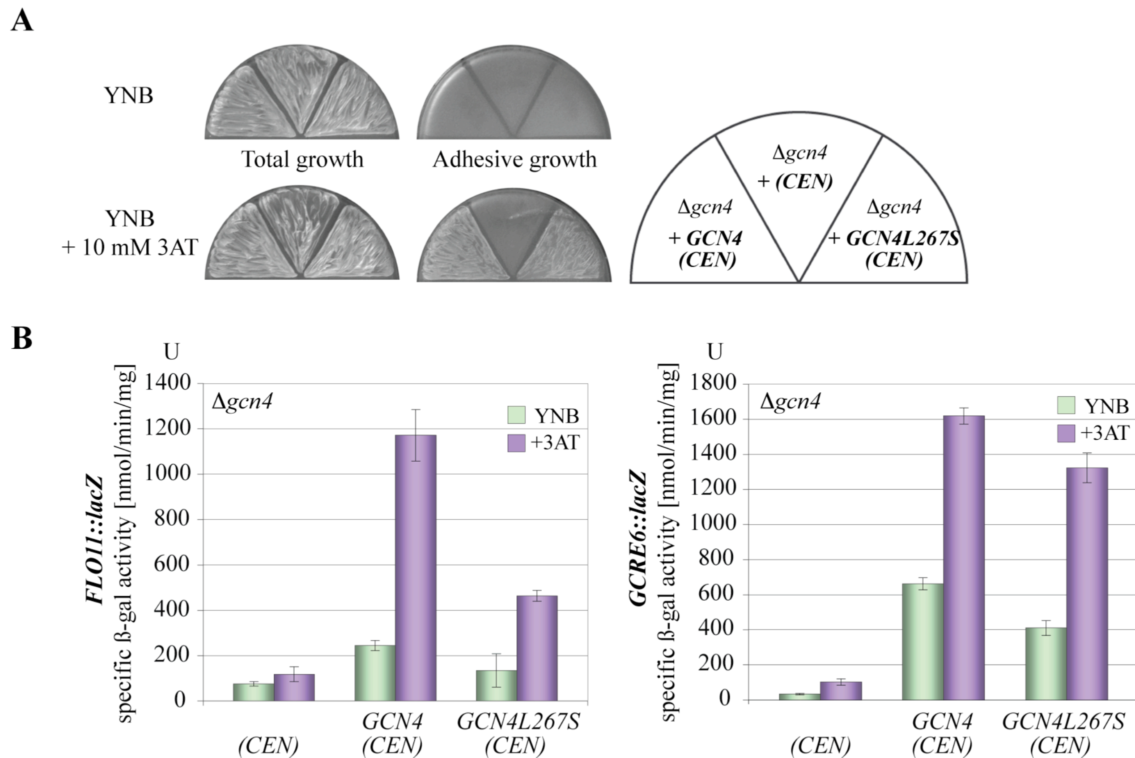


Fig. 9: ***GCN4**^{L267S}* **mediates adhesive growth of haploid yeasts, which are starved for amino acids.**

(A) Haploid yeast Σ1278b strain RH2693 (Δ*gcn4*) expressing wild type *GCN4* (pME1092), *GCN4*^{L267S} (pME2901) or the empty vector (pRS314) as control (Δ*gcn4*) was grown on solid YNB medium in the absence or presence of 10 mM 3AT to induce amino acid starvation. Plates were incubated and documented as described in Fig. 7A.

(B) Expression of *FLO11::lacZ* reporter gene was measured with the same yeast strains as described in (A) carrying a *FLO11::lacZ* reporter. Yeast strain RH2697 (Δ*gcn4*) carrying a *GCRE6::lacZ* reporter transformed with the same plasmids was used for measurement of *GCRE6::lacZ* reporter gene expression. Specific β-galactosidase activities were analyzed as described in Fig. 8.

3.1.4 Dimerization of Gcn4p^{L267S} is reduced compared to wild type Gcn4p

Gcn4p consists of several domains for transcriptional activation, stability, DNA binding or dimerization (Landschulz *et al.*, 1988; Kornitzer *et al.*, 1994; Drysdale *et al.*, 1995). The substitution Leu267Ser is located in the C-terminus in the third of four leucines of the leucine zipper dimerization region of Gcn4p (Fig. 10A). Due to this localization the impact of this substitution on Gcn4p dimer formation was analyzed.

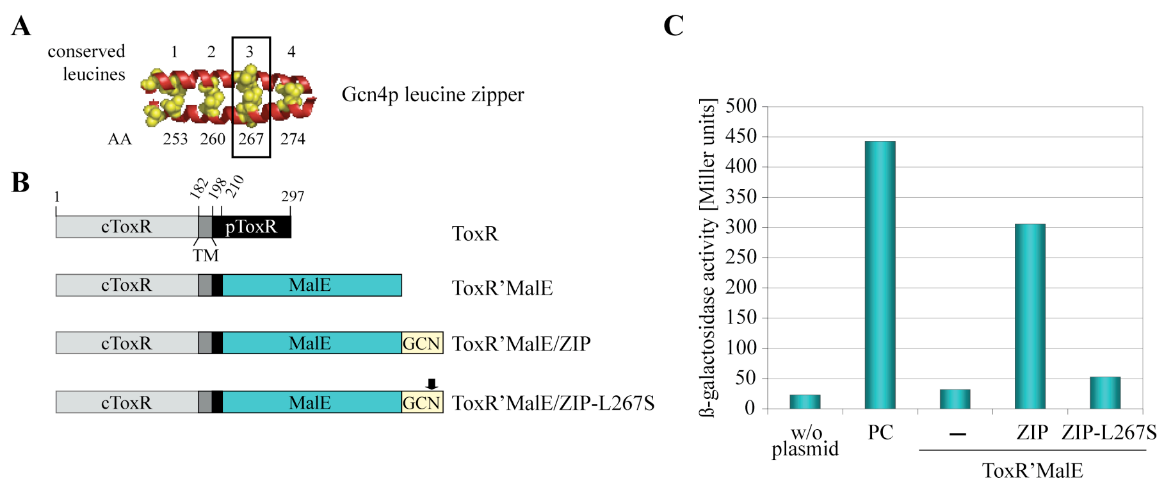


Fig. 10: Decreased dimerization of Gcn4p^{L267S}.

(A) Structure of the Gcn4p leucine zipper modified according to O'Shea *et al.* (1991). Upper numbers indicate the position of the four conserved leucines in the leucine zipper of Gcn4p, lower numbers the amino acid order in Gcn4p (AA). L267 is framed.

(B) Modular organization of the *V. cholerae* signal transduction protein ToxR. Numbers indicate amino acid positions. cToxR, N-terminal cytoplasmic ToxR domain (amino acids 1 – 182); TM, transmembrane segment (amino acids 183 – 198); pToxR, C-terminal, periplasmic domain (amino acids 199 – 297). Amino acids 1 – 210 of ToxR serve as the reference module in all constructs. The periplasmic domain of ToxR was replaced by the monomeric maltose binding protein MalE (ToxR'MalE). By fusing the 33 amino acids of the leucine zipper of Gcn4p to its C-terminus it can be converted from a monomer to a homodimer (ToxR'MalE/ZIP). ToxR'MalE/ZIP-L267S is mutated and contains the amino acid substitution Leu267Ser.

(C) Transcription activation at the *ctx* promoter in *E. coli* mediated by the ToxR derivates shown in (B). Specific β-galactosidase activities of *E. coli* strain FHK12, which contains a chromosomally integrated *lacZ* gene under *ctx* control, expressing ToxR'MalE without dimerization domain as control (-), ToxR'MalE with wild type dimerization domain (ToxR'MalE/ZIP) or mutated (ToxR'MalE/ZIP-L267S) (pME3389) are shown in Miller units. Additionally, the plasmid free reporter strain FHK12 (w/o plasmid) was used as a control for strains without ToxR function and *E. coli* strain FHK12 expressing pHKToxR'REI-T39K served as control (PC) which is noted for a strong increase in dimerization (Kolmar *et al.*, 1995a).

The heterologous bacterial ToxR-system was applied which is based on the *Vibrio cholerae* protein ToxR (Fig. 10B) (Miller *et al.*, 1987). ToxR is an integral membrane protein that acts as transcriptional activator of the *ctx* promoter. Therefore a *ctx* driven *lacZ* reporter, which depends strictly on dimerization of the C-terminal periplasmic ToxR domains gives the opportunity to analyze the dimerization by β-galactosidase assays in an *E. coli* indicator strain (FHK12). The ToxR periplasmic domain was replaced by a fusion of the monomeric maltose binding protein (Richarme, 1982; Duplay *et al.*, 1984) and the Gcn4p dimerization domain. The 33 amino acids of the leucine zipper of Gcn4p (pHKToxR'MalE/ZIP) (Blondel and Bedouelle, 1991; Kolmar *et al.*, 1995b) or the corresponding part of the Leu267Ser mutant variant (pHKToxR'MalE/ZIP^{L267S}) were compared in their ability to dimerize and thereby

mediating transcription of the *lacZ* reporter gene. Comparison of the Gcn4p mutant and wild type leucine zipper revealed an approximately six-fold decrease in dimerization ability in the mutant Gcn4p^{L267S} compared to wild type, which is caused by the amino acid substitution in the leucine zipper (Fig. 10C).

These data suggest that the reduced transcriptional activator potency of the mutant Gcn4p carrying a substitution of leucine to serine at position 267 in the zipper region is due to reduced dimerization ability.

3.1.5 Various amino acid substitutions of zipper leucines lead to a separation of the metabolic and developmental Gcn4p function

The leucine at position 267 of Gcn4p contributes to dimerization as a prerequisite for the full transcriptional activity function of the protein. Full activity neither seems to be required for growth during amino acid starvation nor for the haploid adhesive life style, but it is specifically required for diploid pseudohyphal development and adhesive growth. We introduced other amino acid substitutions at the same position as well as at another conserved leucine of the zipper to analyze whether reduced dimerization is sufficient to separate the metabolic and the developmental Gcn4p function. These Gcn4p zipper substitutions resulted in a broad range of 1.5- to 13-fold decreased *FLO11* expression (41 U – 5 U) in comparison to wild type Gcn4p (*GCN4*) (66 U) during amino acid starvation (Fig. 8).

Fig. 11A shows that the substitution of Leu267 to alanine (L267A) resulted in 60% wild type *FLO11::lacZ* expression (41 U) when treated with 3AT, whereas a substitution of two leucines (L253G and L267G) with a helix breaking glycine nearly abolished *FLO11::lacZ* expression (5 U – 6 U) comparable to a *gcn4* deletion strain (5 U). A substitution with threonine (L267T) or cysteine (L267C) was similar for *FLO11* expression upon amino acid starvation like Leu267Ser. The adhesion phenotypes of each mutant strain scored by biofilm formation in wells of polystyrene plates correlated to these results (Fig. 11B). The drastic effect of the glycine substitutions on the zipper leucines was also corroborated by measuring the transcriptional activity of the Gcn4p variants onto the metabolic target *GCRE6::lacZ* reporter. Upon 3AT treatment *GCRE6::lacZ* induction by the glycine variants displayed only to 7 – 10% (55U and 83U) of wild type induction (782 U) (Fig. 11C). Consistently, the Gcn4 mutant proteins containing a glycine in its leucine zipper were no more able to complement a *gcn4*

deletion phenotype or to survive amino acid starvation in contrast to Gcn4p^{L267S}, Gcn4p^{L267T}, Gcn4p^{L267A} and Gcn4p^{L267C}, respectively (Fig. 12B). In contrast, cells expressing these four *GCN4* variants allowed a 46 – 71% (358 – 555U) activation of Gcn4p-specific target genes upon 3AT treatment (Fig. 11C) and therefore survived amino acid starvation (Fig. 12B).

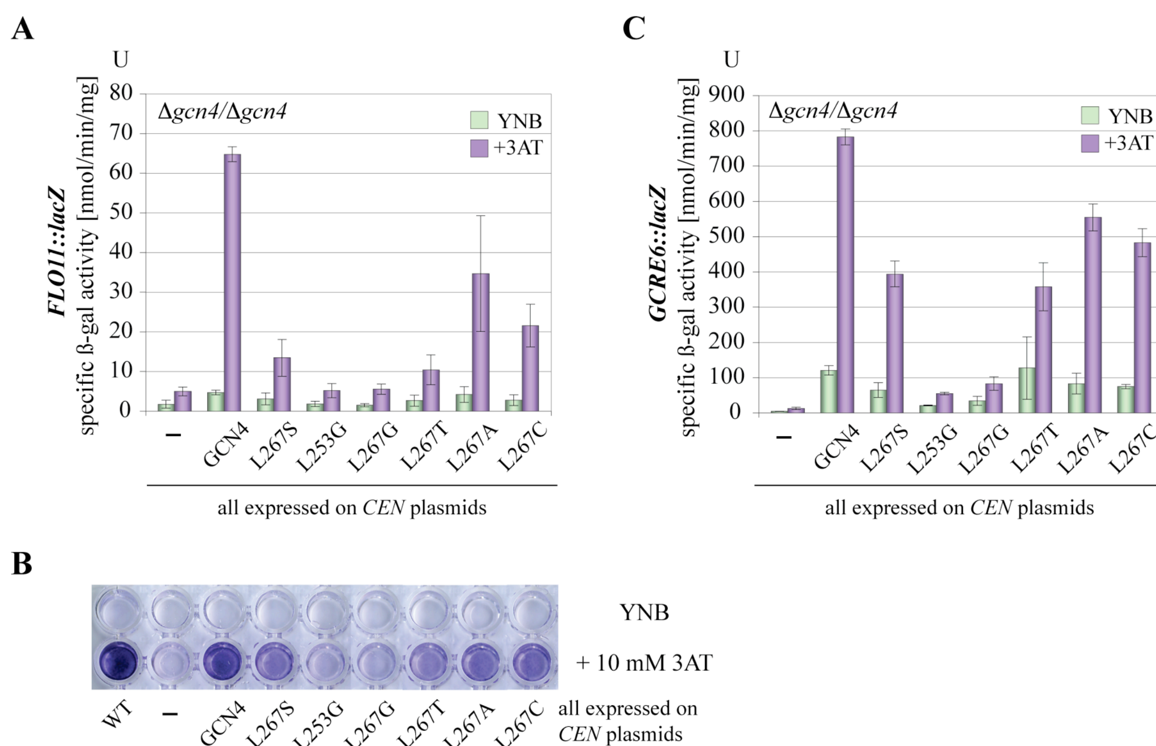


Fig. 11: Impairment of diploid adhesive growth and *FLO11* expression in yeast strains expressing *GCN4* with various amino acid substitutions of zipper leucines.

(A) The diploid yeast Σ1278b strain RH2695 (Δ*gcn4*/Δ*gcn4*) carrying a *FLO11::lacZ* reporter was transformed with *CEN* plasmids to express wild type *GCN4* (pME1092), *GCN4*^{L267S} (pME2901), *GCN4*^{L253G} (pME3378), *GCN4*^{L267G} (pME3379), *GCN4*^{L267T} (pME3380), *GCN4*^{L267A} (pME3381), *GCN4*^{L267C} (pME3382) or the empty vector (pRS314) as control (-) and specific β-galactosidase activities were assayed.

(B) For testing amino acid starvation induced adhesive growth, same diploid yeast strains as described in (A), and additionally the diploid Σ1278b wild type strain RH2656 as control (WT) were grown in liquid YNB media up to an optical density of 0.6 before 300 µl of each culture were transferred in a microtiter well. Cells were grown in absence or presence of 10 mM 3AT to induce starvation dependent adhesive growth. After incubation for 2 days at 30°C, sedimented cells were dyed with crystal violet and carefully washed. Finally plates were photographed to document adhesive growth.

(C) *GCRE6::lacZ* expression was measured as described in Fig. 8. The diploid yeast strain RH2398 (Δ*gcn4*/Δ*gcn4*) carrying a chromosomally integrated *GCRE6::lacZ* reporter construct expressing the different Gcn4p mutant plasmids described in (A).

Next, diploid Δ*gcn4*/Δ*gcn4* yeast strains expressing one of the *GCN4* mutant alleles or wild type *GCN4* as control were analyzed for pseudohyphal development upon nitrogen starvation. Cells expressing *GCN4*^{L267S}, *GCN4*^{L267T} or *GCN4*^{L267G} showed a reduced ability to form pseudohyphae (Fig. 7B and 12A).

