CsnA Dependent Development and Regulation of Amino Acid Biosynthesis of the Filamentous Ascomycete *Aspergillus nidulans*

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Für meine Eltern und meine kleine Rose

Abbreviations

AD .....activation domain
Af .....*A. fumigatus*
AICAR.....5'-phosphoribosyl-5-aminoimidazole-4-carboxamide
AMP .....adenosinemonophosphate
An .....*A. nidulans*
Ao .....*A. oryzae*
ATP .....adenosinetriphosphate
bp(s).....base pair(s)
C .....Cullin
CAAD.....central acidic activation domain
CDK .....cyclin-dependent kinase
cpc .....cross-pathway control of amino acid biosynthesis
CSN .....COP9 signalosome
C-terminal.....carboxy terminal
eIF.....elongation initiation factor
F .....F-box protein
GABA.....γ-amino-butyric acid
gc .....general control of amino acid biosynthesis
GDP .....guanosinediphosphate
GFP.....green fluorescent protein
GKA .....generelle Kontrolle der Aminosäurebiosynthese
GTP .....guanosinetriphosphate
HAM.....resembling HEAT and Armadillo repeats
HisRS .....histidyl-tRNA synthase
hs .....*Homo sapiens*
JAMM .....Jab1/MPN metalloenzyme motif
kb.....1000 base pairs
m .....mouse
MED.....mediator complex
MPN .....Mpr1p, Pad1 N-terminal
MPN+.....JAMM motif
N.....Nedd8 protein
Nc .....*N. crassa*
NLS .....nuclear localisation signal
nt.....nucleotide(s)
NTAD.....N-terminal activation domain
N-terminal .....amino terminal
ORF .....open reading frame
PCI .....proteasome, COP9 signalosome, eIF3
PK.....Protein kinase
PRPP.....phosphoribosylpyrophosphate
R .....RING-finger protein
RNAi .....RNA interference
S.....Svedberg units
Sc .....*S. cerevisiae*
SLC.....solute carrier
sub .....substrate
TAF .....TATA associated factor
TBP.....TATA binding protein
TOR.....target of rapamycin
U / Ubi.....Ubiquitin
uORF .....(small) upstream ORF
UV (light).....ultra violet light
WH .....winged-helix

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Summary

Aspergilli comprise a heterogenous group of filamentous fungi. The ascomycete *Aspergillus nidulans* represents a well studied eukaryotic model system for metabolism and development. *Aspergillus oryzae* and the opportunistic human pathogen *A. fumigatus* are deuteromycetes with significant impact on asian food industry and medical research respectively. This work focusses on the COP9 signalosome as an essential regulator of development in higher eukaryotes and the cross-pathway control of amino acid biosynthesis which regulates the cellular response to amino acid starvation conditions. Both networks are well conserved from yeast to human.

The COP9 signalosome is a multiprotein complex with two major associated enzyme activities - associated kinase and de-ubiquitination activities and an additional intrinsic deneddylation activity. In this thesis, the relevance of the first subunit CsnA of the COP9 signalosome is investigated by expression of truncated CsnA peptides in *csnA* deletion strains. A deletion in *csnA* results in multiple pleiotrophic phenotypes. Expression of the truncated CsnA proteins made it possible to separate functions of the COP9 holoenzyme from CsnA functions. The C-terminal part of CsnA seems to be essential for integration into the COP9 signalosome and maintaining the structural integrity and activities of the complex. The N-terminal of CsnA seems to play a role in regulation of formation of aerial hyphae.

During the course of the manual annotation of the genomes of the three fungi *A. nidulans*, *A. fumigatus* and *A. oryzae* the constituent genes of the cross-pathway control of amino acid biosynthesis (*cpc*) in comparison to higher and lower eukaryotes were investigated. The results show that basic mechanisms of the *cpc* of these filamentous fungi resemble those of yeast and are less complex than in higher eukaryotes. The external and internal amino acid sensing and uptake system of amino acids rather resembles that of mammals than of unicellular yeasts. This indicates a possible role of amino acid uptake systems in regulation of cellular growth and development similar to that of mammalian cells.

The transport of the central transcription factor CpcA into the nucleus was investigated in *S. cerevisiae* and in *A. nidulans*. Efficient transport to the nucleus requires the nuclear localisation signal. In *S. cerevisiae* two importins Srp1p and Kap95p were identified to be essential for transport of CpcA into the yeast nucleus. *In silico* investigations in the *Aspergillus* genomes revealed the highly similar proteins SrpA and KapA that might be involved in nuclear transport of CpcA in *A. nidulans*.

Zusammenfassung

Die Familie der *Aspergillen* ist eine sehr heterogene Gruppe filamentöser Pilze. *Aspergillus nidulans* ist ein wichtiger eukaryotischer Modellorganismus für Metabolismus- und Entwicklungsstudien. *Aspergillus oryzae* ist von großer biotechnologischer Bedeutung in der asiatischen Lebensmittelindustrie, wohingegen der opportunistische humanpathogene Pilz *A. fumigatus* von wachsender medizinischer Bedeutung ist. Diese Arbeit konzentriert sich auf das COP9 Signalosom, einen essentiellen, eukaryontischen Entwicklungsregulator und die generelle Kontrolle der Aminosäurebiosynthese (gc). Die generelle Kontrolle regelt die zelluläre Antwort auf Aminosäuremangel. Beide Netzwerke sind von der Bäckerhefe bis zum Menschen hoch konserviert.

Das COP9 Signalosom ist ein Multiproteinkomplex, dessen wichtigste zugehörige enzymatische Aktivitäten aus Kinase- und De-ubiquitinierungsaktivitäten und einer intrinsischen Deneddylaseaktivität bestehen. Diese Arbeit untersucht die Relevanz der ersten Untereinheit des COP9 Signalosoms CsnA anhand der Expression von verkürzten Proteinvarianten. *csnA* Deletionsmutanten weisen diverse pleiotrophe Phänotypen auf. Die Expression von verkürzten CsnA Peptiden ermöglichte es, die Funktionen des COP9 Signalosoms von Aktivitäten des CsnA Proteins zu trennen. Der CsnA C-Terminus ist wichtig für die Aufrechterhaltung des Komplexzusammenhalts und der COP9 Aktivitäten. Der CsnA N-Terminus hingegen scheint eine wichtige Rolle in der Regulation der Ausbildung von Lufthyphen zu spielen.

Im Zuge der manuellen Annotation der Genome der Pilze *A. nidulans*, *A. fumigatus* und *A. oryzae* wurden grundlegende Gene der generellen Kontrolle der Aminosäurebiosynthese (cpc) im Vergleich zu niederen und höheren Eukaryonten untersucht. Die Resultate der Untersuchung zeigen, daß die grundlegenden Mechanismen der cpc der filamentösen Pilze eher denen der Hefen ähneln und etwas weniger komplex als bei höheren Organismen sind. Die externen und internen Sensoren und die Aufnahmesysteme für Aminosäuren hingegen scheinen eher denen höherer Eukaryonten zu ähneln als denen einzelliger Hefen. Die Ergebnisse weisen darauf hin, daß die Aufnahme- und Sensorsysteme für Aminosäuren einen Einfluß auf das Wachstum und die Entwicklung der Pilze haben könnten, wie man sie aus Säugersystemen kennt.

Die Untersuchung des Transports des zentralen Transkriptionsfaktors der cpc CpcA in den Zellkern wurde in *S. cerevisiae* und *A. nidulans* untersucht. Für einen effizienten Transport in den Nukleus ist das Kernlokalisierungssignal von CpcA notwendig. In der Bäckerhefe wurden zwei Importine Srp1p und Kap95p identifiziert, die essentiell für den Kerntransport von CpcA sind. Diese wurden auch hoch konserviert in *in silico* Untersuchungen in den Aspergillengenomen wiedergefunden. Die hypothetischen Aspergillenproteine SrpA und KapA könnten auch in *Aspergillus* notwendig für den Kerntransport von CpcA sein.

Chapter 1

Introduction

1.1 Genetic control of COP9 dependent development and cross-pathway control

In living organisms cellular actions need to be closely regulated. This chapter will focus on two aspects of genetic regulation in cells. Each cell needs amino acids as essential building blocks for protein synthesis. Amino acids can either be taken up from the diet or culture medium or synthesized de novo by the cells. The de novo synthesis of amino acids in fungi is tightly controlled by the general or cross-pathway control of amino acid biosynthesis. Another way of obtaining amino acids is the degradation of proteins in the cell. The degradation needs to be highly controlled as well to degrade only proteins that are not longer needed. One of the major regulators of the protein degradation machinery is the COP9 signalosome, a multi-subunit complex that controls the activity of the SCF and thus the targeting of proteins for degradation.

1.1.1 Composition of the COP9 signalosome

The COP9 signalosome, or CSN, was originally identified in a screen for altered light response loss-of-function mutants of *Arabidopsis thaliana* and later in other higher and lower eukaryotes (Bech-Otschir *et al.*, 2002; Busch *et al.*, 2003; Chamovitz and Segal, 2001; Harari-Steinberg and Chamovitz, 2004; Irniger and Braus, 2003; Kim *et al.*, 2001; Schwechheimer and Deng, 2001; Seeger *et al.*, 2001; Wei *et al.*, 1994). In the screen two groups of mutants were found: the ones that exhibit light-grown seedling characteristics in the absence of light, a constitutive photomorphogenesis (*cop*) and the other group of mutants that showed de-ethiolation (*det*) (Wei and Deng, 1992). Lethal mutants in this class are allelic to *fusca* mutants which accumulate anthocyanin, a purple pigment, in the mature seed coat and the embryonic leaves (Gusmaroli *et al.*, 2004; Misera *et al.*, 1994). These mutants were classed as *cop*/*det*/*fus* mutants in *A. thaliana* and later on it could be shown that their respective gene loci coded for six of the eight subunits of the COP9 signalosome (Schwechheimer and Deng, 2001; Wei *et al.*, 1998). The mammalian CSN complex is also known as the JAB containing signalosome according to the fifth subunit JAB1 (Carrabino *et al.*, 2004). It was originally

identified as a copurifying byproduct of the 26S proteasome (Scheel and Hofmann, 2005; Seeger *et al.*, 1998).

The overall identification of multi-species CSN complexes from yeast to mammals revealed evolutionary evidence of the conservation of this complex and other PCI complexes. The CSN shares high homology to the catalytic 19S subunit proteins of the proteasome lid and to the eukaryotic translation initiation factor eIF3 subunits. The 26S proteasome, which is required for the degradation of ubiquitinated proteins, consists of the 20S core particle and two 19S lid components located at the exterior ends of the core particle. The eIF3 function is to prevent premature association of the 40S and 60S ribosomal subunits. It facilitates the loading of the 40S subunit onto the ternary eIF2-tRNA-Met-GTP complex and interacts with other translation factors. These three complexes contain proteins with a similar interaction domain, the so-called PCI domain (proteasome, COP9 signalosome, eIF3), which makes these three complexes to PCI protein complexes. A unified nomenclature of the CSN subunit orders them by decreasing size from CSN1 to CSN8 (Deng *et al.*, 2000) (Table 1). In CSN six of the eight subunits harbor PCI domains, whereas two of the subunits harbor MPN domains (Mpr1p, Pad1 N-terminal). The average size of PCI domains is about 140 amino acids and 200 amino acids for MPN domains. Each of the COP9 signalosome subunits share pair-wise similarities to the subunits of the 19S proteasome lid, indicating that they are homologous with a common evolutionary ancestor. The eIF3 complex, on the other hand, contains three PCI and two MPN proteins among its 11 components (Kim *et al.*, 2001).

1.1.2 PCI complex subunit interaction

Subunit interactions of the CSN are largely conserved. Investigations on subunit interactions in several species made it possible to map the interactions of the subunits, summarized in Figure 1 (Fu *et al.*, 2001; Kapelari *et al.*, 2000; Serino *et al.*, 2003; Tsuge *et al.*, 2001). Electron microscopy studies on the hsCSN and lid complex revealed a similar shape, that lacks symmetry and has a central groove structure (Kapelari *et al.*, 2000). Apart from the eight subunit CSN complex smaller partial complexes, whose relevance is so far unknown, have been identified. Small portions of CSN4 and CSN7 were found independently of CSN1 in *Arabidopsis* (Karniol *et al.*, 1999; Serino *et al.*, 1999; Wang *et al.*, 2002) and *Drosophila* (Oron *et al.*, 2002). In *S. pombe* complexes containing CSN4 and CSN5 were found (Mundt

et al., 2002). Additionally, a cytoplasmic-localized subcomplex consisting of CSN4 to CSN8 was found in mammals (Tomoda *et al.*, 2002).

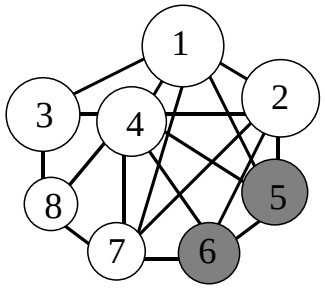


Fig. 1: Subunit interactions in the CSN complex
The figure shows a summary of reported interactions of the CSN subunits. The PCI domain containing subunits are displayed in white, MPN domain containing subunits are displayed in grey (Wei and Deng, 2003).

The interaction between CSN subunits was shown to require PCI domains, which assumably play a scaffolding role in the assembly of the multiprotein complex. So far PCI domain proteins have only been reported in eukaryotic organisms not in prokaryotes, whereas MPN domain proteins were also found in prokaryotes. The MPN domains are also known as JAMM (Jab1/MPN domain metalloenzyme) or MPN+ motif. The JAMM motif is responsible for the catalytic cleavage of the Nedd8-cullin conjugate by the CSN (Ambroggio *et al.*, 2004). The dynamic and cleavage of Nedd8 is responsible for activity regulation of the SCF complex (Pan *et al.*, 2004). The JAMM domain itself is embedded in the large MPN domain. These two types of domains are found predominantly among components of the three large protein complexes, the CSN, the proteasome lid and the eukaryotic translation initiation factor eIF3 (Ambroggio *et al.*, 2004; Glickman *et al.*, 1998; Hofmann and Bucher, 1998; Wei *et al.*, 1998). The subunits of the three complexes not only show similarities, but it was found that the CSN subunits even interact physically with the other PCI complexes, which was shown in copurifications and two-hybrid assays (Karniol *et al.*, 1998; Seeger *et al.*, 1998; Wei and Deng, 2003). Subunit CSN1 of *A. thaliana* was found to bind to RPN6 and eIF3c in yeast two-hybrid assays (Karniol *et al.*, 1998; Kwok *et al.*, 1999). eIF3i was shown to interact strongly with CSN3, CSN6 and CSN7, but only weakly with CSN1 and CSN8 (Hoareau Alves *et al.*, 2002; Yahalom *et al.*, 2001). In *S. cerevisiae* the lid subunit Rpn5p was found to bind stably to Csn5p, additionally Pci8p (CSN11) interacts with eIF3 subunits (Peng *et al.*, 2001c; Shalev *et al.*, 2001). Due to the strong similarities between the single subunits and their interactions it has been proposed that the CSN might constitute an alternative lid for the proteasome (Schwechheimer and Deng, 2001).

Table 1: Relations between subunits of the CSN, 26S proteasome and eIF3

Present nomenclature	Original nomenclature						Similar to		Domain
	H. sapiens	*M. musculus*	*D. melanogaster*	*A. thaliana*	*S.pombe*	*S. cerevisiae*	proteasome	eIF3 subunit	
CSN1	Sgn1, GPS1	COPS1, Mfh	DCH1	COP11, FUS6	Caa1, SpSgn1	CSN9	Rpn7p	P170, p110, p48	PCI
CSN2	Sgn2, TRIP15, hAlien	COPS2	DCH2, Alien	FUS12	SpSgn2	CSN10/RRI2	Rpn6p		PCI
CSN3	Sgn3	COPS3	DCH3	FUS11		CSN11/PCI8	Rpn3p		PCI
CSN4	Sgn4	COPS4	DCH4	COP8, FUS4	SpSgn4	CSN12	Rpn5p		PCI
CSN5	Sgn5, JAB1	COPS5, Jab1	DCH5	AJH1, AJH2	Subunit 5	RRI1	Rpn11p	p47, p40	MPN
CSN6	Sgn6, HVIP	COPS6	DCH6	AtCsn6A, AtCsn6B		CSI1	Rpn8p		MPN
CSN7	Sgn7	COPS7a, COPS7b	DCH7	Csn7i, FUS5, CSN7ii	Subunit7	RPN5/NAS5	Rpn9p	P170, p110, p48	PCI
CSN8	Sgn8, hCOP9	COPS8	DCH8	COP9, FUS7			Rpn12p		PCI

1.1.3 Characteristics of PCI and MPN domains

PCI or PINT domains are found in three multiprotein complexes, the proteasome lid, the COP9 signalosome and the eukaryotic translation initiation factor eIF3. There are several hints, that suggest that the PCI subunits are crucial for complex formation, so far no catalytic activity was described for them (Freilich *et al.*, 1999; Lier and Paululat, 2002; Tsuge *et al.*, 2001; Valasek *et al.*, 2001). The MPN subunits are well conserved and the bioinformatic detection of the domains and their boundaries is relatively simple. In contrast the detection of PCI domains is troublesome due to their high degree of divergence.

The sequence similarities which suggest homology between single subunits of CSN and proteasome lid are easy to spot, whereas the detection of similarities between other paralogous PCI subunits requires advanced approaches, like the generalized profile method (Bucher *et al.*, 1996; Hofmann and Bucher, 1998). Due to these difficulties it is to be expected that a portion of PCI domain proteins remains hidden in the depth of eukaryotic genomes up to now.

A second difficulty arises when it comes to assigning boundaries to the PCI domains. In general one regards a homology domain as a structural unit. This can be regarded true for the C-terminal part of PCI domains, but the amino acid sequence forming the N-terminal part of the PCI domain rather gradually fades instead of providing a sharp drop to provide a sharp boundary. Therefore various domain databases define the N-terminal boundary for PCI domains differently. Recent bioinformatic studies revise the point that a PCI domain should be regarded as an homology domain, but rather consists of two structural distinct domains (Scheel and Hofmann, 2005). The boundaries of the C-terminal part of the PCI domain are well defined by a notable loss of sequence similarities, whereas the boundaries of the N-terminal portion of the PCI domain are difficult to define, because the sequence conservation of this region is low and different families of PCI domain proteins lose their similarity at different positions (Scheel and Hofmann, 2005).

The actual PCI domain can be separated into two domains, the C-terminal WH-domain and the N-terminal HAM domain. The WH-domain is a globular α/β structure with an "αβααββ"-arrangement, which can be classified as a winged-helix (WH) motif. The HAM domain is entirely helical with a core of six regularly-spaced helices that form three antiparallel helical hairpins. It resembles structurally mainly HEAT and Armadillo-repeats, creating the name HAM-domain. Scheel and Hofmann found that TPR-like (tetratrico-peptide repeats) repeats precede many PCI domains, which consist of short bi-helical segments. The

data provided by Scheel and Hofmann and Tsuge *et al*. indicate that different parts of the PCI domain are responsible for binding of different binding partners: a truncated Csn1 protein of *A. thaliana* containing the full PCI domain was able to bind to Csn2, Csn3 and Csn4, whereas a truncated Csn1 protein lacking the WH-region was only able to bind to Csn4 and a truncated protein lacking the helical-repeat region was only able to bind to Csn3. Similar results have been found for proteasome subunits, where amino acid substitutions as a result of point mutations in the WH-portion or the helical-repeat portion of PCI domain proteins were found to abrogate binding to other subunits (Isono *et al.*, 2004).