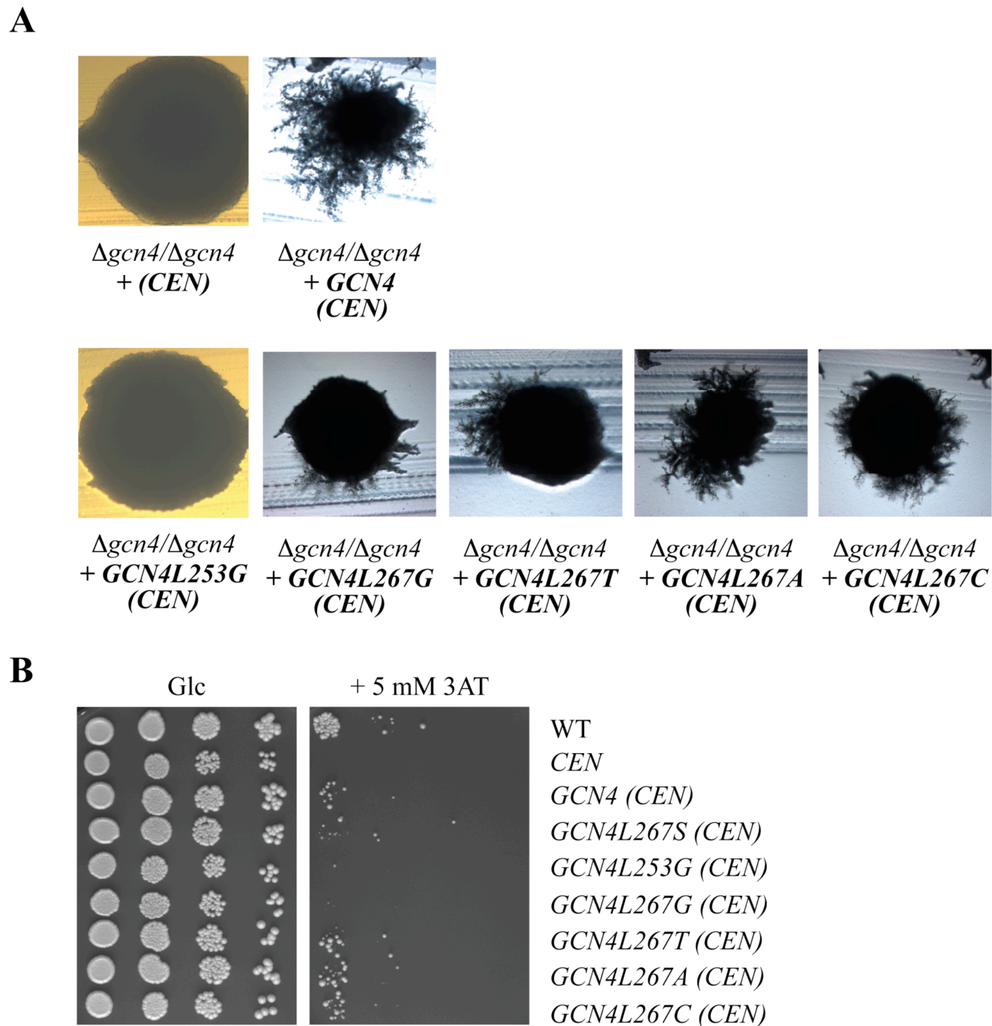


Fig. 12: Constricted pseudohyphal growth of diploid yeast strains expressing different alleles of *GCN4*.

(A) The diploid *gcn4* deletion strain RH2695 (Δ*gcn4*/Δ*gcn4*) expressing wild type *GCN4* (pME1092), the substituted *GCN4*^{L253G} (pME3378), *GCN4*^{L267G} (pME3379), *GCN4*^{L267T} (pME3380), *GCN4*^{L267A} (pME3381) or *GCN4*^{L267C} (pME3382) from *CEN* plasmids with *GCN4* promoter and terminator or as control the empty vector (*CEN*) (pRS314) was streaked out on SLAD medium and after incubation for five days at 30°C pseudohyphal colonies were visualized.

(B) The same yeast strains as described in (A) and additionally the diploid strain RH2695 (Δ*gcn4*/Δ*gcn4*) carrying plasmids with *GCN4*^{L267S} (pME2901) were spotted in 5-fold dilutions on either glucose medium as control or glucose medium containing 5 mM 3AT to induce amino acid starvation. The diploid wild type strain RH3278 (WT) served as growth control. After incubation for 3 – 4 days at 30°C plates were photographed.

As expected, a substitution of Leu253 with glycine resulted in a complete loss of pseudohyphae formation upon nitrogen starvation comparable with a *gcn4* deletion strain (Fig. 12A). Pseudohyphal development is less affected in diploid cells expressing $GCN4^{L267A}$ or $GCN4^{L267C}$. In comparison to wild type cells, pseudohyphae are shorter and less branched. These two mutant proteins equally showed the highest *FLO11* expression and ability to grow adhesively in response to amino acid starvation when compared with the other Gcn4 mutant proteins (Fig. 11A and B).

These results further support, that developmental function of Gcn4p in diploids requires full transcriptional activity of Gcn4p and does not tolerate defects in dimerization. Diploid cells expressing a *GCN4* mutant allele like $GCN4^{L267S}$, which is partially impaired in dimerization, are unable to perform adhesive growth or to form pseudohyphae upon nutrient starvation. However, the same mutants are still able to complement a *gcn4* deletion phenotype and protect the cells against amino acid starvation. Abolishment of the metabolic function is achieved by impairing dimerization by replacing leucines of the zipper by helix breakers like glycine which results in an almost inactive transcription factor.

3.1.6 Protein stability of Gcn4p^{L267S} and other dimerization variants is increased in comparison to the wild type protein

Gcn4p is under tight protein stability control. Under non-starvation conditions the instable Gcn4p has a half life of a few minutes that increases up to 20 minutes when cells are starved for amino acids. Gcn4p^{L267S} dimerization is impaired, suggesting that there is an increased molecule subpopulation which is present as monomer. We analyzed how the stability of Gcn4p^{L267S} or other Gcn4p variants is affected by the protein degradation pathway in comparison to wild type Gcn4p.

A three-fold myc-tagged version of wild type *GCN4* or $GCN4^{L267S}$ was expressed from the inducible *GAL1* promoter in a *gcn4* mutant strain. Protein levels were determined after *GAL1* promoter shut-off in sated and amino acid starved cells. Western hybridization revealed that both wild type Gcn4p and Gcn4p^{L267S} were rapidly degraded in sated cells (Fig. 13). In response to amino acid starvation the substitution of Leu267 to serine was significantly more stabilized than wild type Gcn4p. This stabilization was less pronounced when myc^3-$GCN4^{L267A}$ was expressed. Consistently, expression of $GCN4^{L267A}$ led to the weakest effects on 3AT-induced *FLO11* expression

and adhesive growth when compared with the other *GCN4* alleles (Fig. 11A and B). Even the poorly active Gcn4p^{L253G} mutant protein was more stable under amino acid starvation conditions, compared to wild type Gcn4p, and actually more stable under non-starvation conditions since it was still detectable after 90 minutes under non-starvation conditions (Fig. 13).

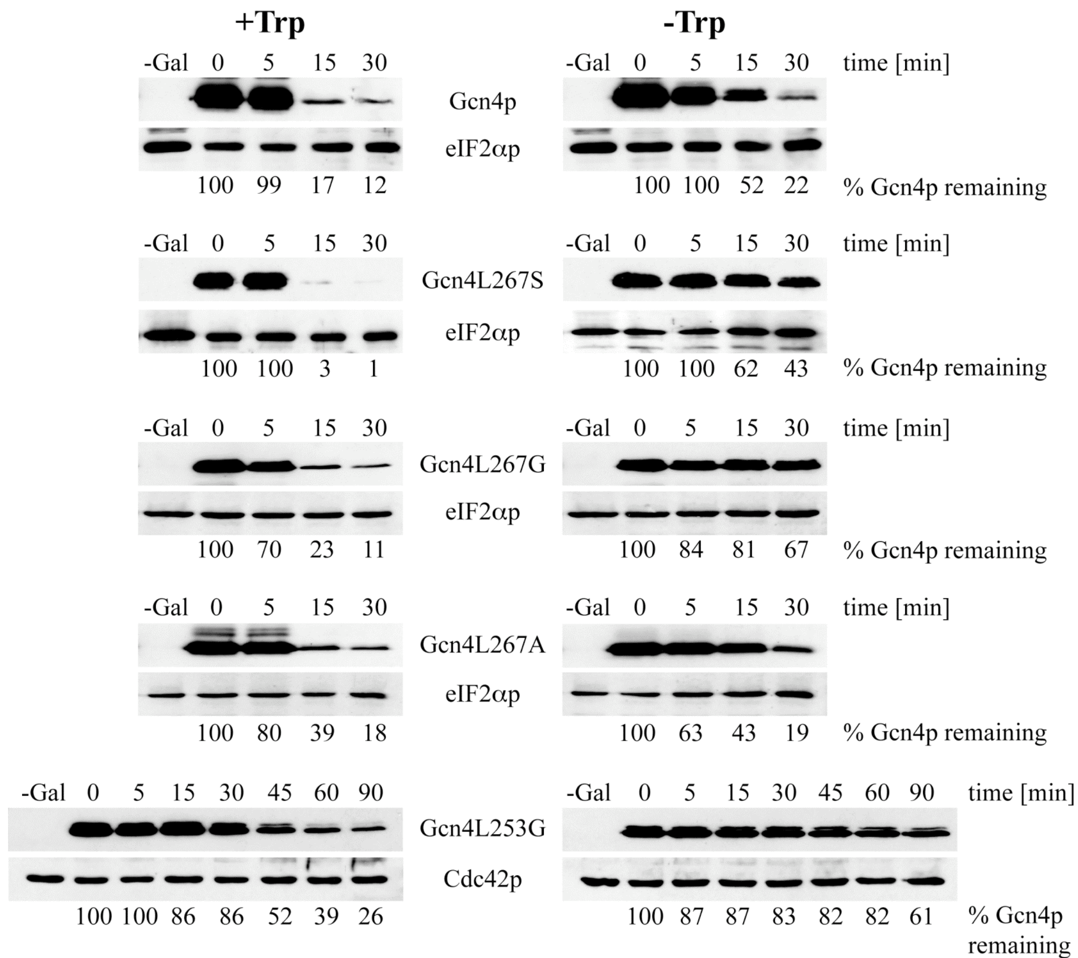


Fig. 13: Increased protein stability of Gcn4p variants compared to wild type Gcn4p.

The diploid Σ1278b strain RH2694 (Δ*gcn4*/Δ*gcn4*) was transformed to express the *GAL1*-driven *myc*^{3}-*GCN4* (pME2923), *myc*^{3}-*GCN4*^{L267S} (pME2925), *myc*^{3}-*GCN4*^{L267G} (pME3384), *myc*^{3}-*GCN4*^{L267A} (pME3386) or *myc*^{3}-*GCN4*^{L253G} (pME3383) on a high copy plasmid. Protein levels of myc^{3}-tagged Gcn4p variants in sated (+Trp) and amino acid starved cells (-Trp) were determined by immunoblotting at the indicated time points after shutting-off the *GAL1* promoter by adding 3% glucose (0-min time point). eIF2αp or Cdc42p were used as loading controls. Numbers given below indicate remaining Gcn4p percentages when compared to eIF2αp or Cdc42p as internal standards.

These data indicate that Gcn4p variants compromised in dimerization and transcriptional activation are more stable than wild type yeast Gcn4p. Increased amino acid starvation-dependent stabilization is achieved when the hydrophobic Leu267 of the zipper is substituted for a polar amino acid. The exchange of zipper amino acid Leu253 by the helix breaker glycine even leads to a stabilization of Gcn4p under non-starvation conditions.

3.1.7 Gcn4p activates its own destruction and therefore Gcn4p transcriptional activity reciprocally correlates to its own protein stability

The results with the different variants of Gcn4p demonstrate that a decreased degree of Gcn4p dimerization correlates with increased Gcn4p stability but at the expense of a reduced transcriptional activation potential. The reason could be that the degradation pathway recognizes a dimer more efficiently than a monomer and therefore a monomeric Gcn4p is a poor target for degradation. Alternatively, full transcriptional activity of Gcn4p could be a prerequisite for an efficient Gcn4 protein degradation pathway. The initial step of the Gcn4p degradation pathway is the cyclin dependent phosphorylation by Pho85p-Pcl5p. The *PCL5* gene encoding the cyclin contains a GCRE in its promoter and is therefore a Gcn4p target gene.

Stability of Gcn4p^{L253G} was analyzed when *PCL5* was constitutively expressed to discriminate between the two possibilities. Gcn4p^{L253G} represents the most stable and transcriptional inactive variant of mutated Gcn4p. Promoter shut-off experiments were performed with a *GAL1* driven *myc*^{3}-*GCN4*^{L253G} in the presence of high amounts of *PCL5* (*MET25-PCL5-GFP).* The presence of Pcl5p resulted in Gcn4p^{L253G} which became significantly instable (Fig. 14A) and the protein turnover was similar to that of Gcn4 wild type protein (Fig. 13).

The same yeast strains were further analyzed for Gcn4p toxicity. Gcn4p synthesis was repressed (glucose) or induced (galactose) by the *GAL1* promoter in the presence or absence of amino acid starvation conditions. The diploid wild type strain (RH3278) and control strains expressing wild type *myc*^{3}-*GCN4* either along with an empty vector or with independently overexpressed *PCL5-GFP* were used.

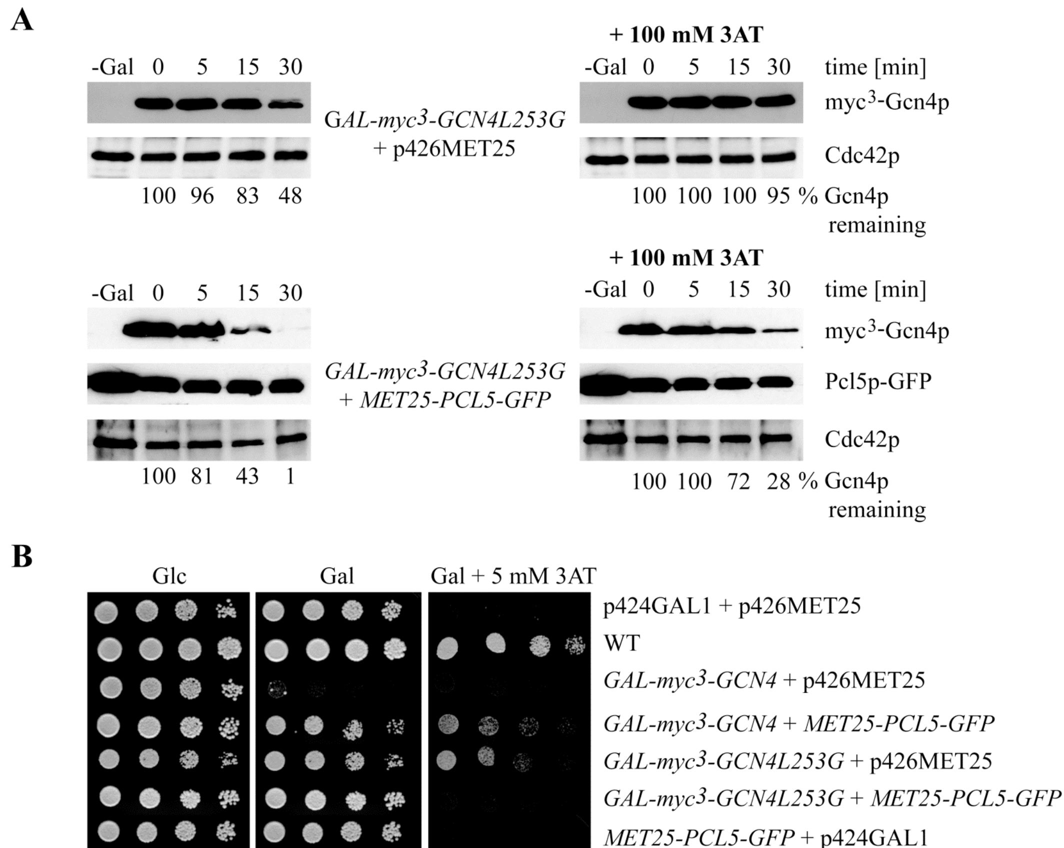


Fig. 14: Correlation between transcriptional activity of Gcn4p and protein stability.

(A) The diploid Σ1278b strain RH2694 (Δ*gcn4*/Δ*gcn4*) was transformed to express *GAL1*-driven *myc*^{3}-*GCN4*^{L253G} together with either independently overexpressed *PCL5-GFP* (pME3388 + pME2846) or an empty vector as control (pME3388 + p426MET25). 100 mM of 3AT was used to induce amino acid starvation. Protein levels of myc^{3}-Gcn4p or Pcl5p-GFP were determined by Western blotting at the indicated time points after shutting-off the *GAL1* promoter by the addition of 3% glucose (0-min time point). Cdc42p was used as loading control. Numbers given below indicate remaining Gcn4p percentages when compared to Cdc42p as internal standard.

(B) The same yeast strains as described in (A), and additionally strain RH2694 transformed with *GAL1*-driven wild type *myc*^{3}-*GCN4* in combination with either independently overexpressed *PCL5-GFP* (pME2918 + pME2846) or an empty vector (pME2918 + p426MET25), were spotted in five-fold dilutions on glucose (repressing conditions) and galactose medium (inducing conditions) to induce expression of *GCN4*. Serving as further controls, strain RH2694 was transformed with either both empty vectors (p424GAL1 + p426MET25) or independently overexpressed *PCL5-GFP* along with an empty vector (pME2846 + p424GAL1). The diploid strain RH3278 was used as wild type control (WT). Furthermore, strains were spotted on galactose medium containing 5 mM 3AT to induce amino acid starvation. After incubation for 3 – 4 days at 30°C the plates were photographed.

Yeast cells expressing wild type *GCN4* from the *GAL1* promoter grew neither under inducing conditions (Gal) nor under inducing conditions and additional amino acid starvation (Gal + 5 mM 3AT) due to the overexpression toxicity of Gcn4p (Fig. 14B) (Tavernarakis and Thireos, 1995; Shemer *et al.*, 2002). Growth was reconstituted when *PCL5* was co-expressed. In contrast, cells expressing *GCN4*^{L253G} along with an

empty vector were able to grow in the presence of 5 mM 3AT, due to the significantly reduced transcriptional activity of this Gcn4p variant (Fig. 11C). Additional overexpression of Pcl5p caused degradation of Gcn4p^{L253G} (Fig. 14A) and cells were unable to grow upon amino acid starvation induced by 5 mM 3AT, similar to cells expressing wild type *GCN4* along with an empty vector (Fig. 14B).

These results revealed that the identified Gcn4p variant Leu267Ser is not sufficiently active to induce its own destruction similar to wild type. Gcn4p^{L253G} exhibits its phenotype due to a feedback loop which connects its protein stability to its reduced transcriptional activation potential.

3.2 The UPR transcription factor Hac1p mediates Flo11p-dependent adhesion and dimorphism in diploid yeasts

Amino acid starvation triggers the activation of the 'general control of amino acid biosynthesis' (GAAC) in *S. cerevisiae*. The transcriptional activator Gcn4p represents the key regulator of this network and activates hundreds of genes (Natarajan *et al*., 2001). The 'unfolded protein response' (UPR) represents a second system and is activated by unfolded proteins, which accumulate in the ER. The central regulator of this system is the transcription factor Hac1p, however, Gcn4p is also required for the activation of elements of the UPR (Patil *et al*., 2004). Therefore we have analyzed the role of Hac1p for the control of Gcn4p specific target genes.

3.2.1 Hac1p is required for inducing a general control reporter gene whereas it is repressed by ER stress

We examined the influence of a *hac1* deletion using a Gcn4p specific reporter gene which contains six GCRE-binding sites for Gcn4p upstream of the *CYC1::lacZ* minimal promoter. Thus, expression of *GCRE6::lacZ* could be quantified by β-galactosidase assays. The *GCRE6::lacZ* gene was chromosomally integrated into the *URA3* locus of haploid wild type strain as well as into Δ*hac1* and Δ*gcn4* single or double mutant strains. β-galactosidase activities of resulting strains were monitored under four different growth conditions as follows: non-starvation (YNB), amino acid starvation induced by the histidine analogon 3-amino-1,2,4-triazole (3AT) (Klopotowski and Wiater, 1965), ER stress by the addition of the UPR stressor tunicamycin (Tm) that inhibits protein folding by inhibiting N-linked glycosylation (Back *et al.*, 2005), and starvation for nitrogen by the reduction of the ammonium sulfate concentration from 50 mM to 50 µM (Fig. 15A).

Basal expression of the *GCRE6::lacZ* reporter was almost identical in wild type and Δ*hac1* cells under sated conditions (YNB). In contrast, upon amino acid starvation (3AT) Δ*hac1* cells were unable to induce the general control system of amino acid biosynthesis and remained at the basal level as in wild type cells in the presence of amino acids (Fig. 15A). Both strains containing a *gcn4* deletion were not able to activate *GCRE6::lacZ* expression neither under non-starvation conditions nor in response to amino acid starvation.

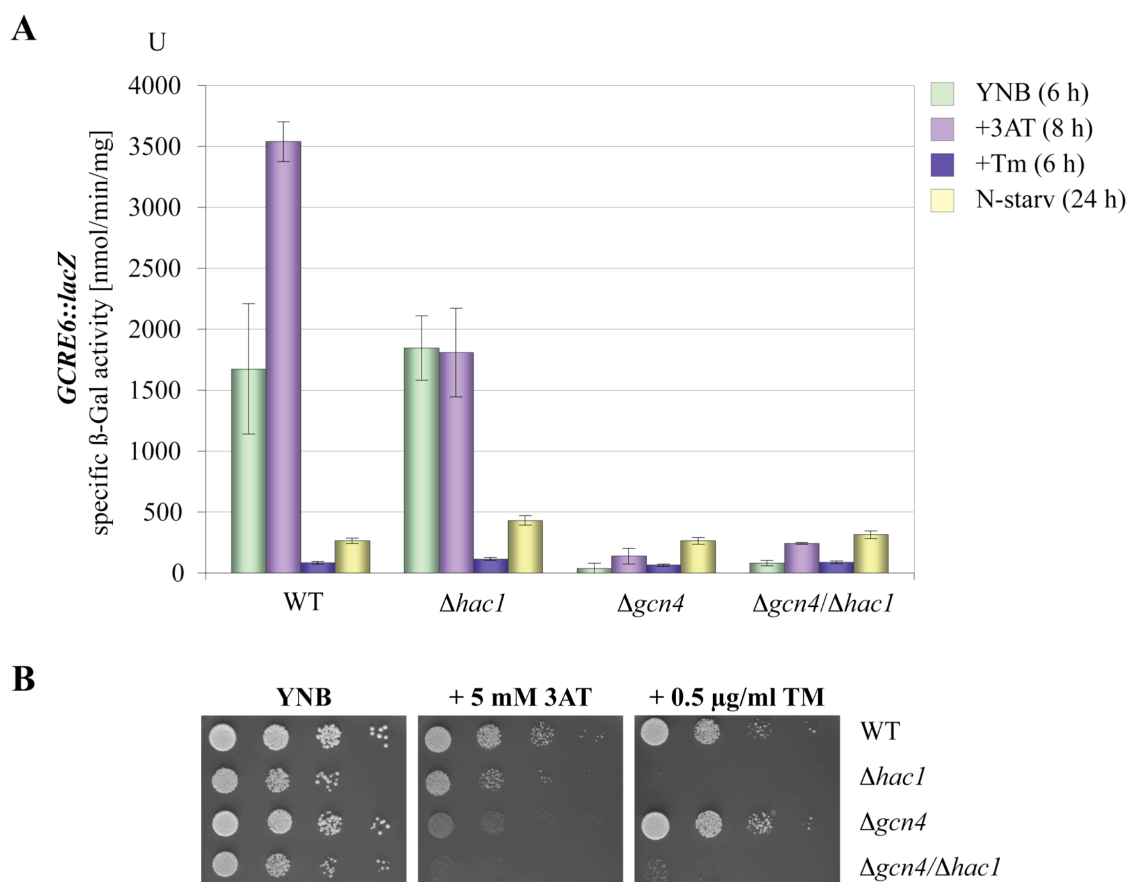


Fig. 15: Gcn4p-dependent reporter gene expression is governed by Hac1p and repressed by ER stress in haploids.

(A) Expression of *GCRE6::lacZ* was determined in haploid Σ1278b wild type strain (WT) (RH3409) as well as in Δ*hac1* (RH3363), Δ*gcn4* (RH3410) and Δ*gcn4*/Δ*hac1* (RH3411) mutant strains each carrying a chromosomally integrated *GCRE6::lacZ* reporter. Expression was measured under different nutritional conditions. Starting from one overnight culture, strains were diluted into fresh medium and further cultivated for 6 – 24 h in the respective media before specific β-galactosidase activities were assayed. Cultures were grown to log-phase in YNB under non-starvation conditions (green bars, YNB (6 h)). Amino acid starvation and ER stress conditions were induced by addition of either 10 mM 3AT (purple bars, +3AT (8 h)) or 1 µg/ml tunicamycin (dark blue bars, Tm (6 h)). For nitrogen starvation (N-starv) yeast cells were washed twice with 2% glucose before incubating for 24 h in minimal medium containing only 50 µM ammonium sulfate as the sole nitrogen source (yellow bars, N-starv (24 h)). Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. The bars represent the mean value of at least three independent measurements.

(B) Haploid Σ1278b wild type strain (WT) (RH2816) as well as Δ*hac1* (RH3351), Δ*gcn4* (RH2676) and Δ*gcn4*/Δ*hac1* (RH3402) mutant strains were spotted in 10-fold dilutions on either glucose medium as control (YNB) or glucose medium containing 5 mM 3AT to induce amino acid starvation. Furthermore, the cells were spotted on glucose medium containing 0.5 µg/ml Tm to mimic ER stress. After incubation for 3 – 4 days at 30°C plates were photographed.

Gcn4p is a very unstable protein under non starvation conditions and half life of Gcn4p increases when starved for amino acids, however, the unstable Gcn4p is sufficient to ensure basal levels of biosynthetic genes in wild type cells. In contrast, yeasts deleted for *GCN4* are no longer in a position to enable this basal activity in sated cells. In addition, expression was repressed in all cells when starved for nitrogen which was already demonstrated by Grundmann *et al.* (2001). Interestingly, the GCRE-driven gene expression was drastically reduced upon ER stress conditions. Expression actually more decreased when compared to starvation for nitrogen. Consistent results were obtained by growth assays under non-starvation, amino acid starvation, and ER stress conditions. All parental haploid strains grew under non-starvation conditions (YNB) whereas growth of Δ*hac1* cells is reduced upon amino acid starvation (+ 5 mM 3AT) when compared with wild type cells (Fig. 15B). Cells containing a *gcn4* deletion neither were able to activate Gcn4p specific target genes under both conditions nor to grow in the presence of amino acid starvation. As expected, cells containing a *hac1* deletion disabled growth under ER stress conditions (+ 0.5 µg/ml Tm).

These data suggest that not only Gcn4p is required for the activation of elements of the UPR upon ER stress but also Hac1p influences activation of Gcn4p specific target genes in response to amino acid starvation. In contrast, ER stress represses this activation similar to nitrogen starvation.

3.2.2 ER stress represses *GCN4* mRNA translation

Our results suggest that activation of Gcn4p specific target genes is repressed by ER stress. Since Patil and co-workers documented an up-regulation of Gcn4p levels in response to ER stress, especially 15 and 30 minutes post tunicamycin treatment (Patil *et al.*, 2004), we were interested whether we can confirm this short UPR-dependent up-regulation of Gcn4p. First of all, we measured the expression of the *GCRE6::lacZ* reporter in haploid wild type cells under eight different conditions whereupon four were described in Fig. 15A. To analyze the activation of the reporter gene at different time points post ER stress induction cells were cultivated corresponding to non-starvation conditions before ER stress was induced for different time intervals (Fig. 16A). In fact, we could measure a short up-regulation of GCRE-driven gene expression, which is Gcn4p-dependent. When cells were treated longer time intervals with tunicamycin,

however, expression of *GCRE6::lacZ* reporter gene was reduced *a fortiori* the longer cells were grown under ER stress conditions.

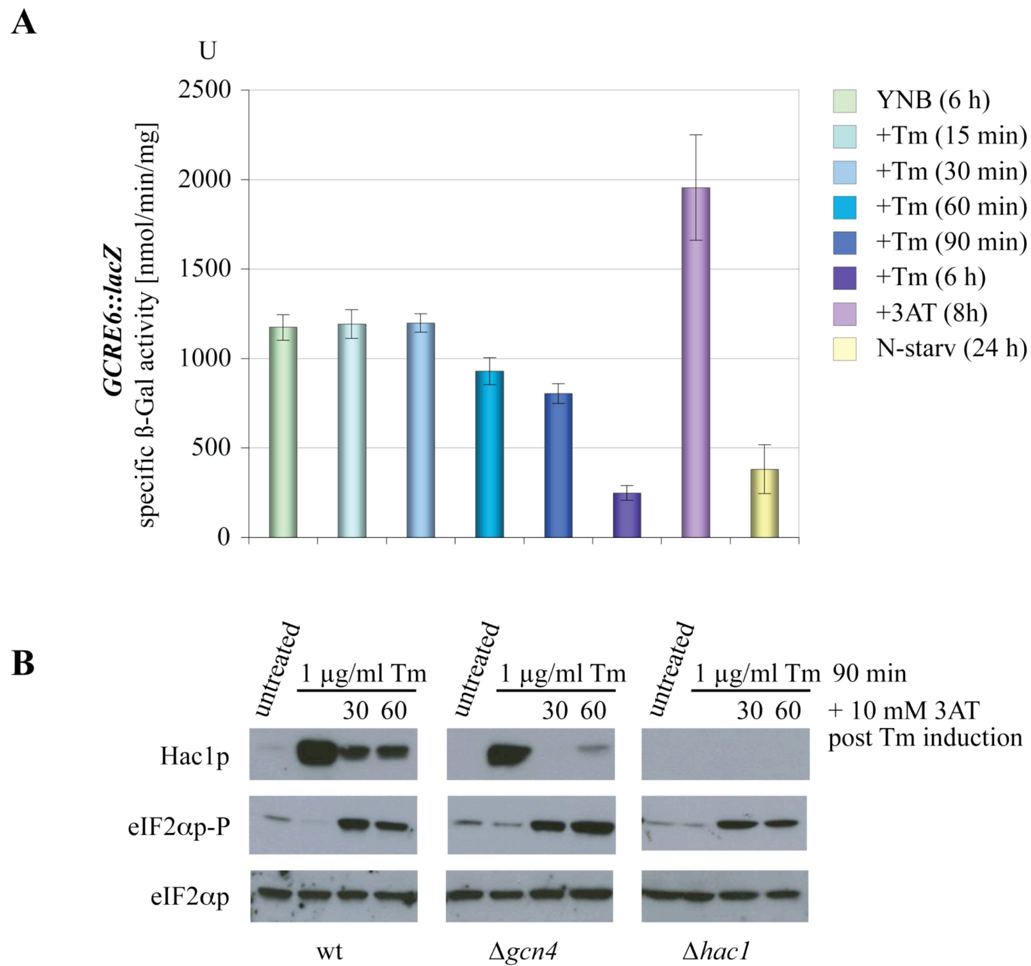


Fig. 16: Gcn4p-dependent reporter gene expression and eIF2αp phosphorylation are reduced upon ER stress.

(A) Expression of the *GCRE6::lacZ* reporter gene was determined in the haploid Σ1278b wild type strain RH3409 under non-starvation (YNB, green bar) in comparison to different starvation or stress conditions. Amino acid (3AT, purple bar) and nitrogen starvation (N-starv, yellow bar) were induced as described in Fig. 15A. For ER stress conditions cells were incubated either in the presence of 1 µg/ml Tm for 6 h (Tm, dark blue bar) or cells were initially grown for 6 h under non-stress conditions before incubating for indicated time points with 1 µg/ml Tm (blue bars; the longer Tm induction, the deeper is the color). Specific β-galactosidase activities were analyzed as described in Fig. 15A.

(B) Crude protein extracts were prepared from haploid Σ1278b wild type cells (WT) (RH2816) as well as from Δ*gcn4* (RH2676) and Δ*hac1* (RH3351) mutant strains, respectively. Cells were grown either under normal conditions (untreated) or in presence of ER stress conditions induced by 1 µg/ml tunicamycin (Tm). Additional amino acid starvation was obtained by adding 10 mM 3AT. Starting from one main culture with OD_{600} ~ 0.8 at 30°C cultures were quartered and cultivated further 90 min under indicated conditions. Protein levels of Hac1p and eIF2αp-P were analyzed by immunoblotting using specific antibodies. eIF2αp was used as loading control.

Hac1p is expressed in both haploid wild type and Δ*gcn4* cells under ER stress conditions (Fig. 16B). This expression is reduced by concurrent presence of 3AT, which in turn is Gcn4p-dependent. Furthermore, phosphorylation of eIF2αp was weak in all untreated strains and weakened once more upon ER stress. In contrast, amino acid starvation overruled the suppressed influence of tunicamycin and de-repressed phosphorylation of eIF2αp. These findings confirm our assumption that Gcn4p-dependent target gene expression is finally repressed upon ER stress conditions, which in turn is caused by reduced eIF2αp phosphorylation.

Additionally, we measured translational efficiency of *GCN4* mRNA using a *GCN4::lacZ* fusion construct which represents an accurate measure for this to further corroborate our hypothesis (Hinnebusch, 1985). Haploid wild type strain as well as Δ*hac1* and Δ*gcn4* single and double mutant strains were transformed with the *GCN4::lacZ* fusion plasmid and β-galactosidase activities were measured likewise under eight conditions as described for *GCRE6::lacZ* expressions (Fig. 16A).