Whilst no catalytic activity was assigned to PCI domain proteins so far, these proteins must have at least three distinct functions: maintaining the structural integrity of the PCI complexes, attaching the MPN subunit to the complexes and binding to other complexes like eIF3 subunits or the proteasome lid (Karniol *et al.*, 1998; Kwok *et al.*, 1999; Peng *et al.*, 2003; Shalev *et al.*, 2001).

The MPN domain appears to be more ancient and diverse than the PCI domain and was found in non-complexed independent proteins and even in prokaryotes (Maytal-Kivity *et al.*, 2002b). MPN domain proteins harbor the JAMM or MPN+ motif (JAB1/MPN/Mov34 metalloenzyme). The MPN+ motif contains five polar residues that resemble the active site residues of hydrolytic enzyme classes, particularly that of metalloproteases of the following composition EX_nHS/THX_7SXXD, coordinating a zinc ion, whereas the glutamic acid residues forms hydrogen-bonds to a water ligand (Ambroggio *et al.*, 2004). The MPN+ domain is embedded in the larger JAMM domain in some MPN proteins. The CSN harbors two MPN domain proteins, CSN5 and CSN6, the proteasome harbors Rpn11p and Rpn8p (Maytal-Kivity *et al.*, 2002b). CSN5 and Rpn11p, but not CSN6 and Rpn8p contain a JAMM/MPN+ motif in their respective MPN domain (Maytal-Kivity *et al.*, 2002b). The two MPN domain proteins of the eukaryotic translation initiation factor eIF3 do not have the JAMM/MPN+ motifs. The function of MPN proteins lacking the MPN+ motif so far remains unclear, but they are obviously necessary for interactions between subunits of these complexes (Maytal-Kivity *et al.*, 2002b).

The MPN+ motif in CSN5 constitutes for the catalytic isopeptidase activity for the cleavage of Nedd8 protein from Cul1 by the CSN or CSN-like complexes (Cope *et al.*, 2002), whereas Rpn11p, a subunit of the proteasome, constitutes the major de-ubiquitination activity of the 26S proteasome (Verma *et al.*, 2002; Yao and Cohen, 2002). DNA point mutations leading to changes in conserved amino acid residues of either of the proteins MPN+ domains lead to a loss of function of the respective isopeptidase activity (Ambroggio *et al.*, 2004; Maytal-Kivity

et al., 2002b). Interestingly the two proteins exhibit their isopeptidase activity only if they are assembled to their respective complexes, allowing an efficient regulation of the catalytic activities of the proteins (Cope *et al.*, 2002).

1.1.4 Regulation of protein degradation through CSN

Since its initial discovery as a regulator of light-morphogenesis in *A. thaliana* many studies in different eukaryotic species have shown the CSN to be a key regulator of cellular mechanisms and development. The CSN exhibits its regulation mainly through its CSN5 associated metalloprotease activity and signalosome associated kinase activities play a regulatory role in protein stabilisation and destabilisation through phosphorylation (Figure 2).

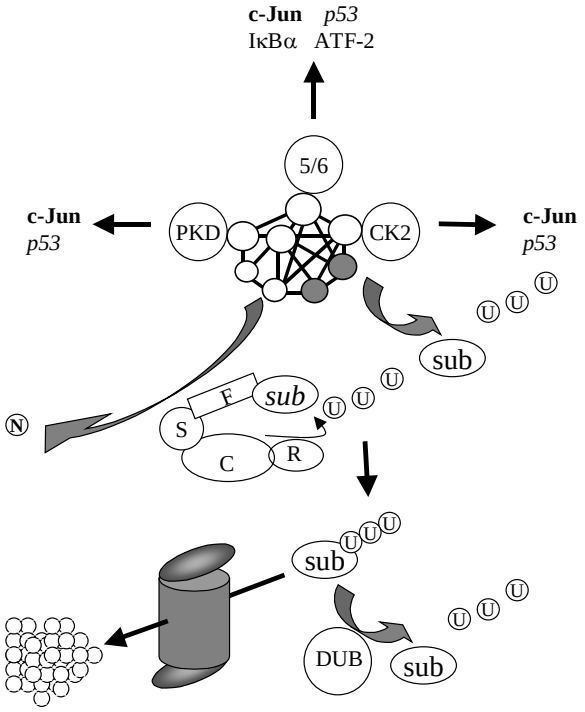


Fig. 2: The CSN is a central regulator of protein degradation

Associated CSN kinases phosphorylate protein targets like the transcription factors c-JUN and p53 and mark them either for degradation (*italic*) or stabilisation in the cell (**bold**) (Uhle *et al.*, 2003; Zheng *et al.*, 2002). Further on the CSN deneddylates and thus activates the SCF complex (Hoffmann *et al.*, 1999; Hoffmann *et al.*, 2000; Tomoda *et al.*, 2004; Valerius *et al.*, 2001). The SCF complex ubiquitinates bound substrates and marks them for degradation in the 26S proteasome. De-ubiquitylation enzymes (DUBs) and the CSN are able to rescue the substrate from destruction by removing the ubiquitin marking (Berndt *et al.*, 2002; Grundmann *et al.*, 2001; Zhou *et al.*, 2003). A more detailed description is found in the text.

The major target of the CSN is the cullin of the SCF ubiquitin ligase complexes. The SCF is an E3 enzyme that conjugates ubiquitin to its target proteins and thus targets them for degradation in the 26S proteasome. A typical SCF complex consists of CUL1, a member of the cullin family, a small RING-finger protein Rbx1/Roc1/Hrt1, Skp1 and an F-box protein (Deshaies, 1999). So far three other cullin proteins next to the CUL1 homologue Cdc53p have been found in *S. cerevisiae*: Apc2p mediating the securin ubiquitination at the onset of sister-chromatid separation in mitosis (Tang *et al.*, 2001), Cul8p which is needed for anaphase progression (Michel *et al.*, 2003) and Cul3p with so far unknown function (Laplaza *et al.*, 2004; Michel *et al.*, 2003). F-box proteins specifically bind substrates following their phosphorylation in response to activation of various signaling pathways (Meimoun *et al.*, 2000; Tyers and Jorgensen, 2000). In addition to the CUL1, five other cullins have been found in human, two of which can also be found in *S. pombe*, but do not have orthologs in *S. cerevisiae* where three cullins are found (Kominami *et al.*, 1998). All human cullins interact with the HRT1/RBX1/ROC1 RING-finger proteins and have a ubiquitin ligase activity *in vitro* (Ohta *et al.*, 1999; Ohta and Xiong, 2001).

The activity of the SCF complexes is regulated through covalent modification of the CUL1 subunit through attachment of the ubiquitin-like peptide Nedd8/Rub1. Covalent binding of Nedd8 or Rub1 to the cullin 1 subunit of the SCF, or neddylation, occurs through a pathway very similar to the ubiquitin ligating pathway: it is catalyzed by an enzymatic cascade involving Nedd8-activating enzymes APP-BP1 and Uba3 (E1) and the conjugating enzyme Ubc12 (E2) (Hershko and Ciechanover, 1998; Hochstrasser, 2000). The neddylation pathway is essential in yeast, worm and mouse and plays a significant role in auxin response in plant (del Pozo *et al.*, 2002; Osaka *et al.*, 2000; Tateishi *et al.*, 2001).

Removal or deneddylation of the Nedd8 peptide from the cullin subunit is carried out by the metalloprotease activity of the CSN5 subunit of the COP9 signalosome, interestingly non-complexed CSN5 subunits do not exhibit this catalytic activity (Cope *et al.*, 2002). The CSN was found to bind to CUL1 and Rbx1 via CSN2, CSN6 and CSN1´s N-terminal domain (Lyapina *et al.*, 2001; Schwechheimer *et al.*, 2001; Yang *et al.*, 2002) and promotes the SCF function *in vivo*. Paradoxically deneddylation of the cullin 1 subunits inhibits SCF activity *in vitro* (Cope and Deshaies, 2003; Wei and Deng, 2003; Wolf *et al.*, 2003). A possible solution for this paradoxon is that the SCF tends to auto-ubiquitination of the SCF components and thus leads to degradation of the SCF instead of the target proteins (Figure 3). In wildtype cells the SCF-bound substrate is poly-ubiquitinated and further degraded at the

26S proteasome, during this time the cullin subunit remains neddylated (He *et al.*, 2005; Wee *et al.*, 2005).