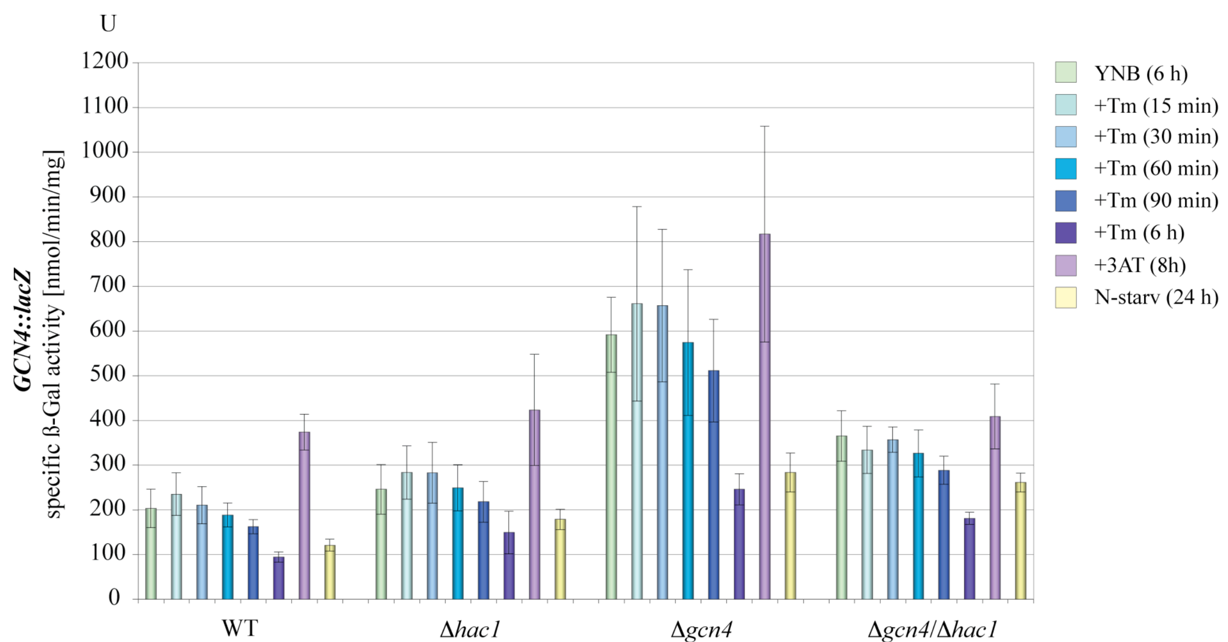


Fig. 17: Repression of *GCN4* mRNA translation by ER stress is independent of Hac1p.

Expression of a *GCN4::lacZ* fusion gene (p180) was measured in a haploid Σ1278b wild type strain (WT) (RH2816) as well as in Δ*hac1* (RH3351), Δ*gcn4* (RH2676) and Δ*gcn4*/Δ*hac1* (RH3402) mutant strains under different nutritional conditions. Bars depict means of at least three independent measurements of β-galactosidase activities.

GCN4::lacZ expression decreased when cells were starved for ammonium (Fig. 17) (Grundmann *et al.*, 2001). Consistent with our previous findings, *GCN4::lacZ* expression increased in all strains for a short time (15 and 30 min) post ER stress induction before decreasing after 6 hours onto the expression achieved by nitrogen starvation. However, with the exception of the Δ*gcn4* strain, no prominent differences between the other strains were measured. The general increased data of the Δ*gcn4* are traced back to an induced general control system and in agreement with our previous findings that nitrogen starvation represses amino acid starvation-induced activation of *GCN4* expression on its translation level (Grundmann *et al.*, 2001). We further measured the expression of the *GCN4::lacZ* fusion construct in haploid S288c wild type cells in which a higher activation of *GCN4* expression upon amino acid starvation but not for nitrogen starvation is documented (Grundmann *et al.*, 2001).

Amino acid starvation led to a significant induction of β-galactosidase activity in haploid S288c wild type cells. In contrast, both nitrogen starvation and ER stress abolished *GCN4::lacZ* expression (Fig. 18).

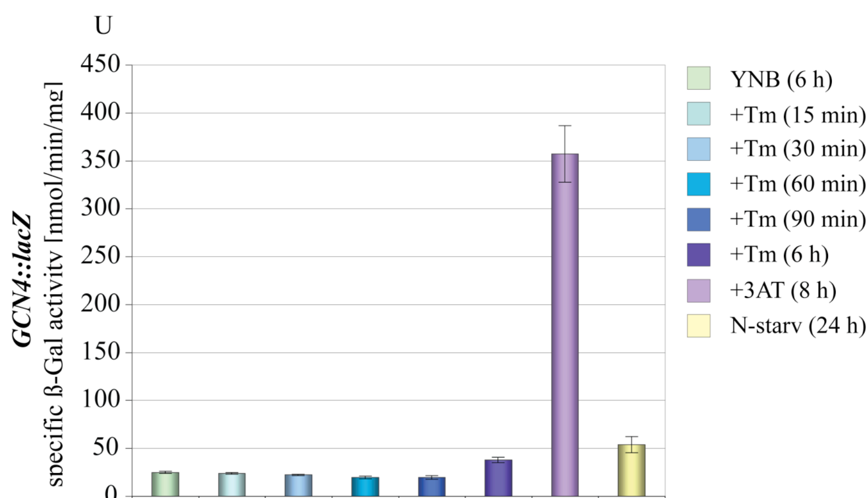


Fig. 18: Repression of *GCN4* expression by ER stress in haploid S288c cells.

Haploid S288c wild type strain RH2520 was transformed with plasmid p180 to express the *GCN4::lacZ* reporter gene. Cells were cultivated as described in Fig. 16 before specific β-galactosidase activities were assayed. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. The bars represent the mean value of at least three independent measurements.

These data suggest that ER stress is a further signal beside nitrogen starvation, which leads to repression of eIF2αp phosphorylation followed by repressed *GCN4* mRNA translation and target gene expression. Indeed, Hac1p itself does not influence eIF2αp phosphorylation and *GCN4* mRNA translation but is responsible fur full activation of Gcn4p target gene expression upon amino acid starvation, which suggests an independent role of Hac1p in activation of GCREs. It is demonstrated that Gcn4p directly interacts with UPRE-1 (CAGNGTG) and UPRE-2 (TACGTG) (Patil *et al.*, 2004). Both share 'half-site' similarity having a three base identity at the 3' end (GTG) and for Gcn4p it is known that it can bind to half-sites (Hollenbeck and Oakley, 2000) and the potential UPRE-half-site is present in the GCRE consensus sequence (RRRWGASTCA; with R = purine, W = T or A, and S = G or C) (Natarajan *et al.*, 2001). Therefore, it could be possible, that both Gcn4p and Hac1p, regulate the activation of the other target genes by binding to half-sites in their promoter regions. In contrast, Gcn4p influences UPR-activated Hac1p expression in concurrent presence of 3AT.

3.2.3 Repression of Gcn4p-dependent gene expression in diploid homozygous Δ*hac1* and Δ*gcn4* strains

Furthermore, we were interested whether our findings concerning the reduced Gcn4p target gene expression upon amino acid starvation in haploid Δ*hac1* cells could be confirmed in diploids, since most natural yeasts are dimorphic and diploid (Mortimer, 2000). Therefore, the *GCRE6::lacZ* reporter gene containing haploid *MAT**a*** strains were crossed with respective *MATα* strains to obtain homo- and heterozygous diploid strains deleted for *HAC1*, *GCN4* or both. Expression of Gcn4p reporter gene was monitored in sated and amino acid starved cells (Fig. 19).

With the exception of the heterozygous Δ*gcn4*/Δ*hac1* strain, we found that expression of the *GCRE6::lacZ* reporter is insignificantly reduced in heterozygous Δ*hac1* and Δ*gcn4* strains under both conditions when compared to the expression obtained in diploid wild type cells. As expected, expression was strong decreased in diploid homozygous strains deleted for *GCN4*. However, GCRE-driven gene expression also was significantly reduced in homozygous Δ*hac1* cells. No intrinsic activation upon amino acid starvation could be detected as indicated for haploid Δ*hac1* cells.

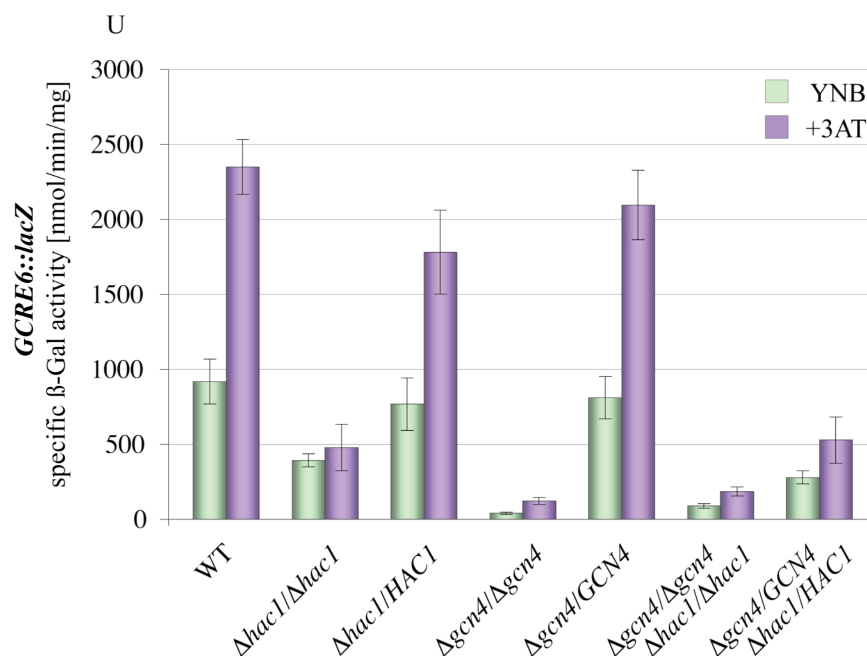


Fig. 19: Gcn4p-dependent reporter gene expression is repressed in diploid homozygous Δ*hac1* and Δ*gcn4* cells.

Diploid wild type yeast strain (WT) (RH3421) as well as homo- and heterozygous Δ*hac1*/Δ*hac1* (RH3422), Δ*hac1/HAC1* (RH3423), Δ*gcn4*/Δ*gcn4* (RH2398), Δ*gcn4/GCN4* (RH3424), Δ*gcn4*/Δ*gcn4*/Δ*hac1*/Δ*hac1* (RH3350) and Δ*gcn4/GCN4*/Δ*hac1/HAC1* (RH3425) mutant strains each carrying a chromosomally integrated *GCRE6::lacZ* reporter were grown to log-phase in YNB in absence (green bars, YNB) or presence of 10 mM 3AT (purple bars, +3AT) before specific β-galactosidase activities were assayed.

These findings further confirm our assumption that not only Gcn4p is required for the activation UPREs upon ER stress but also Hac1p influences activation of Gcn4p specific target genes in response to amino acid starvation in haploids and as well in diploids. However, heterozygous diploids compensate the decreased expression.

3.2.4 Hac1p reduces *FLO11* expression and adhesive growth in haploid cells

Beside this activation of target genes by direct binding to specific Gcn4p-response elements in their promoter regions Gcn4p evokes a strong adhesion of yeast cells on surfaces or on each other upon amino acid starvation, which is mediated by the floccolin Flo11p (Braus *et al.*, 2003). Flo11p is classified as indirect target gene of Gcn4p since the promoter of *FLO11* does not possess direct binding sites for Gcn4p and direct binding has not been shown so far. We investigated whether Hac1p also influence expression of Gcn4p unspecific target genes using a *FLO11::lacZ* reporter construct. Therefore, the *FLO11::lacZ* reporter that contains 3,500 base pairs of the *FLO11*

promoter in front of a *CYC1::lacZ* minimal promoter was integrated into the *URA3* locus of haploid wild type as well as into Δ*hac1* and Δ*gcn4* single or double mutant strains, respectively. β-galactosidase activities of resulting strains were determined under non-starvation conditions (YNB) and in amino acid starved cells (+ 3AT).

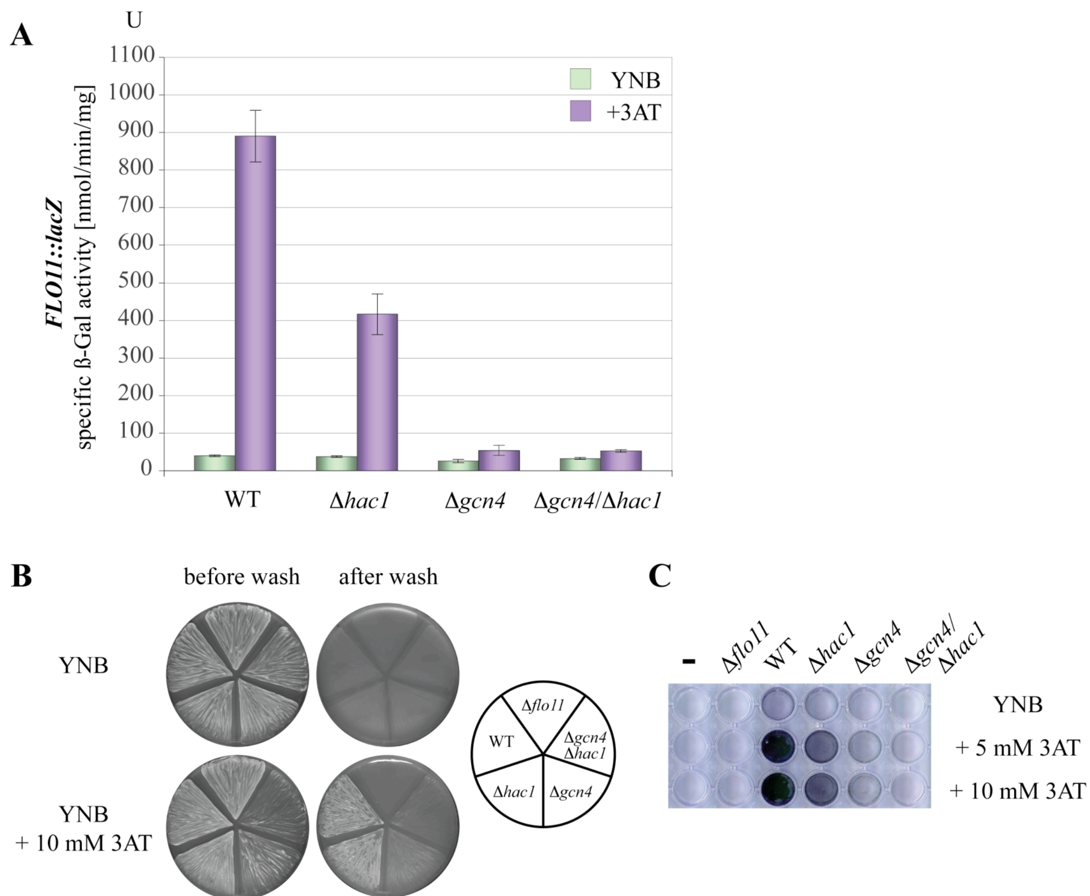


Fig. 20: *FLO11::lacZ* expression and adhesive growth are partially reduced in haploid Δ*hac1* cells.

(A) Expression of *FLO11::lacZ* was assayed in haploid Σ1278b wild type yeast strain (WT) (RH3406) as well as in Δ*hac1* (RH3360), Δ*gcn4* (RH3407) and Δ*gcn4*/Δ*hac1* (RH3408) mutant strains each carrying a chromosomally integrated *FLO11::lacZ* reporter. Cultures were grown to log-phase in YNB in absence (green bars, YNB) or presence of 10 mM 3AT (purple bars, +3AT) before specific β-galactosidase activities were measured.

(B) Haploid Σ1278b Δ*flo11* (RH2681), wild type (WT) (RH2816), Δ*hac1* (RH3351), Δ*gcn4* (RH2676) and Δ*gcn4*/Δ*hac1* (RH3402) yeast strains were streaked out on solid YNB medium (non-starved cells) and with 10 mM 3AT (histidine starved cells), respectively. After incubation for three days at 30°C adhesive growth was determined. Plates were photographed prior (before wash) and after washing under a stream of water (after wash) to document remaining cells on the agar surface.

(C) The same yeast strains as described in (B) were grown in liquid YNB media until an optical density of 0.6 before 300 µl of each culture were transferred in a microtiter well. Cells were grown in absence or presence of 5 or 10 mM 3AT to induce starvation dependent adhesive growth. After incubation for 24 h at 30°C, sedimented cells were dyed with crystal violet and carefully washed. Finally, plates were photographed to document adhesive growth.

Amino acid starvation led to a significant induction of β-galactosidase activity in haploid wild type cells (891 U) (Fig. 20A). In contrast, only a partial induction could be detected in Δ*hac1* cells (416 U) corresponding to ~ 50% of the *FLO11::lacZ* expression measured in wild type cells when treated with 3AT. As expected, haploid cells containing a *gcn4* deletion showed no induction upon amino acid starvation (54 U for Δ*gcn4*, 52 U for Δ*gcn4*/Δ*hac1*). Similar results were obtained for the activation of the *GCRE6::lacZ* reporter whereas basal activity was higher which is due to the Gcn4p-dependent basal expression of genes involved in different amino acid biosynthetic pathways regulated by the general control system. However, Flo11p is highly up-regulated when haploid cells are starved for either glucose or amino acids (Cullen and Sprague, 2000; Braus *et al.*, 2003), which enables cells to grow adhesively. We investigated whether the reduced *FLO11::lacZ* expression of Δ*hac1* cells is sufficient to grow adhesively when starved for amino acids. Adhesion was analyzed either on agar or on plastic. As expected, all strains did not adhere to the agar substrate or to plastic under non-starvation conditions (Fig. 20B and C). Consistent to the *FLO11::lacZ* expression, haploid wild type and Δ*hac1* cells became adhesive when treated with 3AT whereas cells deleted for *GCN4* were not able to grow adhesively comparable to the Δ*flo11* strain. Furthermore, Δ*hac1* cells showed a constricted adhesive growth on plastic which reflected the halved *FLO11::lacZ* expression. The reduced growth of strains lacking *GCN4* was already investigated in Fig. 15 and is due to an inactivated general control system, which is regulated by Gcn4p. We conclude that Hac1p is not only responsible for activation of Gcn4p specific target genes but also for Gcn4p unspecific target genes such as Flo11p, nevertheless, is over a threshold which enables haploid Δ*hac1* cells to grow adhesively.

3.2.5 Diploid homozygous *hac1* and *gcn4* deletion strains repress *FLO11::lacZ* expression and adhesive growth in response to amino acid starvation

Since we have shown that the effects of a *hac1* deletion on *GCRE6::lacZ* expression are heightened in diploid cells, we analyzed whether this was also valid for the expression of the *FLO11::lacZ* reporter. Therefore, the *FLO11::lacZ* reporter gene containing haploid *MAT**a*** strains were crossed with respective *MATα* strains to obtain homo- and heterozygous diploid strains deleted for *HAC1*, *GCN4* or both. Expression of

FLO11::lacZ was determined in sated and amino acid starved cells, respectively. As documented for the expression of *GCRE6::lacZ* (Fig. 19), we found that starvation-dependent *FLO11::lacZ* expression is strongly decreased in diploid homozygous Δ*hac1* and Δ*gcn4* strains (Fig. 21A) and cells were not able to grow adhesively (Fig. 21B). Expression was fully restored in heterozygous Δ*hac1* cells, which allowed adhesive growth. Indeed, *FLO11::lacZ* expression of heterozygous Δ*gcn4* containing cells was reduced in comparison to the expression obtained in wild type or rather heterozygous Δ*hac1* cells but was still sufficient to grow adhesively. However, adhesive growth is reduced when compared with wild type and heterozygous Δ*hac1* cells, respectively.

Starvation for nitrogen is a further nutritional signal in diploid cells, which triggers developmental changes. In response to nitrogen starvation diploid yeast cells can switch to a filamentous growth form, which is called pseudohyphae (Gimeno *et al.*, 1992; Mösch, 2000). Due to the fact that the flocculin Flo11p is required for adhesive growth in response to amino acid starvation as well as for filamentous growth in response to nitrogen starvation in diploids (Gimeno *et al.*, 1992), we examined pseudohyphal growth. Commensurate to our previous findings, all homozygous deletion strains were suppressed in forming pseudohyphae comparable with a Δ*flo11*/Δ*flo11* mutant strain (Fig. 21C). In contrast, all heterozygous strains developed pseudohyphae.

The results of this section show, that not only direct target genes of Gcn4p are influenced by Hac1p but also indirect target genes such as Flo11p. As shown in previous section, consequences are less intense in haploids. Effects caused by diploid homozygous mutants can be compensated in respective heterozygous strains.

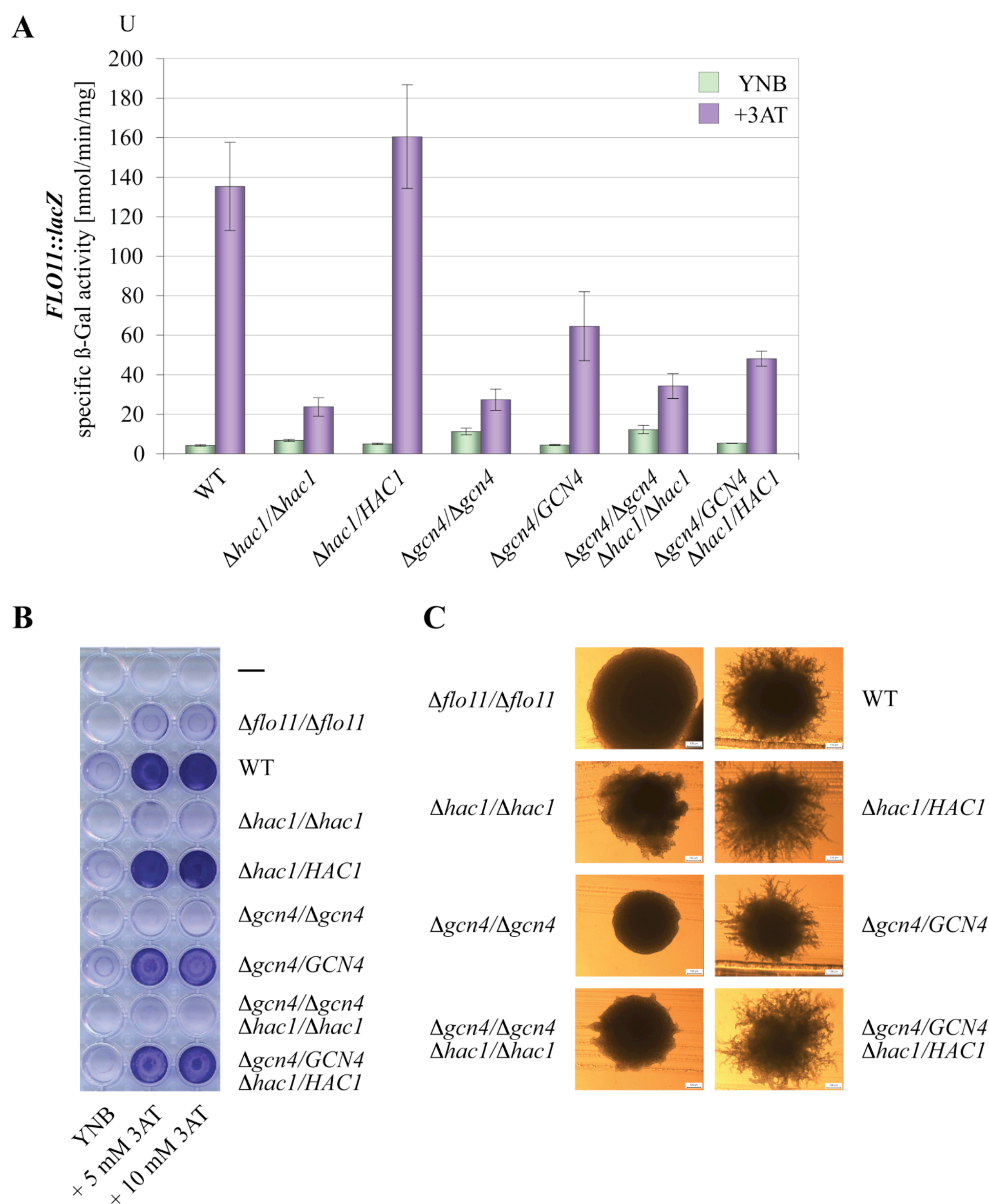


Fig. 21: *FLO11::lacZ* expression, adhesion and pseudohyphal growth are repressed in diploid homozygous Δ*hac1* and Δ*gcn4* strains.

(A) *FLO11::lacZ* expression was determined in diploid Σ1278b wild type yeast strain (WT) (RH3417) as well as in diploid homo- and heterozygous Δ*hac1*/Δ*hac1* (RH3362), Δ*hac1/HAC1* (RH3418), Δ*gcn4*/Δ*gcn4* (RH2695), Δ*gcn4/GCN4* (RH3419), Δ*gcn4*/Δ*gcn4*/Δ*hac1*/Δ*hac1* (RH3349) and Δ*gcn4/GCN4*/Δ*hac1/HAC1* (RH3420) mutant yeast strains each carrying a chromosomally integrated *FLO11::lacZ* reporter.

(B) For testing amino acid starvation induced adhesive growth, diploid wild type yeast strain (WT) (RH2656) as well as Δ*flo11*/Δ*flo11* (RH2661), Δ*hac1*/Δ*hac1* (RH3412), Δ*hac1/HAC1* (RH3413), Δ*gcn4*/Δ*gcn4* (RH2658), Δ*gcn4/GCN4* (RH3414), Δ*gcn4*/Δ*gcn4*/Δ*hac1*/Δ*hac1* (RH3415) and Δ*gcn4/GCN4*/Δ*hac1/HAC1* (RH3416) mutant strains were used and assay was performed as described in Fig. 20.

(C) The same yeast strains as described in (B) were streaked out on solid nitrogen starvation medium (SLAD) containing only 50 µM ammonium sulfate as nitrogen source to induce pseudohyphal growth. After incubation for five days at 30°C pseudohyphal colonies were visualized with an Axiovert microscope and photographed. Bar equals 100 µm.

3.2.6 Identification of *FLO11* promoter elements mediating regulation by Hac1p and Gcn4p in response to amino acid starvation

Our previous analysis of the *FLO11* promoter, which spans approximately 3.6 kb and therefore is presumably the longest promoter in *S. cerevisiae,* has identified one UAS (upstream activation site) and one URS (upstream repression site) element which are necessary for regulation of *FLO11* expression under amino acid starvation (Braus *et al.*, 2003). Since our results indicate that beside Gcn4p, Hac1p is also required for activation of *FLO11* expression upon amino acid limitation we asked whether this influence was due to a specific region in the *FLO11* promoter. Therefore a set of 14 reporter constructs containing individual 400 bp *FLO11* promoter fragments that overlap by 200 bp and were cloned in front of a *CYC1::lacZ* fusion gene (Rupp *et al.*, 1999) were used. They were transformed into the diploid wild type strain as well as into the diploid strains deleted for either *GCN4* or *HAC1*, respectively.

In wild type cells this series of reporter constructs confirmed our previous findings where we identified four strong UAS elements in the segments *FLO11-3/2*, *FLO11-6/5*, *FLO11-7/6* and *FLO11-10/9* (Table IV) (Braus *et al.*, 2003). Additionally, the element present in *FLO11-11/10* increased the expression of *CYC1::lacZ* at least five-fold compared with the reporter without any insert (Table IV). Due to the fact that the elements present in *FLO11-3/2* (base pairs -620 to -182), *FLO11-7/6* (base pairs -1421 to -981) and *FLO11-11/10* (base pairs -2220 to -1781) were not regulated by amino acid starvation they indicate for basal UAS elements (UAS^B). In contrast, activity in response to amino acid starvation was at least 2.3-fold induced in segments *FLO11-6/5* (base pairs -1120 to -779) and *FLO11-10/9* (base pairs -2020 to -1573) suggesting that these segments contain UAS elements that confer regulation by amino acid starvation (UAS^R).

Deletion of *HAC1* only led to a more than three-fold reduction in the expression of *FLO11-7/6* in response to amino acid starvation when compared with the activity of this element in the wild type strain whereas induction by amino acid starvation was not observed (Table IV and Fig. 22). These results suggest that the element present in *FLO11-7/6* (base pairs -1421 to -981) represents a Hac1p dependent UAS which is not inducible by 3AT in principle but activation upon amino acid starvation was 3.6-fold decreased when compared to the same segment expressed in wild type cells.

Table IV. β-Galactosidase assays of individual 400 bp *FLO11* promoter elements cloned into the UAS of a *CYC1::lacZ* reporter construct.

	Wild type (*Mata/α*) Units (nmol/min/mg)			Δ*hac1*/Δ*hac1* Units (nmol/min/mg)		Expression relative to WT WT/Δ*hac1*		Δ*gcn4*/Δ*gcn4* Units (nmol/min/mg)		Expression relative to WT WT/Δ*gcn4*	
	YNB	+3AT	3AT/ YNB	YNB	+3AT	YNB	+3AT	YNB	+3AT	YNB	+3AT
FLO11	27	227	8,4	27	75	1,0	**3,0**	12	28	2,3	**8,1**
No insert	19	39	2,0	22	50	0,9	0,8	8	17	2,4	2,3
2/1	36	61	1,7	40	82	0,9	0,7	15	30	2,4	2,0
3/2	**107**	**121**	1,1	123	138	0,9	0,9	33	69	**3,2**	1,8
4/3	61	78	1,3	46	106	1,3	0,7	12	25	**5,1**	**3,1**
5/4	36	60	1,7	35	64	1,0	0,9	8	23	**4,5**	2,6
6/5	**674**	**1530**	2,3	336	768	2,0	2,0	120	176	**5,6**	**8,7**
7/6	**806**	**920**	1,1	323	254	2,5	**3,6**	108	158	**7,5**	**3,6**
8/7	58	69	1,2	34	50	1,7	1,4	9	15	**6,4**	**4,6**
9/8	70	92	1,3	60	121	1,2	0,8	21	44	**3,3**	2,1
10/9	**100**	**333**	3,3	69	211	1,4	1,6	27	72	**3,7**	**4,6**
11/10	**287**	**255**	0,9	822	537	0,3	0,5	172	463	1,7	0,6
12/11	92	99	1,1	95	94	1,0	1,1	23	55	**4,0**	1,8
13/12	33	64	1,9	31	59	1,1	1,1	11	28	**3,0**	2,3
14/13	33	64	1,9	29	81	1,1	0,8	12	30	2,8	2,1
15/14	28	68	2,4	35	101	0,8	0,7	11	34	2,5	2,0

The diploid wild type strain (RH2656) as well as diploid Δ*hac1*/Δ*hac1* (RH3412) and Δ*gcn4*/Δ*gcn4* (RH2658) mutant strains were transformed with indicated *CYC1::lacZ* reporter constructs (Rupp *et al.*, 1999) and expression was assayed under non-starvation conditions (YNB) compared to amino acid starvation induced by adding 10 mM 3AT (3AT). The received β-galactosidase units (nmol/min/mg) are given for each sequence element in each strain. Values with at least five-fold elevation of the reporter over a plasmid without an insert expressed in the wild type strain are highlighted in bold. In the column 3AT/YNB, the ratio of activities obtained for a given reporter construct in the wild type strain in the presence or absence of 3AT is presented. Additionally, the values for the diploid homozygous *hac1* and *gcn4* deletion strains are listed relative to the respective wild type data and marked in bold when it is repressed at least three-fold. The segment which is regulated by both, Gcn4p and Hac1p, is framed.

Deletion of *GCN4* led to more than three-fold reduction in the expression of almost all reporter constructs whereas upon amino acid starvation only expression of *FLO11-4/3*, *FLO11-6/5*, *FLO11-7/6*, *FLO11-8/7* and *FLO11-10/9* was more than three-fold reduced. However, induction of these elements by amino acid starvation was not considerable and stretched from 1.5 for elements *FLO11-6/5* and *FLO11-7/6* to maximum 2.6 for element *FLO11-10/9*. Since the *FLO11* promoter neither contains a specific binding site for Gcn4p nor direct binding was observed so far, Flo11p represents an indirect target gene of Gcn4p. Therefore, the influence of Gcn4p on most constructs could be due to its indirect influence over other transcription factors involved

in Flo11p regulation. Element *FLO11-7/6* was targeted both by Gcn4p and Hac1p upon amino acid starvation which provides existence of comprehensive domains in the *FLO11* promoter influenced by more than one transcription factor and therefore indicates for an associated function on *FLO11* expression.