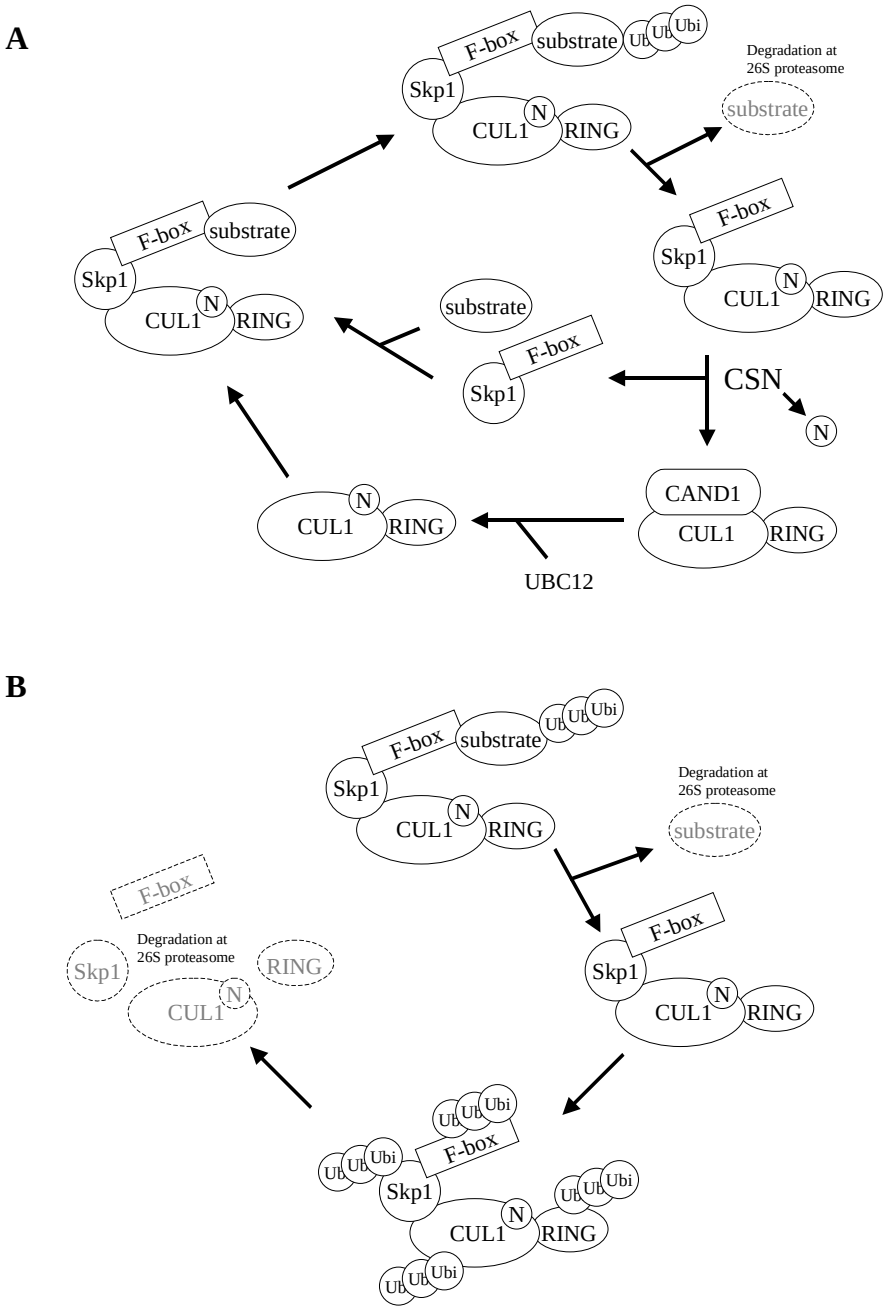


Fig. 3: Regulation of the SCF cycle
(A) In a wild type eukaryotic cell the assembled, substrate bound SCF ubiquitinates its substrate, which is then degraded in the 26S proteasome. Subsequently the SCF is disassembled after deneddylation by the CSN and CAND1 binds to the cullin subunit rendering the ligase complex inactive. During re-neddylation of the cullin and dissociation of CAND1 from the cullin subunit the Skp1/F-box subunits are recharged with a new substrate and the SCF is reassembled. In *csn* mutants (B) the complex fails to disassemble after ubiquitination of the substrate and its subunits are subject to auto-ubiquitination and destruction in the 26S proteasome (He *et al.*, 2005).

In HeLa cells it was found, that the deneddylated cullin and RING-finger part of the SCF are bound by CAND1 (cullin associated and Nedd8 dissociated). This leads to dissociation of the Skp1/F-box part from the SCF and inactivation of the ubiquitin ligase (Zheng *et al.*, 2002). During the dissociated state of the Skp1/F-box proteins these are recharged with new substrates for ubiquitination. Ubc12 neddylates the cullin subunit, which in turn leads to dissociation of CAND1 and re-association of the substrate bound Skp1/F-box proteins. A defect in the deneddylation activity leads to degradation of the target protein, but leaves the neddylated SCF intact. The SCF is now subject to auto-ubiquitination and subsequent degradation which leads to accumulation of SCF substrates in yeast and *Neurospora* (He *et al.*, 2005; Wee *et al.*, 2005). This shows that the neddylation and deneddylation of cullins is a highly dynamic and important process, on the other hand misregulation of substrate degradation leads to severe consequences for a living organism.

Interestingly the CSN does not only show deneddylation activity but it also displays a ubiqitin isopeptidase activity. The CSN can through the metalloprotease domain of CSN5 either depolymerize ubiquitin chains or de-ubiquitinate mono-ubiquitinated substrates, which suggests that the mechanisms of deneddylation and de-ubiquitination are similar from fission yeast to human (Groisman *et al.*, 2003; Zhou *et al.*, 2003).

Another important feature of the CSN are associated kinase activities. To date three associated kinase activities have been found. The first identified kinase is a inositol 1,3,4-triphosphate 5/6-kinase or short 5/6-kinase of *Arabidopsis* (Wilson *et al.*, 2001). It was shown that the 5/6-kinase physically interacts with the CSN1 subunit of the CSN and overexpression of CSN1 was shown to repress this kinase activity (Wilson *et al.*, 2001). Further on in HeLa cells the kinases CK2 and PKD were found associated with the CSN and able to phosphorylate subunits of the CSN and c-Jun and p53, thus directly regulating the ubiquitin conjugation of these transcription factors (Uhle *et al.*, 2003). All three kinases were found to be inhibited by curcumin (Sun *et al.*, 2002; Uhle *et al.*, 2003; Wilson *et al.*, 2001). Phosphorylation of c-Jun, a part of the AP-1 transcription complex, leads to stabilisation of the protein in proliferating cells (Dunn *et al.*, 2002), the phosphorylation of the tumor suppressor p53 leads to destabilisation of the protein (Sharpless and DePinho, 2002), and disruption of the CSN leads to accumulation of p53 and eventually to cell cycle arrest and cell death (Bech-Otschir *et al.*, 2001).

1.1.5 The role of CSN in control of cellular functions

The CSN is a global regulator of development in higher and lower eukaryotes. Its effect on development and physiology has been studied in depth in recent years in numerous organisms like mammals, worm and fly, plant and in fungi (Busch *et al.*, 2003; Freilich *et al.*, 1999; Mundt *et al.*, 2002; Serino and Deng, 2003; Wee *et al.*, 2002; Yan *et al.*, 2003; Zhou *et al.*, 2001). Generally, the role of the CSN is diverse in these organisms, it reaches from light-related signal transduction (He *et al.*, 2005; Wei and Deng, 1992; Wei *et al.*, 1994), oogenesis (Doronkin *et al.*, 2003), immune response (Boussiotis *et al.*, 2000; Kleemann *et al.*, 2000), apoptosis (Yan *et al.*, 2003), cell cycle control (Mundt *et al.*, 1999), checkpoint control (Liu *et al.*, 2003) to DNA repair (Groisman *et al.*, 2003).

The cellular function, development and maintenance of (multi-)cellular organisms strongly relies on the proper complexation of the eight subunits of the CSN. A loss of single subunits of the CSN leads to a loss of the whole complex and thus severe cellular and developmental defects or even cell death (Busch *et al.*, 2003; Freilich *et al.*, 1999; Oron *et al.*, 2002; Smith *et al.*, 2002; Tomoda *et al.*, 2004).

In mammals embryonic development relies, among other factors, on the regulation of cyclin E and p53 protein stability by the COP9 signalosome through the SCF. Misregulation or loss of Uba3 (Tateishi *et al.*, 2001) and Cul1 (Dealy *et al.*, 1999), as parts of the neddylation machinery and SCF, or the loss of single subunits of the CSN leads to a stabilisation of p53 following apoptosis and on the other hand to dysregulation of cyclin E (Lykke-Andersen *et al.*, 2003; Wang *et al.*, 1999). Cyclin E in complex with Cdk2 mediates phosphorylation and subsequent ubiquitination and degradation of the Cdk inhibitor p27 during late G1 impeding progression to S phase (Sherr and Roberts, 1999; Slingerland and Pagano, 2000). The precise mechanism of p27 regulation during development through the CSN remains to be fully uncovered, though. In human the Smith-Magenis Syndrom (SMS) has been assigned to a 1,5-2,0MB hemizygous deletion on chromosome 17, containing approx. 20 genes including CSN3 (Elsea *et al.*, 1999; Potocki *et al.*, 1999; Potocki *et al.*, 2000). Patients suffering from SMS display genital anomalies and mental retardation in addition to a phase shift of circadian rhythm leading to severe sleep disturbances (De Leersnyder *et al.*, 2001; Greenberg *et al.*, 1996).

CSN has essential functions in *Drosophila* oogenesis and embryogenesis, whereas *csn* mutants are lethal at early larval stages (Freilich *et al.*, 1999; Oron *et al.*, 2002). Mutation analyses of the gene for the CSN5 subunit have shown that the CSN is needed for

photoreceptor R cell differentiation and promotion of laminal glial cell migration and axon targeting, indicating that deneddylation is needed during development (Suh *et al.*, 2002) (Cope *et al.*, 2002). Eggs of *Drosophila csn* mutants display characteristic disruptions in anteroposterior and dorsoventral axis formation due to activation of a DNA damage checkpoint (Doronkin *et al.*, 2002). In *C. elegans* knockdowns of CSN5 by RNAi result in a sterile phenotype with small gonads and no oocytes (Smith *et al.*, 2002).

In *Arabidopsis*, *csn* mutants survive embryogenesis, but die after germination at seedling stage with characteristic phenotypes: the mutants are small and accumulate high levels of the anthocyanin pigment. They display a constitutive light-morphology with a gene expression profile similar to that of seedlings under high-light intense stress (Ma *et al.*, 2003). *Csn* mutants with decreased expression of single subunits on the other hand are viable but display various abnormalities in adult plants (Peng *et al.*, 2001a, b; Schwechheimer *et al.*, 2002; Wang *et al.*, 2002). Some of the development defects occur because of mismodulation of SCF E3 ligases by the CSN. It could be shown, that a direct interaction of CSN with SCF^{TIR1} influences the activity of this specific SCF complex in mediating auxin response, hindering SCF^{TIR1} from degrading target proteins and abolishing the auxin response (Schwechheimer *et al.*, 2002). The CSN was also found to interact directly with SCF^{UFO} regulating SCF^{UFO}-mediated flower development (Wang *et al.*, 2003). In a third case the CSN was found to interact with SCF^{COI1} regulating the plant defense-response (Feng *et al.*, 2003). The CSN seems to modulate a variety of SCF activities in *Arabidopsis* in diverse development pathways.