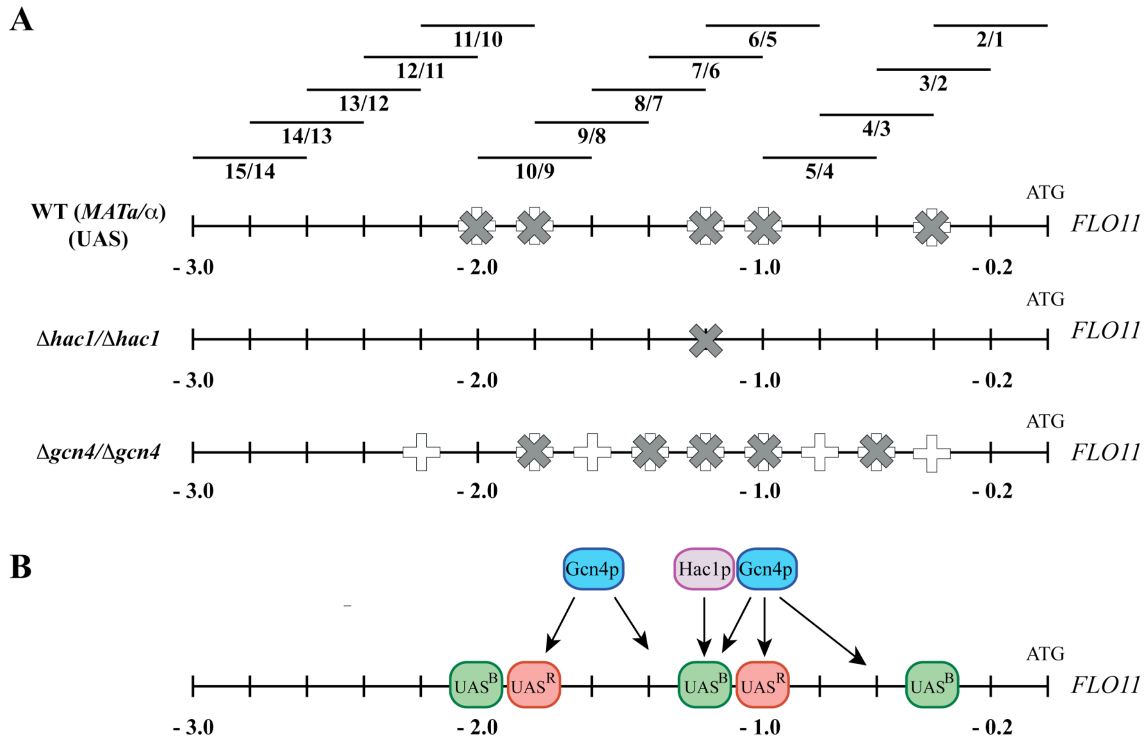


Fig. 22: Sequence elements involved in regulation of *FLO11*.

(A) The β-galactosidase activity of 14 isolated 400 bp elements of the *FLO11* promoter region was assayed in diploid wild type cells or in corresponding cells deleted for the transcription factors *HAC1* or *GCN4* (Table IV). The activity was determined under non-starvation conditions (YNB) or in the presence of amino acid starvation (3AT) induced by the addition of 10 mM 3AT. Each line represents the *FLO11* promoter in the indicated genetic background. The symbols (white cross = non-starvation conditions; gray cross = starvation conditions) are placed on a line in a position that indicates which of the 400 bp fragments stimulated β-galactosidase activity. The first row (WT *MAT**a**/α* UAS) denotes sequence elements showing a >5-fold elevation of the reporter over a plasmid without an insert (pME2212) as measured in wild type cells. The next two lines (*Δhac1/Δhac1* and *Δgcn4/Δgcn4*) represent sequence elements showing a >3-fold reduction of the β-galactosidase activity in the mutants as compared with the activity of the same element in wild type strain.

(B) Summary of the identified elements, which were regulated by Gcn4p and Hac1p in response to amino acid starvation. Both, Gcn4p and Hac1p, influenced element *FLO11-7/6,* which represents a basal upstream activation sequence (UAS^B). Elements *FLO11-6/5* and *FLO11-11/09* where at least 2.3-fold induced when starved for amino acids and therefore represent amino acid starvation dependent upstream repression sites (UAS^R).

3.2.7 Reduced Hac1 protein levels upon amino acid starvation are independent of the Pho85p-Pcl5p complex

Rapid turnover of Gcn4p and Hac1p is regulated by phosphorylation by the cyclin-dependent kinase Srb10p and that followed ubiquitination by the SCF^{Cdc4} ligase complex, which results in degradation of both, Gcn4p and Hac1p, by the 26S proteasom (Irniger and Braus, 2003; Pal *et al.*, 2007). Additionally, stability of Gcn4p is regulated by the CDK Pho85p in interaction with Pcl5p (Irniger and Braus, 2003). To investigate the role of the Pho85p-Pcl5p complex in Hac1p degradation, we analyzed the decay of Hac1p in haploid Δ*pho85* and Δ*pcl5* strains by synthesis shut-off assays. Additionally, stability of Hac1p in haploid strains deleted for *GCN4* was determined. Furthermore, we examined Hac1p turnover under amino acid starvation conditions, which leads to a stabilization of Gcn4p (Bömeke *et al.*, 2006).

The half life of Hac1p in Σ1278b wild type cells was ~ 1.5 min (Fig. 23A). Degradation in Δ*pcl5* was not influenced whereas Hac1p was minimally stabilized in Δ*gcn4* cells, nevertheless, is very insignificant compared to the stabilization documented in Δ*srb10* cells (Pal *et al.*, 2007). Deletion of *PHO85* pointed into the opposite direction and caused a destabilization of Hac1p. This could be explained by either general growth deficit of Δ*pho85* cells (Huang *et al.*, 2007) or Pho85p usually stabilizes Hac1p. Amino acid starvation induced by 100 mM 3AT repressed Hac1p expression in all cells. Similar results were obtained when eIF2αp phosphorylation was determined (Fig. 16B), however with less intense. This could be due to either 10-fold concentrations of 3AT or the time span or both, respectively. As shown in Fig. 16B longer treatment (for 60 min, lane 30 min post Tm induction) with 10 mM 3AT repressed Hac1p expression in haploid Δ*gcn4* cells whereas after 30 min Hac1p was still detectable. Cells were further tested for resistance to ER stress wherein no significant growth differences were obtained (Fig. 23B). Since Δ*pho85* cells are characterized by a general growth deficit no precise conclusion could be observed. Similar results were achieved using S288c wild type and Δ*gcn4* or rather Δ*pho85* deletion strains of the Euroscarf strain collection (Brachmann *et al.*, 1998).

The findings of this section indicate a Pho85p-Pcl5p independent degradation of Hac1p whereas the other components (CDK Srb10p and SCF^{Cdc4} ubiquitin ligase) of Gcn4p stability regulation are involved in the turnover of Hac1p. Neither stabilization of Hac1p in Δ*gcn4* cells nor destabilization of Hac1p in Δ*pho85* cells are well defined

so that no precise conclusion is possible. The CDK Pho85p can interact with ten different cyclins and possibly Hac1p will be regulated by Pho85p together with another cyclin. However, amino acid starvation, a signal for up-regulation of Gcn4p, results in repression of Hac1p. Similar results were shown for *GCN4* mRNA translation upon ER stress conditions.

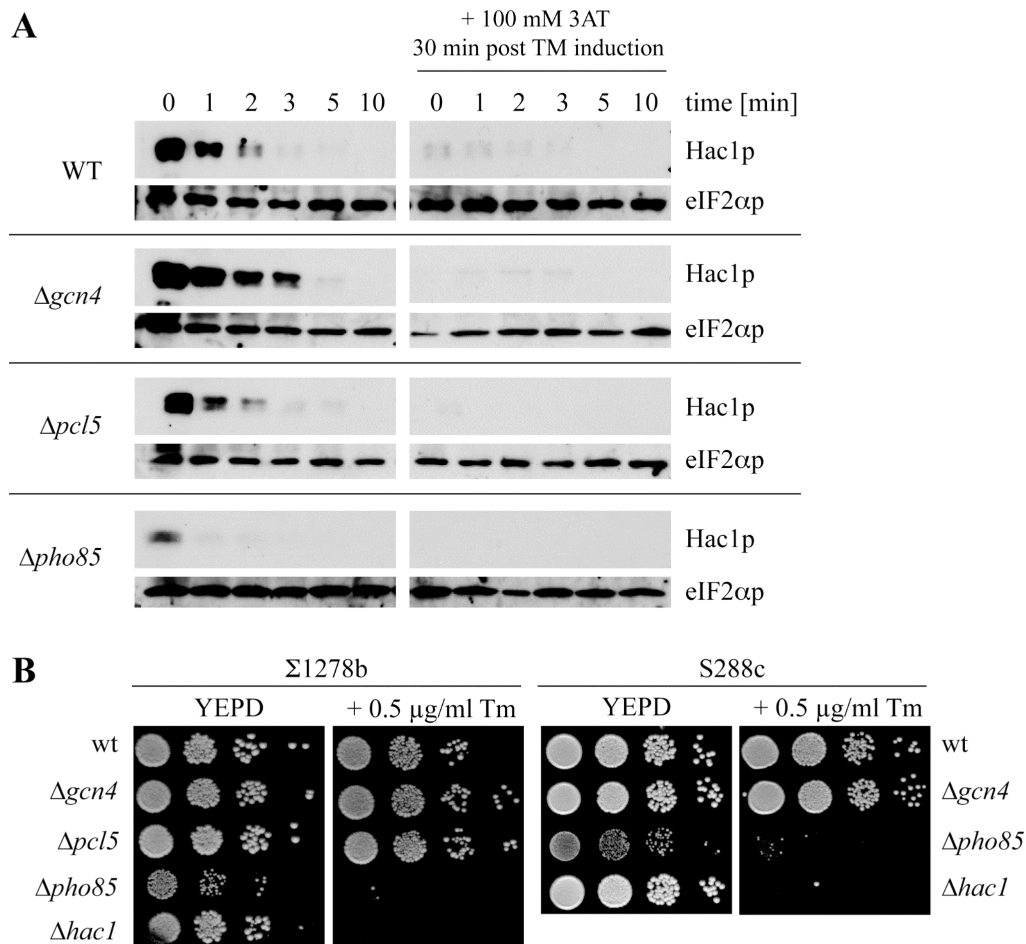


Fig. 23: Hac1p is hardly detectable upon amino acid starvation.

(A) Haploid Σ1278b wild type strain (WT) (RH2816) as well as haploid Δ*gcn4* (RH2676), Δ*pcl5* (RH3279) and Δ*pho85* (RH3426) mutant strains were grown in YNB to OD_{600} ~ 0.6 before dividing in two independent cultures, respectively. ER stress was mediated in all cultures by 5 µg/ml tunicamycin (Tm) to induce synthesis of Hac1p. After 30 minutes post tunicamycin treatment, additional amino acid starvation was achieved by the addition of 100 mM 3AT. 90 minutes post tunicamycin induction 1 mg/ml cycloheximide was added to all cultures to halt protein synthesis und samples were taken at the indicated time points after addition. Protein levels of Hac1p were analyzed by Western hybridisation and eIF2αp was used as loading control.

(B) The same haploid Σ1278b strains described in (A) and additional haploid S288c wild type strain (BY4741) as well as Δ*gcn4* (Y00249), Δ*pho85* (Y02797) and Δ*hac1* (Y05650) mutant strains were spotted in 10-fold dilutions on either solid YEPD medium alone or in presence of 0.5 µg/ml tunicamycin to induce ER stress. After incubation for 3 – 4 days at 30°C plates were photographed.

3.2.8 Stabilization of Hac1p by sumoylation

Recent publication predicts sumoylation of XBP1 which encodes for the mammalian homologue of Hac1p (Chen and Qi, 2010). Actually, a predict SUMO consensus site (ψKXD/E) is located in the C-terminus of Hac1p. To determine a possible Hac1p sumoylation in *S. cerevisiae* we analyzed its stability in a haploid W303 wild type strain in comparison to strains carrying temperature sensitive mutations in either *UBC9* or *SMT3* gene. Smt3p encodes for the yeast SUMO protein whereas Ubc9p represents the SUMO-conjugating enzyme.

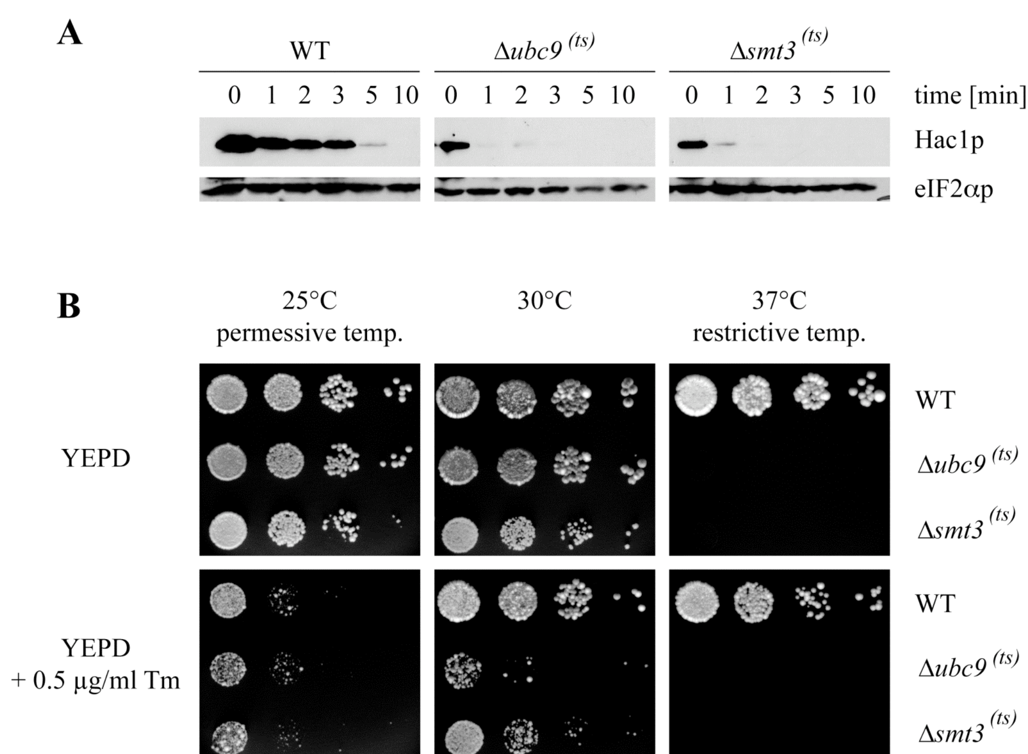


Fig. 24: Hac1p stability is regulated by sumoylation.

(A) Haploid W303 temperature sensitive strains Δ*ubc9*$^{(ts)}$ (S099) and Δ*smt3*$^{(ts)}$ (S542) as well as haploid W303 wild type strain (S001) were grown in liquid YEPD media at 23°C or rather 30°C to an optical density of 0.6 before ER stress was induced by addition of 5 µg/ml tunicamycin. After 90 minutes post induction temperature sensitive cultures were shifted to the restrictive temperature of 37°C for 30 minutes. Protein synthesis was blocked by adding 1 mg/ml cycloheximide and samples were taken at the indicated time points after addition. Protein levels of Hac1p were determined by immunoblotting. eIF2αp was used as loading control.

(B) The same yeast strains as described in (A) were spotted in 10-fold dilutions on either solid YEPD medium alone or in presence of 0.5 µg/ml tunicamycin to induce ER stress. After incubation for 3 – 4 days at 23°C, 30°C and 37°C plates were photographed.

The half life of Hac1p in the parental W303 cells was ~ 3 min (Fig. 24A). By contrast, the rate of degradation of Hac1p was significantly increased in temperature sensitive Δ*ubc9*$^{(ts)}$ and Δ*smt3*$^{(ts)}$ cells indicating a possible stabilization of Hac1p by SUMO. Consistent with this instability of Hac1p, Δ*ubc9*$^{(ts)}$ and Δ*smt3*$^{(ts)}$ cells were more sensitive to ER stress than were wild type cells (Fig. 24B). We conclude that Hac1p is activated and stabilized by sumoylation. This function is contrary to the role of SUMO modification of XBP1 in mammalian where sumoylation represses transcriptional activity.

Taken together, our findings indicate a so far unknown function of Hac1p in metabolic and developmental processes regulated by Gcn4p. Not only Gcn4p is able to activate Hac1p specific target genes but also Hac1p is involved in Gcn4p target gene expression. Indeed, haploid Δ*hac1* cells are able to survive amino acid starvation and to activate Gcn4p specific and unspecific target genes upon amino acid starvation but is partial reduced in both cases. This partial reduction affects resistance and adhesive growth when compared to wild type cells. Since diploids are more complex, effects are stronger in homozygous Δ*hac1* cells, which are compensated in heterozygous Δ*hac1* cells. Furthermore, our data indicate for a possible regulation of Hac1p by Gcn4p in response to amino acid starvation whereas Gcn4p-regulation is independent of Hac1p. Transcription factor specific stress situation (amino acid starvation for Gcn4p and ER stress for Hac1p) results finally in repression of the respective antagonist. First results suggest regulation of Hac1p by SUMO/Smt3p.