In several fungal species the cellular role of the CSN has been investigated. In contrast to higher eukaryotes, fungal mutants with mutations in one or more genes for the subunits of the CSN are viable. In *S. pombe* mutations in the genes for subunits CSN1 and CSN2 exhibit an elongated cell phenotype, slow growth and sensitivity to UV and gamma irradiation (Liu *et al.*, 2003). The reason for this is that Csn1 and Csn2 are required to regulate ribonucleotide reductase (RNR) through the degradation of a small protein, Spd1, that acts to anchor the small RNR subunit in the nucleus. Spd1 destruction correlates with the nuclear export of the small RNR subunit, which, in turn, correlates with a requirement for RNR in replication and repair (Liu *et al.*, 2003). Mutations in the genes for subunits CSN3-5 do not produce any phenotypes, but the mutants are not capable of deneddylation (Mundt *et al.*, 2002; Zhou *et al.*, 2001). In *S. cerevisiae* COP9 mutants display enhanced pheromone response and increased mating efficiency (Maytal-Kivity *et al.*, 2002a). Recently, He *et al.* discovered that in *N. crassa* the COP9 signalosome regulates the circadian clock by controlling the stability of the

SCF^{FWD-1} complex. FWD-1 is an F-box protein that is specific for FREQUENCY (FRQ) degradation, a circadian clock protein that is critical for clock function. A mutation in *csn-2* leads to degradation of SCF^{FWD-1} and stabilisation of FRQ, thus abolishing a natural light-dark dependent conidiation rhythm. Another example of developmental regulation by CSN is the fungus *Aspergillus nidulans*. Csn deletion strains of *A. nidulans* are viable but strains are impaired in cell polarity and accumulate a red pigment (Busch *et al.*, 2003). Furtheron the mutants enter the sexual cycle and form fruitbodies that are blocked in development at the stage of primordia formation. The development and maturation from primordia to fertile cleistothecia does not take place (Busch *et al.*, 2003).

1.2 Control of amino acid biosynthesis in fungi

1.2.1 The transcriptional activator of the general/cross-pathway control

Amino acids are important building blocks for protein production in the cell. A rapid response to nutritional and other external stimuli is of great importance for the cell to adopt and allow transcriptional reprogramming in case of a lack of amino acids or carbon or nitrogen sources (Davis *et al.*, 2005). Most fungi prefer to take up amino acid from their diet, but are able to produce amino acids themselves in times of hardship. When amino acids are present in the medium, no further specific enzyme activities are needed. On the other hand under starvation conditions counter-actions of the cell need to be imposed and orchestrated. In case of starvation on one or more amino acids the biosynthesis of all amino acids is turned on. To regulate amino acid biosynthesis a transcription factor regulates transcription in the cells. This control network is called general control (gc) of amino acid biosynthesis in *S. cerevisiae* or cross-pathway control (cpc) in filamentous fungi (for an overview see Figure 4) (Hinnebusch, 1986; Sachs, 1996). Gcn4p of *S. cerevisiae* or CpcA of *A. nidulans* and CPC-1 of *N. crassa* are the central regulatory transcription factors of these pathways (Braus *et al.*, 2004; Hinnebusch and Natarajan, 2002). The C-terminus of the proteins consists of a basic leucine zipper, commonly seen in transcription factors of the AP-1 family of basic leucine zippers (bZIP) (Kouzarides and Ziff, 1989). These approximately 60 amino acids are sufficient for homo-dimerization and binding to the DNA (Hope and Struhl, 1986). Other transcription factors of the AP-1 family like c-jun and c-Fos are in contrast to fungal members of this family not restricted to homo-dimerization (Turner and Tjian, 1989). Two nuclear localisation

signals (NLS) have been found for Gcn4p, whereas *A. nidulans* CpcA only contains one NLS in the C-terminal region, overlapping some amino acids of the binding function (Pries *et al.*, 2004). The NLS signal is essential for translocation of the transcription factor into the nucleus independent of amino acid availability (Pries *et al.*, 2002). The activation domains, needed for efficient stimulation of transcription, of the transcription factors of the gc/cpc are not was well conserved as the dimerization domains. In yeast this activation domain (AD) is divided further in two subdomains, the central acidic activation domain (CAAD) and the N-terminal activation domain (NTAD), this concept of divided activation domains seems to be conserved among other fungi (Drysdale *et al.*, 1995). In yeast the two activation domains are divided by a characteristic PEST region, named after their amino acid content (Rechsteiner and Rogers, 1996). This PEST region harbors phosphorylation and ubiquitination sites responsible for the instability of the protein which is finally degraded in the 26S proteasome (Kornitzer *et al.*, 1994). Under starvation conditions the transcription factor binds to defined nucleotide sequences in the promoter regions of amino acid biosynthesis genes and increases the transcription of those genes. This specific sequence is called Gcn4p protein response element (GCRE) in yeast or CpcA protein response element (CPRE) in filamentous fungi, but is also found as a binding site for mammalian AP-1 counterparts (Arndt and Fink, 1986; Hoffmann *et al.*, 2001; Hope and Struhl, 1985; John *et al.*, 1996; Oliphant *et al.*, 1989). The sequence of the binding site is palindromic and contains 9bp of the following sequence: 5´-ATGA(C/G)TCAT-3´. It is found upstream of the promoters of numerous target proteins and upstream of the promoters of *cpc-1* and *cpcA*, where under starvation conditions transcriptional auto-regulation takes place (Ebbole *et al.*, 1991; Hoffmann *et al.*, 2001). The transcription factors Gcn4p, CpcA and CPC-1 are fully interchangeable, indicating a highly conserved mechanism for gc/cpc between yeast and filamentous fungi (Hoffmann *et al.*, 2001).

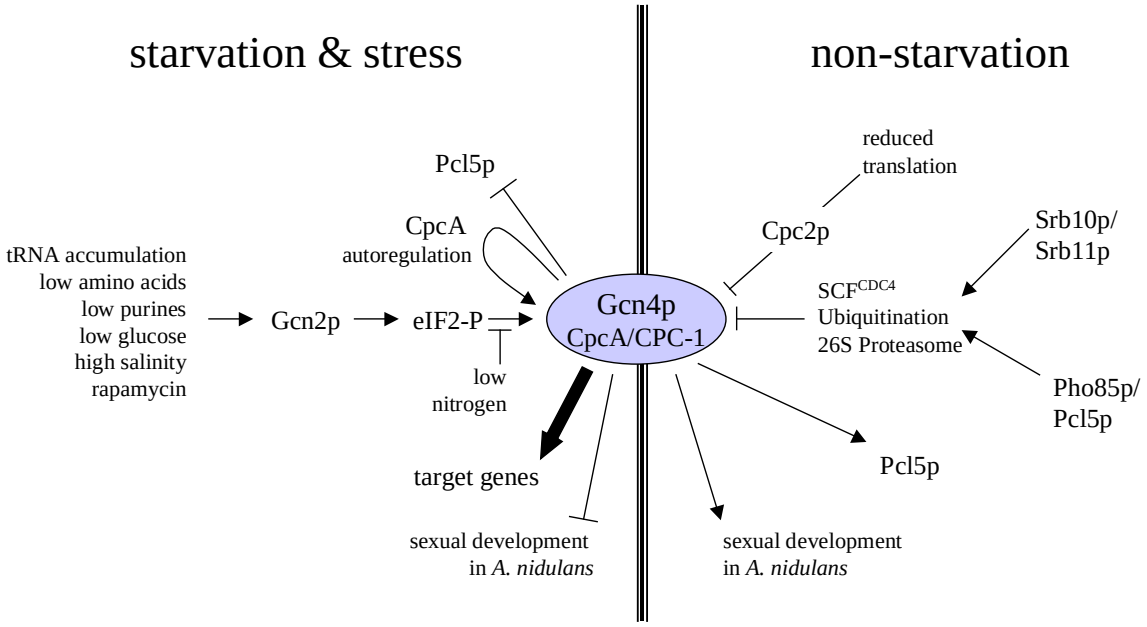


Fig. 4: Summarizing overview of the general control/cross-pathway control of fungi

The complex network induced by Gcn4p and presumably its counterparts in other fungi is displayed according to Hinnebusch and Natarajan, 2002; Irniger and Braus, 2003; Shemer *et a.l*, 2002; Grundmann *et al.*, 2001; Pries *et al.*, 2002; Valerius *et al.*, 2001; Ebbole *et al.*, 1991 and Hoffmann *et al.*, 2000/2001. Details see text.

1.2.2 Translational control of Gcn4p synthesis in *S. cerevisiae*

Due to the fact that Gcn4p is a global transcription factor regulating diverse cellular pathways its activity needs to be strongly regulated. In the case of *S. cerevisiae* this happens on translational as well as post-translational levels through degradation of the protein (Braus *et al.*, 2004). Cells need to decide or sense whether amino acids are present or not, then decide whether the biosynthesis of amino acids needs to be switched on or not. If the fungal cell senses a lack of one or more amino acids, the biosynthesis of amino acids is globally turned on in contrast to bacteria where only the biosynthesis of the respective amino acid(s) is activated (Cahel and Rudd, 1987). To sense the presence of amino acids *S. cerevisiae*, *N. crassa* and *Aspergillus* (see Chapter 3) have amino acid sensorkinases, called Gcn2p or CPC-3 or CpcC, respectively (Carsiotis and Jones, 1974; Carsiotis *et al.*, 1974; Piotrowska *et al.*, 1980). The sensor is combined with a kinase and is located in the cytoplasm attached to the ribosome (Dong *et al.*, 2000). The sensor kinase monitors the availability of amino acid charged tRNAs and regulates the overall translation performance.