4. DISCUSSION

4.1 Dissection of the dual function of Gcn4p

Gcn4p of *S. cerevisiae* encodes the founding member of a global transcriptional metabolic activator for a genetic system that secures the appropriate response to amino acid starvation. Gcn4p of yeast is conserved and corresponds to ATF4 in human (Hinnebusch, 2005; Ameri and Harris, 2008). In diploids, the metabolic function of yeast Gcn4p is combined with adhesive growth and pseudohyphal development upon nutrient starvation (Braus *et al.*, 2003). The metabolic and the developmental functions of the transcription factor Gcn4p could be dissected by abolishing the developmental function, which allows pseudohyphae formation in yeast and maintaining the potential to survive in amino acid starvation conditions. Fig. 25 illustrates our findings schematically.

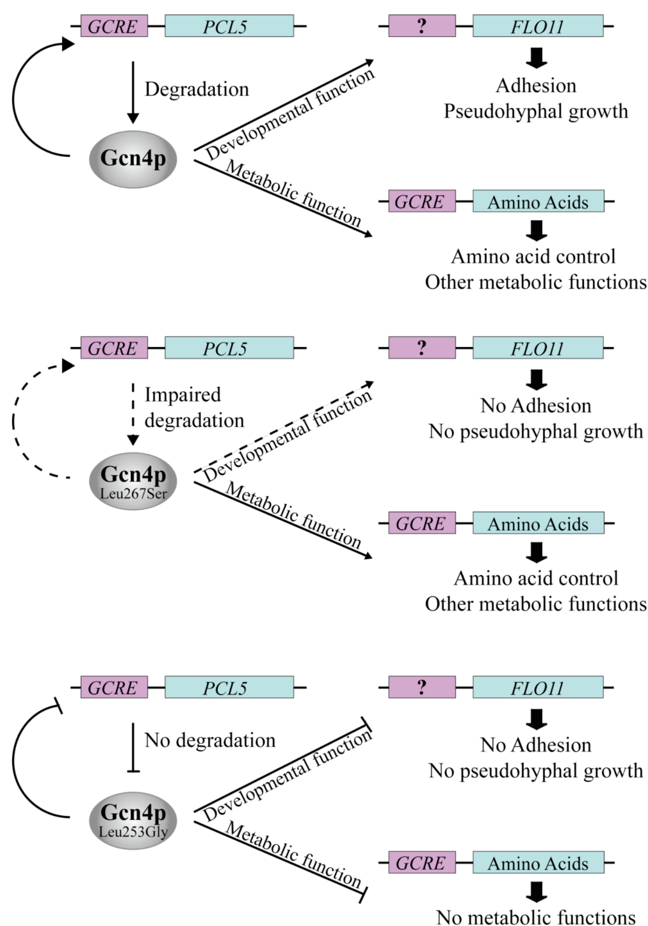


Fig. 25: Model for self-regulation of Gcn4p.

Shown is the self-controlled degradation of Gcn4p. Transcriptional activity of wild type Gcn4p enables to fulfill its dual function as metabolic and developmental regulator. Furthermore, Gcn4p regulates its own degradation by activating Pcl5p. Substitution of Leu267Ser results in a partial active but more stable transcription factor, which only fulfills metabolic processes. Substitution with helix breakers lead to a strong stabilized Gcn4p because Gcn4p is completely transcriptionally inactive.

Leu267Ser represents a single mutant allele of *GCN4,* which was isolated in a screen for a separation of the dual function. Analogous *in vitro* designed derivatives have similar consequences in diploids: (i) they have a reduced affinity for dimerization, which decreases transcriptional activation, (ii) this results in a reduced activation of the Gcn4p destruction pathway and therefore an increased protein stability, (iii) partial Gcn4p activity is sufficient to provide growth under amino acid starvation conditions, but is below the threshold, which is necessary to activate *FLO11* sufficiently to grow adhesively or as pseudohyphae. The impact on haploid yeasts is similar, nevertheless, the observed effects are less pronounced than those determined in diploids.

4.1.1 Gcn4p as conserved global transcription factor

A combination of metabolic and developmental functions is not only a feature of yeast Gcn4p. Various Gcn4p counterparts in other eukaryotic organisms have a similar metabolic function but are additionally involved in different developmental processes. The cross pathway control (CPC) of amino acid biosynthesis in filamentous fungi is similar to the general control system of yeast and functions as a general stress response system (Sachs, 1996). In the filamentous fungus *Aspergillus nidulans* the Gcn4p-related CpcA provides sufficient amino acid during starvation but can also induce an arrest in fruit body formation in response to amino acid starvation, indicating a connection between metabolism and sexual development (Hoffmann *et al.*, 2000). CpcA of *Aspergillus fumigatus* also controls amino acid supply but plays an additional important role in the virulence of this opportunistic human fungal pathogen (Krappmann *et al.*, 2004). Similarly, the Gcn4p homologue *VlCpcA* of the fungal plant pathogen *Verticillium longisporum* is induced in infected plants (Singh *et al.*, 2010). The dimorphic human pathogen *Candida albicans* is able to switch from a unicellular yeast form to a multicellular hyphal form, which causes disseminated systemic infections in immuno-compromised individuals. This infective hyphal form is also induced by various environmental stimuli, like carbon or nitrogen starvation or elevated temperatures (Ernst, 2000; Sánchez-Martinez and Pérez-Martin, 2001). Homologous proteins for Gcn4p and Pcl5p were identified in *C. albicans* and are necessary for amino acid biosynthesis and the induction of filamentous growth in response to amino acid starvation (Tripathi *et al.*, 2002; Gildor *et al.*, 2005).

The Gcn4p like ATF4 of mammalian cells also combines its metabolic function with a developmental function. ATF4 fulfills its metabolic function to ensure cell survival in response to amino acid starvation, but also to react to different stress signals like ER stress or oxidative stress (Harding *et al.*, 2003; Ameri and Harris, 2008). ATF4s' developmental functions include cell proliferation and eye or rather bone development as well as processes like learning and long-term memory (Masuoka and Townes, 2002; Chen *et al.*, 2003; Costa-Mattioli *et al.*, 2005). It will be interesting to analyze whether these Gcn4p related proteins show a similar pattern of self control mediated by the interplay of transcriptional activation and protein turnover.

4.1.2 Protection against hyperactive *GCN4*

The metabolic function of Gcn4p results in the up-regulation of hundreds of genes in yeast. Misregulation of this global transcriptional activator may therefore result in a liability for the cell. This might require a certain degree of insensitivity to variations in Gcn4p activity. Increased activity is neutralized by reduced Gcn4p stability. Wild type Gcn4p has only a half life of a few minutes in sated cells and therefore, the cell can cope with more active alleles as well as with overexpression of the transcription factor. When a deficient Gcn4p degradation pathway is combined with overexpression of the transcription factor, this results in a toxic effect that causes growth inhibition (Tavernarakis and Thireos, 1995; Shemer *et al.*, 2002).

The rapid decay of Gcn4p is initiated within the nucleus by phosphorylation by two independent cyclin-dependent kinases, Pho85p and Srb10p. Srb10p phosphorylation occurs constitutively and might be necessary for transcriptional activation. One possibility is that Srb10p action is required for promoter clearing after the transcription factor has promoted transcription (Chi *et al.*, 2001; Irniger and Braus, 2003) This could explain why mutations in the E3 ubiquitin ligase SCF^{Cdc4} or inhibition of the proteasome can decrease the transcription of Gcn4p targets. Therefore, a certain turnover rate is necessary for Gcn4p gene activation function (Lipford *et al.*, 2005). The kinase cyclin complex Pho85p-Pcl5p phosphorylates Gcn4p when amino acids are present but is less active when amino acids are limited. The kinase reaction is the committing step in Gcn4p degradation, because phosphorylation at the residue Thr165 is necessary to mark Gcn4p for poly-ubquitination by the E2 ubiquitin-conjugating enzyme Cdc34p together with the E3 SCF^{Cdc4} RING ligase. The final step is the

degradation in the 26S proteasome (Kornitzer *et al.*, 1994; Meimoun *et al.*, 2000; Shemer *et al.*, 2002).

The *PCL5* gene itself is a target gene of Gcn4p, which results in the paradox situation that Gcn4p controls a gene, which is responsible for its own degradation. Under amino acid starvation conditions the Gcn4p mediated increased *PCL5* mRNA levels do not result in increased Pcl5 protein levels in wild type yeasts (Jia *et al.*, 2000; Shemer *et al.*, 2002). This has been explained by the reduced overall translational efficiency during amino acid starvation combined with the even lower protein stability of Pcl5p in comparison to Gcn4p (Kornitzer *et al.*, 1994). In addition, the Pho85p-Pcl5p complex dissociates during amino acid starvation (Bömeke *et al.*, 2006). In wild type cells, Gcn4p is stabilized in the nucleus to an increased half life of 5 min to up to 20 min (Kornitzer *et al.*, 1994; Irniger and Braus, 2003). Although amino acid starvation decreases overall translation, it improves *GCN4* mRNA translation and therefore increases the amount of Gcn4 protein in the cytoplasm (Hinnebusch, 1984; Dever *et al.*, 1992).

Our data suggest that the Gcn4p controlled expression of *PCL5* is a backup system which is important to adapt Gcn4p stability to its own transcriptional activity providing an additional level for genetic robustness against variations in the expression or structure of the *GCN4* gene itself.

4.1.3 A fine-tuned circuit of self-control of a leucine zipper transcription factor

The feedback control of Gcn4p stability and activation function might be necessary because the Gcn4p controlled network comprises several hundreds of genes for the biosynthesis of metabolites as amino acids or purines. The regulation of numerous genes by one transcription factor might require the potential for gradual modification of transcription circuits, in particular, when target genes are involved in so many different processes all over the cell (Tuch *et al.*, 2008). The fact that uncontrolled Gcn4p overexpression is toxic might support this view. Unprotected overexpression might result in the interference of too many transcriptional activation pathways (Tavernarakis and Thireos, 1995; Shemer *et al.*, 2002).

This study revealed that self-controlled degradation of Gcn4p regulated by its transcriptional activity can follow two metabolic possibilities: (i) Gcn4p is partially active but nevertheless more stable or (ii) Gcn4p is more active resulting in a faster

degradation and therefore a less stable protein. We have shown that both options are not only possible, but also enable Gcn4p to fulfill its metabolic function. This is not only feasible due to the circuit of self-control of protein stability and function, but also because of the metabolic excess capacity, which is provided by a high Gcn4p independent basal activity of transcription of metabolic genes (Braus *et al.*, 1988; Paravicini *et al.*, 1988).

The developmental function of Gcn4p differs from the metabolic function in that it has no residual basal activity. Therefore reduced activity provides a phenotype, which consists in impairment of diploid adhesive growth upon amino acid starvation and formation of pseudohypae in response to nitrogen starvation. Only diploid cells expressing wild type *GCN4,* in which transcriptional activity and stability of Gcn4p are optimized, show an intact developmental function. Diploid development might require a distinct decision for a given cell, which is either a yes or a no.

The rewiring of pathways over evolutionary time scales can lead to morphologic and physiologic differences of closely related organisms (Tuch *et al.*, 2008). It is interesting to note that the sequence comparison of *S. cerevisiae GCN4* reveals that only the Gcn4p homologues of *C. albicans* and the human ATF4 feature an adequate leucine zipper structure consisting of four leucines each separated with seven amino acids (Landschulz *et al.*, 1988). Since we were able to separate the metabolic from the developmental function of the global transcription factor Gcn4p in diploid yeasts, it would be interesting to see the consequences of such a mutation in higher eukaryotes.

4.2 Crosstalk between the 'Unfolded Protein Response' and the 'General Amino Acid Control' via their regulators Hac1p and Gcn4p

The 'unfolded protein response' (UPR) and its key regulator XBP1, the mammalian homologue of Hac1p, play important roles in tumorogenesis of mammals and UPR suppressor are proposed as therapeutic agents (Saito *et al.*, 2009; Shajahan *et al.*, 2009; Wang *et al.*, 2010). Cell differentiation and dimorphism, respectively, therefore are of fundamental relevance. Similarly, CaHac1p and HacA, the Hac1p homologues of *C. albicans* and *A. fumigatus*, play important roles in regulating the morphology in both organisms, which in turn is important for virulence of these pathogenic fungi (Wimalasena *et al.*, 2008; Richie *et al.*, 2009). Furthermore, both CaGcn4p and CpcA, the Gcn4p homologues proteins, are also involved in their pathogenicity (Tripathi *et al.*, 2002; Krappmann *et al.*, 2004; Gildor *et al.*, 2005). In *S. cerevisiae*, Gcn4p is required for adhesion and pseudohyphal development upon nutrient starvation (Braus *et al.*, 2003). In contrast, Hac1p is described as repressor of differentiation in *S. cerevisiae,* which is oppositional to its role in higher eukaryotes (Schröder *et al.*, 2000). In this study we could not only disprove this evidence but also were able to define a region in the *FLO11* promoter where Hac1p potentially could act. Flo11p represents the main adhesion in Σ1278b cells required for nutritionally induced cell-cell and cell-surface adhesion during invasive growth, biofilm formation and pseudohyphal development (Lo and Dranginis, 1998; Guo *et al.*, 2000; Reynolds and Fink, 2001).

4.2.1 Analysis of *FLO11* promoter elements

Initially, we found that diploid homozygous Δ*hac1*/Δ*hac1* yeast cells neither were able to activate *FLO11* and therefore adhesive growth in response to amino acid starvation nor to develop pseudohyphae upon nitrogen starvation. Similar results were obtained for Gcn4p, previously (Braus *et al.*, 2003). The *FLO11* promoter spans approximately 3 kb and therefore is presumably the longest promoter in *S. cerevisiae*. Using a set of divers *FLO11* promoter constructs we identified at least one element (*FLO11-7/6*), which is regulated by both, Gcn4p and Hac1p, in response to amino acid starvation (Fig. 26). This element is the only element influenced by Hac1p upon amino

acid starvation whereas Gcn4p reduces activity in five different elements in response to amino acid starvation and mostly all elements under basal conditions. These findings confirm our previous assumption, that expression of *FLO11* might not involve direct binding of Gcn4p but rather in combination with other transcriptional regulators. The overlapping elements, which are influenced by different transcription factors, support this hypothesis. Similar to Gcn4p, we suppose an indirect binding of Hac1p to the *FLO11* promoter since sequence analysis does neither predict any Gcn4p-recognition element nor a Hac1p specific UPR element.

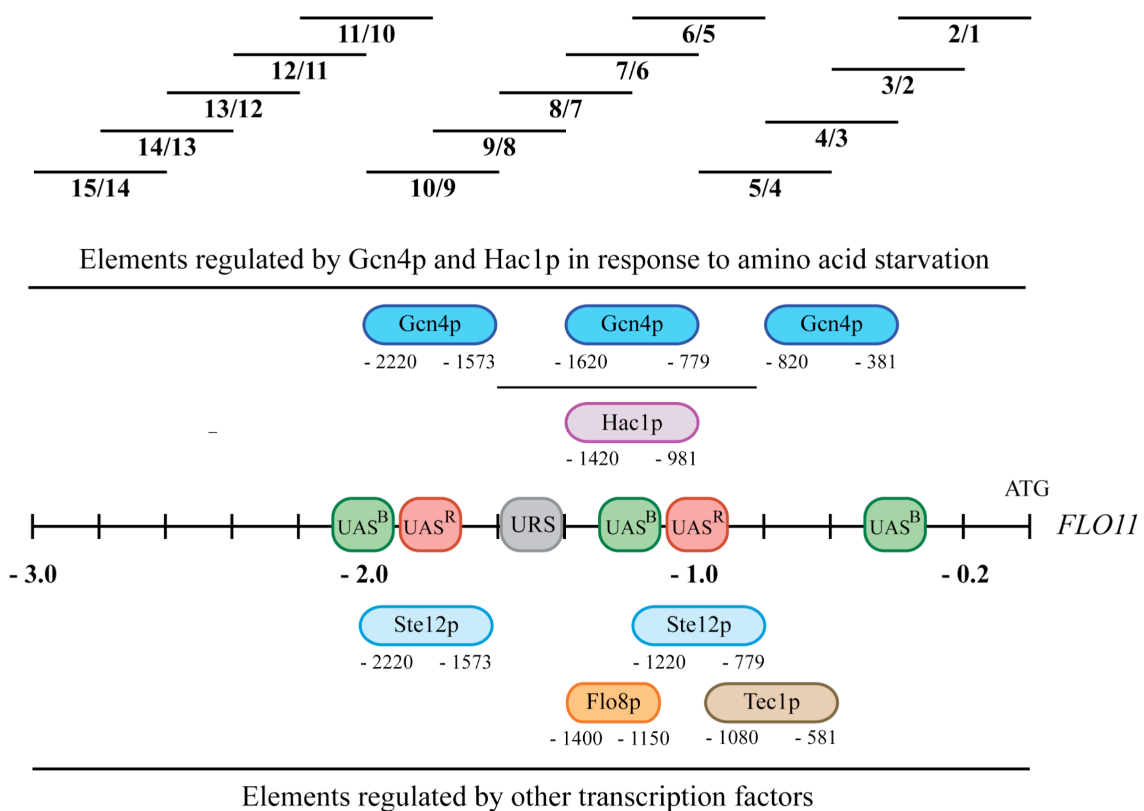


Fig. 26: Summary of *FLO11* promoter elements regulated by Gcn4p and Hac1p in response to amino acid starvation as well as by other transcription factors (see text for details).