Gcn2p/CPC-3 consists of a C-terminal histidyl-tRNA synthetase (HisRS)-related domain and a N-terminal protein kinase domain (Harashima and Hinnebusch, 1986; Zhu *et al.*, 1996). The HisRS kinase responds to uncharged tRNAs by activating the N-terminal protein kinase activity (Dong *et al.*, 2000). The tRNA-binding domain of Gcn2p also contains a dimerization domain that interacts with another monomer, the kinase domain and is needed for kinase activation (Qiu *et al.*, 2001; Wek *et al.*, 1995). *In vivo* the sensor kinase needs to be complexed to Gcn20p/Gcn1p which bind to the N-terminal domain of Gcn2p to function (Garcia-Barrio *et al.*, 2000; Kubota *et al.*, 2000; Kubota *et al.*, 2001). Gcn1p seems to bind near the ribosomal entry site for aminoacyl-tRNAs and promotes Gcn2p kinase activity when uncharged tRNAs bind to the entry site and to Gcn2p (Sattlegger and Hinnebusch, 2000). This leads to activation of the kinase domain of Gcn2p. The kinase then phosphorylates the α subunit of eukaryotic elongation initiation factor eIF-2 resulting in a block of the elongation factor and hence a general repression of translation (Hinnebusch and Natarajan, 2002).

Gcn4p expression is under slight transcriptional control and translated on a basal, low level even under non-starvation conditions (Albrecht *et al.*, 1998). The promoter of *GCN4*, *cpc-1* and *cpcA* contains small open reading frames (uORFs). Four uORFs are found in yeast and two slightly larger ones in *N. crassas* and *A. nidulans* 5´-leader region of the promoter, respectively. Under starvation conditions, where translation in general is repressed, these uORFs allow for efficient translation of the open reading frames of the transcription factors,

whereas they repress translation effectively under non-limiting conditions (Hinnebusch and Natarajan, 2002). At the onset of translation initiation the small ribosomal subunit binds to the 5´-end of the *GCN4*/*cpc-1*/*cpcA*-mRNA and forms an initiation complex with eIF-2α-GTP and the initiation methionine-tRNA at the first AUG START codon that is reached while scanning the mRNA. During initiation of translation the GTP of eIF-2α is hydrolized to GDP. After reaching the STOP codon the ribosome dissociates from the mRNA, leaving the small ribosomal subunit attached. For the next initiation the GDP needs to be replaced by GTP by the GEF. Under non-limiting conditions this takes place prior to the AUG START codon of the fourth uORF, which is subsequently translated and the ribosome dissociates without being able to recharge eIF-2α with GTP and thus translation of the coding sequence of *GCN4*/*cpc-1*/*cpcA* is repressed. Under amino acid limiting conditions the phosphorylation of eIF-2α inhibits the GEF eIF2B, thus the recycling rate of the translation initiation complex is slowed down. In this case the first uORF is translated, but re-initiation of translation does not take place prior to the fourth uORF, but before the coding sequence of the mRNA leading to increased expression of the transcription factors (Hinnebusch, 1997). For *N. crassa* CPC-1 and *A. nidulans* CpcA this means that they can increase their transcription through the CPREs in their respective promoter regions, thus increasing their own production even stronger (Hoffmann *et al.*, 2001). Under nitrogen starvation conditions induction of *GCN4* mRNA translation by amino acid analogues can be overruled in yeast by an additional repression mechanism independent of eIF-2α phosphorylation by Gcn2p (Grundmann *et al.*, 2001).

The ribosome bound WD-repeat proteins Cpc2p/CpcB/CPC-2 incorporate another regulatory mechanism to the expression of the transcription factors. In *cpc2* mutants the transcription of *GCN4* target genes is increased but not the protein level, suggesting no specific role in *GCN4* mRNA translation (Hoffmann *et al.*, 1999). Deletions in *CPC2* lead to increased Gcn4p dependent transcriptional activation of its target genes under non-starvation conditions in wild type cells and under non-starvation and starvation conditions in *gcn2* mutants (Hoffmann *et al.*, 1999). The full cellular function of the protein remains yet unclear, but it was hypothesized that Cpc2p is activated on ribosomes translating at the maximum rate in cells growing and is then deactivated by Gcn2p in starved cells (Hinnebusch and Natarajan, 2002). The corresponding rat protein RACK-1 is an intracellular receptor for activated protein kinase C (PKC) involved in the cellular localisation of PKC (Rotenberg and Sun, 1998) and expression of the mammalian protein complements the general control defect in yeast *cpc2* mutants (Hoffmann *et al.*, 1999).

The yeast TOR (target of rapamycin) proteins have a moderate positive impact on the translation of Gcn4p. It was shown with GCN4 reporter constructs that rapamycin slightly induced *GCN4* translation (Valenzuela *et al.*, 2001). The eIF2a kinase Gcn2p activity is regulated by phosphorylation by the TOR kinase and dephosphorylated by Tap42p under amino acid starvation and non-starvation conditions respectively (Cherkasova and Hinnebusch, 2003). It is speculated that the increased expression of Gcn4p target genes required for acquisition of less-favoured nitrogen sources bases most likely to the fact that a slight starvation on nitrogen leads to a slight starvation on amino acids (Valenzuela *et al.*, 2001).

1.2.3 Post-translational control of Gcn4p expression

After synthesis of the transcription factors Gcn4p/CCP-1/CpcA in the cytosol these need to be transported into the nucleus to increase transcription of their target genes. Their cellular destination is marked by their nuclear localisation sequences (Pries *et al.*, 2002). Since the cytosol and the nucleus are divided by the nuclear membrane, the proteins need to be channeled through nuclear pores (Lusk *et al.*, 2004). This process is driven by GTP hydrolysation by the small GTPase Ran, though the initial step of nuclear import is carried out by the formation of a heterodimeric importin α/β complex in the cytoplasm which recognizes the NLS as part of the cargo protein, translocates the cargo into the nucleus and returns to the cytoplasm (Lusk *et al.*, 2004). The importins necessary to import Gcn4p into the nucleus were identified to be the α-importin Srp1p and the β-importin Kap95p (Pries *et al.*, 2004). Import of the transcription factor Gcn4p seems to be constitutive and independent of the nutritional status (Pries *et al.*, 2004).

Once Gcn4p has entered the nucleus it is subject to phosphorylation through cyclin dependent kinases (CDK) and subsequent degradation in the 26S proteasome (Pries *et al.*, 2002). To date nothing is known about the stability of Gcn4p counterparts in filamentous fungi or if there is a regulation of the stability of CPC-1 or CpcA at all. In *S. cerevisiae* stability of Gcn4p is highly regulated (Irniger and Braus, 2003): under non-limiting conditions the protein is unstable with a half-life of approximately 5 minutes. Under starvation conditions the half-life of the protein increases to approximately 20 minutes (Kornitzer *et al.*, 1994). Cyclins are unstable proteins and are known for their role in various steps of the cell division cycle. The cyclin Pcl5p is known to take part in the degradation of Gcn4p. By assembly of Pcl5p with its

cyclin CDK (cyclin dependent kinase) Pho85p Gcn4p is phosphorylated at Thr165 which leads to ubiquitination and subsequent degradation in the 26S proteasome (Shemer *et al.*, 2002). Under starvation conditions the instable Pcl5p is not found throughout the cell, most likely due to its rapid turnover which can not be counteracted by transcriptional activation through Gcn4p (Jia *et al.*, 2000).

The second important CDK known to destabilize Gcn4p is Srb10p. Srb10p seems to constitutively destablize Gcn4p. The CDK is part of the mediator, a multiprotein complex which is part of the RNA polymerase holoenzyme II and is needed to mediate between transcription factors and the RNA polymerase II (Lusk *et al.*, 2004). Thus Srb10p may be involved in limiting the transcripts (and resulting proteins) for bound Gcn4p at its target promoters enabling a fast turn-down of the gc/cpc if necessary (Chi *et al.*, 2001).

By phosphorylation at residue Thr165 through the CDKs Gcn4p is target to ubiquitination through the SCF (see chapter 3), where Cdc4p is the specificity protein for Gcn4p ubiquitination (Meimoun *et al.*, 2000). Ubiquitination of Gcn4p results in its rapid degradation in the 26S proteasome.

1.2.4 Gc/cpc dependent activation of transcription in fungi

Transcriptional profiling in *S. cerevisiae* showed that at least 539 genes of the genome were targets of Gcn4p under starvation conditions (Natarajan *et al.*, 2001). The target genes encompassed encoded not only for amino acid biosynthesis proteins, but also for vitamin biosynthesis enzymes, peroxisomal components, mitochondrial carrier proteins and also autophagy proteins and other transcription factors (Natarajan *et al.*, 2001). This analysis showed a wide range of pathways regulated by Gcn4p, making Gcn4p a master regulator of cellular functions. Interestingly Gcn4p seems also important for a general stress response like purine or glucose starvation, salt stress or UV light (Engelberg *et al.*, 1994; Goossens *et al.*, 2001; Natarajan *et al.*, 2001; Rolfes and Hinnebusch, 1993) and cellular adhesion in diploid *S. cerevisiae* strains (Kleinschmidt *et al.*, 2005).

Interestingly there seem to be three major mechanisms how Gcn4p activates the transcription of certain sets of genes - reorganisation of chromatin structure, histone modification and recruitment of the transcription machinery (Drysdale *et al.*, 1998; Natarajan *et al.*, 1999).

One function of the Gcn4p activation domain seems to loosen the chromatin structure of promoters and enable transcription. The smallest unit of chromatin is a nucleosome,

consisting of an octamer of the histones H2A, H2B, H3 and H4 wrapped with 146bp of DNA (Luger *et al.*, 1997; Noll and Kornberg, 1977; Shaw *et al.*, 1976). In this structure the DNA is protected against the access of transcription factors resulting in repressed gene expression (Kornberg and Lorch, 1991). Usually the promoter region comprising the TATA element where the transcription factor complex TFIID binds is masked by chromatin. Activator de-repression weakens the DNA-histone interactions and enables transcription factors to access the promoters. The transcription activators do not have the immediate ability to de-repress the histone-bound promoters, but rather recruit other chromatin remodeling or modifying complexes in order to activate their target genes.

The eukaryotic *SWI2/SNF2* complex is a well-characterized model of chromatin remodelers. Chromatin remodeling requires ATP and sequence similarities between the energy-consuming ATPase subunits which are characteristic for this protein family. The *SWI2/SNF2* complex is recruited to target loci and binds independent of any sequence specifity to DNA and nucleosomes (Boeger *et al.*, 2005; Cote *et al.*, 1998; Quinn *et al.*, 1996). The amount of *S. cerevisiaes* SWI2/SNF2 complex is very low and it needs to be recruited specifically to the respective target loci, where it disrupts the nucleosomes and gives the transcription factor access to the now prone promoter region. It is shown for *HIS3* that a physical interaction of *SWI2/SNF2* and Gcn4p is required for efficient transcription activation by Gcn4p (Holstege *et al.*, 1998; Natarajan *et al.*, 1999), though only a subset of all genes requires the activity of *SWI2/SNF2* for transcription activation (Holstege *et al.*, 1998). The hydrophobic clusters of the Gcn4p activation domain are important for the interaction with the *SWI2/SNF2* complex (Neely *et al.*, 2002). Therefore, one way of transcription activation by Gcn4p is to bind to nucleosomal DNA and recruit *SWI2/SNF* to specific promoter regions.

Gcn4p is also able to affect histone modification and thus alteration of chromatin structure to access promoter regions of some genes. Several reversible covalent modifications are known so far that affect the local chromatin structure (Ito, 2003; Verger and Crossley, 2004). The best studied example of chromatin modification is acetylation/deacetylation of histone subunits, mainly H3 and H4 and to a lesser extend the H2 histones (Khan and Krishnamurthy, 2005; Khochbin and Kao, 2001). In fungi Gcn5p is well characterized as a histone acetylase which acetylates H3 and H4. For *HIS3* it was shown that Gcn4p dependent transcription relies on Gcn5p dependent H3 acetylation (Filetici *et al.*, 1998). Gcn5p expresses its acetylase activity as part of the SAGA complex (Spt-Ada-Gcn5 acetyl transferase)(Roberts and Winston, 1997; Timmers and Tora, 2005). As for the SWI2/SNF2 complex SAGA needs to be recruited to the respective promoter regions, where in the case of amino acid biosynthesis

control the transcription activator Gcn4p recruits the complex with the hydrophobic part of its activation domain (Drysdale *et al.*, 1998).

A third mechanism of Gcn4p activating transcription of target genes is direct interaction with the transcription machinery. Gcn4p can bind directly to TFIID which binds to the TATA box. TFIID consists of the actual TATA binding protein (TBP) and several TBP-associated factors (TAFs) with multiple functions in the transcription initiation complex (Green, 2000; Matangkasombut *et al.*, 2004). The interaction of TFIID and Gcn4p is mediated by the multiprotein bridging factor Mbf1p, which is highly conserved from yeast to man (Kabe *et al.*, 1999; Takemaru *et al.*, 1998). Mbf1p seems to bind to the DNA-binding moiety of Gcn4p rather than to the activation domain, indicating that the actual binding of the transcription factor can promote trans-activation (Takemaru *et al.*, 1998). In addition Gcn4p can directly recruit the RNA polymerase II holoenzyme, which is important for the actual transcription of the target genes, via the mediator complex (MED) (Kim *et al.*, 1994). The co-activator MED consists of more than 20 proteins, one of which is Srb10p (see above) (Qiu *et al.*, 2004), and interacts transcription factors with RNA polymerase II subunits.

1.2.5 Impact of the cross-pathway control on *A. nidulans* development

In filamentous fungi the lack of translational precursors has drastic effects on the development of these organisms. The absence of amino acid and hence an active gc/cpc results in a strong impact on *A. nidulans* developmental program. Strains auxotrophic for amino acids are dependent on a suitable supply of amino acids in their medium. Auxotrophic strains defective in the tryptophan pathway are blocked in the formation of conidia and cleistothecia (Eckert *et al.*, 1999; Käfer, 1977; Yelton *et al.*, 1983). A systematic study on four different tryptophan biosynthesis genes showed that fruitbody formation could be restored by high concentrations of tryptophan and was promoted by the addition of indole or auxin, whereas the fertility of sexual fruitbodies could only be partially restored. An increasing amount of tryptophan is necessary to pass the three major steps of development of *A. nidulans*: mycelia growth, conidiation and formation of cleistothecia (Eckert *et al.*, 1999). Deletion in the *trpB* gene showed that these effects are directly linked to the loss of activity itself, similar results were found for the effects of the loss of the *hisB* gene (Busch *et al.*, 2001; Eckert *et al.*, 1999). *argB* mutant strains are deficient in the formation of cleistothecia and on the other hand excessive supply of arginine inhibits ascospore formation in the wild type (Serlupi-Crescenzi *et al.*, 1983).

Auxotrophic *A. nidulans* strains are blocked in fruitbody formation and amino acid limitation results in impaired fruitbody formation (Eckert *et al.*, 1999; Eckert *et al.*, 2000). *A. nidulans* strains growing under amino acid starvation conditions are able to initiate the formation of cleistothecia, but are blocked at the stage of microcleistothecia before the completion of meiosis. The resulting microcleistothecia are filled with hyphae and considerably smaller than cleistothecia. This block in cleistothecia formation can be overcome by addition of the respective amino acids to the medium (Hoffmann *et al.*, 2000).

An artificially simulated amino acid starvation due to overexpression of CpcA as well as the yeast counterpart Gcn4p results in the same block even in the absence of amino acid limitation, suggesting that gc/cpc related signals interfere with the developmental program (Hoffmann *et al.*, 2000). The deletion of *cpcB*, which presumably acts as an inhibitor of the gc/cpc under non-limiting conditions, also results in the formation of microcleistothecia (Hoffmann *et al.*, 2000).

Concluding one could assume that the block of sexual development in *A. nidulans* seems to be an economical consequence of a lack of building material in nature, mediated by elevated expression of the transcription factor CpcA.

1.3 Scope and aim of this work

Well studied examples of biological and biotechnological relevant organisms are species of the gender *Aspergillus*. *Aspergilli* are filamentous ascomycetes. So far more than 185 *Aspergilli* are known. At least 20 Aspergilli are human pathogen. *Aspergillus fumigatus* and *Aspergillus flavus* produce β-Lactam antibiotics and aflatoxin. *Aspergillus fumigatus* has become the most relevant human pathogen causing invasive pulmonary aspergillosis in immunocompromised patients. *Aspergillus oryzae* is of high biotechnological importance in asian countries, being used in the production of soy sauce, sake and miso. *Aspergillus nidulans* is capable of very complex and diverse biosynthesis and differentiation processes where after mating with a compatible partner or “selfing” cleistothecia are formed which contain octades of ascospores. These examples of the *Aspergillus* family are relatively easy genetically manipulated and thus provide suitable model organisms for studying regulatory networks of molecular cross-pathway connections between environmental stimuli, metabolism and development.

This study directs the focus on two possibly interacting regulatory networks: the COP9 signalosome, important for development of the fungus *Aspergillus nidulans*, and the cross-pathway control network necessary for amino acid biosynthesis. Chapter 2 focusses on CsnA which is the largest and therefore first subunit of the COP9 signalosome of *A. nidulans.* The effects of loss of function and partial expression of the gene on development of the fungus are analysed and single protein functions are separated from COP9 signalosome functions. Chapter 3 shows an in depth investigation on cross-pathway control related genes and proteins in all three above mentioned *Aspergilli*, based on the recently released genome sequences of the ascomycete *A. nidulans* and the deuteromycetes *A. fumigatus* and *A. oryzae*. In chapter 4 an analysis of the cross-pathway control transcription factor CpcA is provided, focussing on nuclear import of the protein into the nucleus.

1.4 References

Albrecht, G., Mösch, H.U., Hoffmann, B., Reusser, U., and Braus, G.H. (1998) Monitoring the Gcn4 protein-mediated response in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* **273**: 12696-12702.

Ambroggio, X.I., Rees, D.C., and Deshaies, R.J. (2004) JAMM: A Metalloprotease-Like Zinc Site in the Proteasome and Signalosome. *PLoS Biol* **2**: E2.

Arndt, K., and Fink, G.R. (1986) GCN4 protein, a positive transcription factor in yeast, binds general control promoters at all 5' TGACTC 3' sequences. *Proc Natl Acad Sci U S A* **83**: 8516-8520.

Bech-Otschir, D., Kraft, R., Huang, X., Henklein, P., Kapelari, B., Pollmann, C., and Dubiel, W. (2001) COP9 signalosome-specific phosphorylation targets p53 to degradation by the ubiquitin system. *EMBO J* **20**: 1630-1639.

Bech-Otschir, D., Seeger, M., and Dubiel, W. (2002) The COP9 signalosome: at the interface between signal transduction and ubiquitin-dependent proteolysis. *J Cell Sci* **115**: 467-473.

Berndt, C., Bech-Otschir, D., Dubiel, W., and Seeger, M. (2002) Ubiquitin system: JAMMing in the name of the lid. *Curr Biol* **12**: R815-817.

Boeger, H., Bushnell, D.A., Davis, R., Griesenbeck, J., Lorch, Y., Strattan, J.S., Westover, K.D., and Kornberg, R.D. (2005) Structural basis of eukaryotic gene transcription. *FEBS Lett* **579**: 899-903.

Boussiotis, V.A., Freeman, G.J., Taylor, P.A., Berezovskaya, A., Grass, I., Blazar, B.R., and Nadler, L.M. (2000) p27kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nat Med* **6**: 290-297.

Braus, G.H., Pries, R., Düvel, K., and Valerius, O. (2004) Molecular biology of fungal amino acid biosynthesis regulation. In *The Mycota II*. Kück, U. (ed). Berlin-Heidelberg: Springer-Verlag, pp. 239-269.

Bucher, P., Karplus, K., Moeri, N., and Hofmann, K. (1996) A flexible motif search technique based on generalized profiles. *Comput Chem* **20**: 3-23.