Elements which are regulated by Ste12p, Tec1p and Flo8p, respectively, derive from Rupp *et al.* (1999) and Pan and Heitman (2002). The URS was defined by Braus *et al.* (2003).

However, numerous Gcn4p half-site are distributed over the *FLO11* promoter, which possibly could explain the activation of mostly elements on the one hand and indicate for an indirect regulation together in combination with another transcription factor. By scanning the Yeastract Database (http://www.yeastract.com/) we identified at

least four potential transcription factors, which are documented as indirect targets of Gcn4p (Table V). These transcription factors, namely Ste11p, Ste12p, Tpk2p, and Tpk3p, are all involved in Flo11p regulation by either cAMP or MAPK pathway (for details see section 1.1.4). Furthermore, *STE12* and in addition *TEC1* represent potential target genes of Gcn4p since both carrying at least one Gcn4p recognition element in their promoters. Indeed, neither direct nor indirect regulation by Hac1p is documented but its specific binding consensus sequence (CCAGC) is present in numerous important transcription factors involved in Flo11p regulation, namely Flo8p, Ste11p, Tec1p, Tpk1p and Tpk2p. The promoter of *TEC1* actually contains five independent UPREs whereas three of them are arranged on the complementary strand. These findings enforce our hypothesis that both, Gcn4p and Hac1p, are involved in Flo11p regulation *per se*, however, presumably by binding to another transcription factor. In fact, first Western experiments indicated reduced Tec1p levels in cells deleted for *GCN4* and *HAC1*, respectively (data not shown).

Table V: Documented and potential target genes of Gcn4p and Hac1p.

	Genes regulated by Gcn4p				Genes regulated by Hac1p	
	Document. direct	Document. indirect	Potential direct TGACTMT	Potential direct TGACTC	Document. direct	Potential direct CCAGC
GCN4		X				XX
HAC1			X	X	X	XX
ARO4	X					X
FLO8						X^{C}
HIS7	X					X
STE11		X				X
STE12		X	X			
TEC1			X	X		$XX(XXX)^{C}$
TPK1		X				X^{C}
TPK2		X				XX^{C}
TRP2	X					X^{C}
TRP3	X					$X(XXX)^{C}$

Documented and potential target genes of Gcn4p and Hac1p, which are involved in *FLO11* expression identified by scanning the Yeastract Database (http://www.yeastract.com/). Furthermore, Gcn4p specific target genes are listed. Crosses depict the numbers of specific consensus sequence in the respective promoter. ‘C‘ reflects its localization on the complementary strand.

The conflicting findings of other groups could be explained for instance by strain differences. Schröder and co-workers used yeasts with either W303 or SK1 strain background (Schröder *et al.*, 2000) whereas we used yeasts with Σ1278b strain background, which is the most common background to study differentiation processes such as pseudohyphal development of yeasts. Furthermore, their used strains possess many auxotrophic markers, e.g. uracil, leucine and tryptophan, which could influence pseudohyphal development. The more amino acids you have to supplement the more ammonium gets indirect to your media which can in turn act suppressive or permissive (Gimeno *et al.*, 1992). Finally, the concentration of ammonium is a crucial factor for these processes.

4.2.2 Role of Hac1p in the 'general amino acid control' of *S. cerevisiae*

Patil and co-workers discovered that Gcn4p is required for the activation of the 'unfolded protein response' by direct binding to two of three UPR elements (Patil *et al.*, 2004). We therefore were interested whether this also applies accordingly *vice versa*. We could demonstrate that Hac1p itself is not responsible for basal expression of Gcn4p target genes in haploids but in response to amino acid starvation failed to excess the basal level and therefore haploid cells showed a higher sensitivity adverse 3AT. In diploid homozygous Δ*hac1* cells basal expression was additionally reduced. The question arise whether Hac1p itself activates Gcn4p targets, but than with less intense, or whether activation upon amino acid starvation depends on Hac1p, e.g. by stabilization of Gcn4p. The amino acid biosynthetic genes *ARO4*, *TRP2*, *TRP3* and *HIS7* represent direct target genes of Gcn4p, which are up-regulated upon amino acid starvation. Each gene also contains at least one Hac1p specific consensus sequence in its promoter and therefore a direct binding cannot be excluded (Table V).

A regulation or rather stabilization of Gcn4p by Hac1p, as it is supposed by Patil and co-workers, can be excluded since our data point at opposite direction (Patil *et al.*, 2004). We found that Hac1p was hardly detectable in Δ*gcn4* cells upon both amino acid starvation and ER stress conditions, which could be compensated in wild type cells. Together with the Gcn4p reporter gene assays, where deletion of *GCN4* resulted in a loss of activation we expect that Gcn4p regulates Hac1p under amino acid starvation conditions. Furthermore, Hac1p contains two Gcn4p specific consensus sequences it its promoter, nevertheless, direct interaction has not been proven yet. Furthermore, Gcn4p

has a weak but distinct ribonuclease activity and therefore it might be possible that Gcn4p regulates Hac1p by splicing (Nikolaev *et al.*, 2010). A regulation of Gcn4p by Hac1p can be further excluded since expression of *GCN4::lacZ* fusion gene did not alter in Δ*hac1* cells when compared with wild type cells. Indeed, we can confirm a transient increase of *GCN4* mRNA translation in response to ER stress, nevertheless, *GCN4* mRNA translation and therefore Gcn4p specific target gene expression is finally repressed upon ER stress, namely *a fortiori* the longer. Consistently, phosphorylation of the eukaryotic translational inititiation factor eIF2α is repressed after 90 min ER stress which can be derepressed by additional amino acid starvation. Furthermore, concurrent amino acid starvation reduces Hac1p levels.

We therefore conclude, that transcription factor specific stress situations finally results in repression of the respective antagonist. The fact that higher concentrations of 3AT (mimics amino acid starvation) repress Hac1p expression despite ER stress, support this suggestion. Nevertheless, the dual stress liability could also result in stress-induced apoptosis, which is regulated by both eIF2α phosphorylation and Gcn4p (Tallóczy *et al.*, 2002; Ecker *et al.*, 2010). Recent study demonstrated that the Gcn4p mammalian homologue ATF4 mediates autophagy in response to ER stress and therefore provides a direct mechanistic link between the UPR and the autophagic machinery (Rzymski *et al.*, 2010). As demonstrated, ER stress finally results in repression of eIF2α phosphorylation and thus in decreased *GCN4* mRNA translation, which can be derepressed by additional amino acid starvation. This in turn stabilizes Gcn4p and could result in autophagy.

4.2.3 Consequences of phosphorylation and sumoylation

Post-translational modifications are chemical modifications of proteins after translation and enable cells to adapt to environmental changes, e.g. starvation for nutrients. Different post-translational modifications such as phosphorylation, ubiquitination and sumoylation influence a variety of cellular processes like activity, degradation, cell cycle or transcription. In eukaryotic cells, most instable proteins, such as transcription factors, are degraded via the ubiquitin-proteasome pathway (Varshavsky, 1997; Hershko and Ciechanover, 1998). The regulated turnover of Hac1p shares many features with that of Gcn4p. Both, Gcn4p and Hac1p, are bZIP transcription factors whose cellular levels are regulated on translational level

(Hinnebusch, 1984; Kawahara *et al.*, 1997) and by degradation by the proteasome (Kornitzer *et al.*, 1994; Pal *et al.*, 2007). The initial step of degradation is phosphorlyation by a cyclin-dependent kinase. The CDK Srb10p phosphorylates both, Gcn4p and Hac1p (Irniger and Braus, 2003; Pal *et al.*, 2007), whereas a phosphorylation by Pho85p is only documented for Gcn4p (Irniger and Braus, 2003). Therefore, we determined Hac1p turnover in Δ*pho85* cells as well as in Δ*pcl5* and Δ*gcn4* cells, respectively. Pcl5p represents the specific Pho85p cyclin required for phosphorylation of Gcn4p (Shemer *et al.*, 2002). We could not detect significant alterations of Hac1p turnover in Δ*pcl5* and Δ*gcn4*, however, Hac1p was hardly detectable in Δ*pho85* cells which was in contrast to our assumption that Hac1p is also a target of the Pho85p-Pcl5p complex. One possible explanation for the rapid turnover of Hac1p in Δ*pho85* cells could be either due to a general growth deficit of Δ*pho85* cells (Huang *et al.*, 2007) or Pho85p usually stabilizes Hac1p in interaction with another cyclin, since Hac1p levels did not alter in Δ*pcl5* cells. Due to the fact, that other groups identified Δ*pho85* cells as sensitive towards tunicamycin our second hypothesis that Pho85p in association with another cyclin is involved in Hac1p stability control is supported (Huang *et al.*, 2002). Pho80p represents a further cyclin of Pho85p and Δ*pho80* cells are also sensitive towards tunicamycin (Huang *et al.*, 2002; Chen *et al.*, 2005). The Pho80p-Pho85p complex regulates the transcription factor Pho4p and phosphorylates Pho4p on at least five different sites promoting either nuclear export or nuclear import as well as interaction with another transcription factor can be blocked (O'Neill *et al.*, 1996; Komeili and O'Shea, 1999). Therefore, our hypothesis can be modified as follows: the Pho80p-Pho85p complex possibly phosphorylates Hac1p, this in turn imports Hac1p into the nucleus where it can finally bind to the promoter regions of its target genes. Recent studies verified a direct interaction of Hac1p and Pho80p, which support our hypothesis (Costanzo *et al.*, 2010). Indeed, Pal and co-workers identified a classical nuclear localization sequence (cNLS) in the N-terminus of Hac1p (aa 29 – 35), but further particulars of nuclear import of Hac1p are unknown so far (Pal *et al.*, 2007).

This cNLS in turn could be of further interest since Hac1p was not only hardly detectable in Δ*pho85* cells but also in Δ*ubc9*$^{(ts)}$ and Δ*smt3*$^{(ts)}$ cells. Smt3p encodes for the yeast SUMO (small ubiquitin-like modifier) protein, which alters post-translational modifications and Ubc9p represents the SUMO-conjugating enzyme. SUMO is

structurally related to ubiquititin. In contrast to ubiquitination, sumoylation does not generally direct target proteins to degradation but influences properties such as subcellular localization, protein activity and protein stability (Geiss-Friedlander and Melchior, 2007). Thus, the rapid turnover of Hac1p could be due to either activation or stabilization by sumoylation. Alternatively, sumoylation is a prerequisite for nuclear import or a combination of both. In general post-translational modifications with SUMO are associated with transcriptional repression. In contrast, recent studies identified novel roles of sumoylation in yeast (Rosonina *et al.*, 2010). They demonstrate that sumoylation can also result in both constitutive and activated transcription. Therefore, it is possible that SUMO modifications of Hac1p could alter its activity by stabilization. In contrast, our second hypothesis is supported by the N-terminal cNLS. SUMO modification in yeast plays an important role in nucleocytoplasmic trafficking and cNLS dependent protein import was impaired in mutants with defective enzymes involved in the SUMO conjugation reaction (Stade *et al.*, 2002). Recent publication identified that *Drosophila* Sall proteins can be sumoylated and this modification influences their nuclear localization and therefore modulates its activity (Sánchez *et al.*, 2010). In the case, that sumoylation of Hac1p is required for nuclear import *inter alia*, a prevention of this post-translational modification would result in an instable protein. A possible sumoylation of Hac1p is supported by demonstrated sumoylation of XBP1, the mammalian homologue (Chen and Qi, 2010) and additionally by existence of a predicted SUMO consensus site (ψKXD/E) located in the C-terminus of Hac1p. In contrast to our assumption regarding sumoylation of Hac1p in *S. cerevisiae*, XBP1 sumoylation results in the most common effect, namely transcriptional repression.

A summary of all aspects is given in Fig. 27. Our data indicate a so far unknown function of Hac1p in metabolic and developmental processes generally regulated by Gcn4p in response to amino acid starvation, nevertheless, Gcn4p appears to be involved in Hac1p regulation and thus has an epistatic effect on Hac1p upon amino acid starvation. An oppositional function of Gcn4p in regulating Hac1p target genes was described in the past (Patil *et al.*, 2004). Furthermore, we demonstrate that transcription factor specific stress situation results finally in repression of the respective antagonist. Finally our study establishes novel aspects in Hac1p regulation.

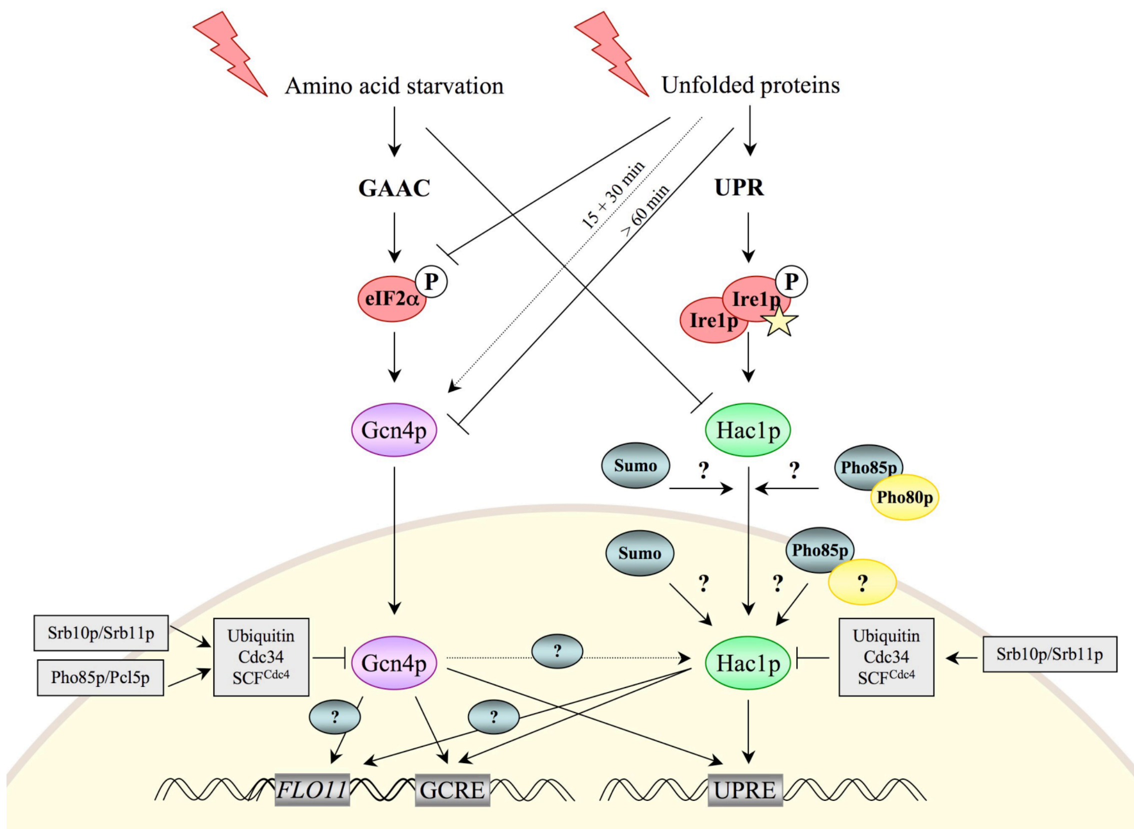


Fig. 27: Current model for the developmental and metabolic regulation conferred by Gcn4p and Hac1p (see text for details).

At least 13 bZIP transcription factor exist in *S. cerevisiae* whereas there are minimum 51 in humans (Amoutzias *et al.*, 2008). This reflects the increasing complexity during evolution. The bakers' yeast *S. cerevisiae* represents an established unicellular model system for the eukaryotic cell since most relevant genes are conserved from yeast to human. Both ATF4 and XBP1, the mammalian homologues of Gcn4p and Hac1p, represent essential genes involved in a multiplicity of metabolic and developmental processes ensuring the survival of the organism. Furthermore, XBP1 plays an important role in tumorogenesis whereas ATF4 mediates ER stress-induced cell death of tumor cells. Understanding the complex regulation of dimorphism, stability control, nuclear trafficking and cell death pathways are relevant for tumor therapy in human and thus this study indicates important aspects, which could be useful for clarification of differentiation processes as well as for the crosstalk between the UPR and the GAAC in yeast and higher organisms.

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