Busch, S., Hoffmann, B., Valerius, O., Starke, K., Düvel, K., and Braus, G.H. (2001) Regulation of the *Aspergillus nidulans hisB* gene by histidine starvation. *Curr Genet* **38**: 314-322.

Busch, S., Eckert, S.E., Krappmann, S., and Braus, G.H. (2003) The COP9 signalosome is an essential regulator of development in the filamentous fungus *Aspergillus nidulans*. *Mol Microbiol* **49**: 717-730.

Cahel, M., and Rudd, K.E. (1987) The stringent response. In *Escherichia coli and Salmonella Typhimurium: cellular and molecular biology*. Neidhardt, F.C., Ingraham, J.L., Low, K.B., NMagasanik, B., Schaechter, M. and Umbareger, H.E. (eds). Washington, DC: American Society for Microbiology, pp. 1410-1438.

Carrabino, S., Carminati, E., Talarico, D., Pardi, R., and Bianchi, E. (2004) Expression pattern of the JAB1/CSN5 gene during murine embryogenesis: colocalization with NEDD8. *Gene Expr Patterns* **4**: 423-431.

Carsiotis, M., and Jones, R.F. (1974) Cross-pathway regulation: tryptophan-mediated control of histidine and arginine biosynthetic enzymes in *Neurospora crassa*. *J Bacteriol* **119**: 889-892.

Carsiotis, M., Jones, R.F., and Wesseling, A.C. (1974) Cross-pathway regulation: histidine-mediated control of histidine, tryptophan, and arginine biosynthetic enzymes in *Neurospora crassa*. *J Bacteriol* **119**: 893-898.

Chamovitz, D.A., and Segal, D. (2001) JAB1/CSN5 and the COP9 signalosome. A complex situation. *EMBO Rep* **2**: 96-101.

Cherkasova, V.A., and Hinnebusch, A.G. (2003) Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev* **17**: 859-872.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev* **15**: 1078-1092.

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V., and Deshaies, R.J. (2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**: 608-611.

Cope, G.A., and Deshaies, R.J. (2003) COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. *Cell* **114**: 663-671.

Cote, J., Peterson, C.L., and Workman, J.L. (1998) Perturbation of nucleosome core structure by the SWI/SNF complex persists after its detachment, enhancing subsequent transcription factor binding. *Proc Natl Acad Sci U S A* **95**: 4947-4952.

Davis, M.A., Askin, M.C., and Hynes, M.J. (2005) Amino acid catabolism by an areA-regulated gene encoding an L-amino acid oxidase with broad substrate specificity in *Aspergillus nidulans*. *Appl Environ Microbiol* **71**: 3551-3555.

De Leersnyder, H., De Blois, M.C., Claustrat, B., Romana, S., Albrecht, U., Von Kleist-Retzow, J.C., Delobel, B., Viot, G., Lyonnet, S., Vekemans, M., and Munnich, A. (2001) Inversion of the circadian rhythm of melatonin in the Smith-Magenis syndrome. *J Pediatr* **139**: 111-116.

Dealy, M.J., Nguyen, K.V., Lo, J., Gstaiger, M., Krek, W., Elson, D., Arbeit, J., Kipreos, E.T., and Johnson, R.S. (1999) Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. *Nat Genet* **23**: 245-248.

del Pozo, J.C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W.M., and Estelle, M. (2002) AXR1-ECR1-dependent conjugation of RUB1 to the *Arabidopsis* Cullin AtCUL1 is required for auxin response. *Plant Cell* **14**: 421-433.

Deng, X.W., Dubiel, W., Wei, N., Hofmann, K., and Mundt, K. (2000) Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. *Trends Genet* **16**: 289.

Deshaies, R.J. (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* **15**: 435-467.

Dong, J., Qiu, H., Garcia-Barrio, M., Anderson, J., and Hinnebusch, A.G. (2000) Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. *Mol Cell* **6**: 269-279.

Doronkin, S., Djagaeva, I., and Beckendorf, S.K. (2002) CSN5/Jab1 mutations affect axis formation in the *Drosophila* oocyte by activating a meiotic checkpoint. *Development* **129**: 5053-5064.

Doronkin, S., Djagaeva, I., and Beckendorf, S.K. (2003) The COP9 signalosome promotes degradation of Cyclin E during early Drosophila oogenesis. *Dev Cell* **4**: 699-710.

Drysdale, C.M., Duenas, E., Jackson, B.M., Reusser, U., Braus, G.H., and Hinnebusch, A.G. (1995) The transcriptional activator GCN4 contains multiple activation domains that are critically dependent on hydrophobic amino acids. *Mol Cell Biol* **15**: 1220-1233.

Drysdale, C.M., Jackson, B.M., McVeigh, R., Klebanow, E.R., Bai, Y., Kokubo, T., Swanson, M., Nakatani, Y., Weil, P.A., and Hinnebusch, A.G. (1998) The Gcn4p activation domain interacts specifically in vitro with RNA polymerase II holoenzyme, TFIID, and the Adap-Gcn5p coactivator complex. *Mol Cell Biol* **18**: 1711-1724.

Dunn, C., Wiltshire, C., MacLaren, A., and Gillespie, D.A. (2002) Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. *Cell Signal* **14**: 585-593.

Ebbole, D.J., Paluh, J.L., Plamann, M., Sachs, M.S., and Yanofsky, C. (1991) cpc-1, the general regulatory gene for genes of amino acid biosynthesis in *Neurospora crassa*, is differentially expressed during the asexual life cycle. *Mol Cell Biol* **11**: 928-934.

Eckert, S.E., Hoffmann, B., Wanke, C., and Braus, G.H. (1999) Sexual development of *Aspergillus nidulans* in tryptophan auxotrophic strains. *Arch Microbiol* **172**: 157-166.

Eckert, S.E., Kübler, E., Hoffmann, B., and Braus, G.H. (2000) The tryptophan synthase-encoding *trpB* gene of *Aspergillus nidulans* is regulated by the cross-pathway control system. *Mol Gen Genet* **263**: 867-876.

Elsea, S.H., Mykytyn, K., Ferrell, K., Coulter, K.L., Das, P., Dubiel, W., Patel, P.I., and Metherall, J.E. (1999) Hemizygosity for the COP9 signalosome subunit gene, SGN3, in the Smith-Magenis syndrome. *Am J Med Genet* **87**: 342-348.

Engelberg, D., Klein, C., Martinetto, H., Struhl, K., and Karin, M. (1994) The UV response involving the Ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. *Cell* **77**: 381-390.

Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W. (2003) The COP9 signalosome interacts physically with SCF COI1 and modulates jasmonate responses. *Plant Cell* **15**: 1083-1094.

Filetici, P., Aranda, C., Gonzalez, A., and Ballario, P. (1998) GCN5, a yeast transcriptional coactivator, induces chromatin reconfiguration of HIS3 promoter in vivo. *Biochem Biophys Res Commun* **242**: 84-87.

Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D., and Chamovitz, D.A. (1999) The COP9 signalosome is essential for development of *Drosophila melanogaster*. *Curr Biol* **9**: 1187-1190.

Fu, H., Reis, N., Lee, Y., Glickman, M.H., and Vierstra, R.D. (2001) Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *EMBO J* **20**: 7096-7107.

Garcia-Barrio, M., Dong, J., Ufano, S., and Hinnebusch, A.G. (2000) Association of GCN1-GCN20 regulatory complex with the N-terminus of eIF2alpha kinase GCN2 is required for GCN2 activation. *EMBO J* **19**: 1887-1899.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94**: 615-623.

Goossens, A., Dever, T.E., Pascual-Ahuir, A., and Serrano, R. (2001) The protein kinase Gcn2p mediates sodium toxicity in yeast. *J Biol Chem* **276**: 30753-30760.

Green, M.R. (2000) TBP-associated factors (TAFIIs): multiple, selective transcriptional mediators in common complexes. *Trends Biochem Sci* **25**: 59-63.

Greenberg, F., Lewis, R.A., Potocki, L., Glaze, D., Parke, J., Killian, J., Murphy, M.A., Williamson, D., Brown, F., Dutton, R., McCluggage, C., Friedman, E., Sulek, M., and Lupski, J.R. (1996) Multi-disciplinary clinical study of Smith-Magenis syndrome (deletion 17p11.2). *Am J Med Genet* **62**: 247-254.

Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A.F., Tanaka, K., and Nakatani, Y. (2003) The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* **113**: 357-367.

Grundmann, O., Mösch, H.U., and Braus, G.H. (2001) Repression of GCN4 mRNA translation by nitrogen starvation in *Saccharomyces cerevisiae*. *J Biol Chem* **276**: 25661-25671.

Gusmaroli, G., Feng, S., and Deng, X.W. (2004) The *Arabidopsis* CSN5A and CSN5B subunits are present in distinct COP9 signalosome complexes, and mutations in their JAMM domains exhibit differential dominant negative effects on development. *Plant Cell* **16**: 2984-3001.

Harari-Steinberg, O., and Chamovitz, D.A. (2004) The COP9 signalosome: mediating between kinase signaling and protein degradation. *Curr Protein Pept Sci* **5**: 185-189.

Harashima, S., and Hinnebusch, A.G. (1986) Multiple GCD genes required for repression of GCN4, a transcriptional activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* **6**: 3990-3998.

He, Q., Cheng, P., and Liu, Y. (2005) The COP9 signalosome regulates the *Neurospora* circadian clock by controlling the stability of the SCFFWD-1 complex. *Genes Dev*. **19**:1518-1531

Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu Rev Biochem* **67**: 425-479.

Hinnebusch, A.G. (1986) The general control of amino acid biosynthetic genes in the yeast *Saccharomyces cerevisiae*. *CRC Crit Rev Biochem* **21**: 277-317.

Hinnebusch, A.G. (1997) Translational regulation of yeast GCN4. A window on factors that control initiator-tRNA binding to the ribosome. *J Biol Chem* **272**: 21661-21664.

Hinnebusch, A.G., and Natarajan, K. (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell* **1**: 22-32.

Hoareau Alves, K., Bochard, V., Rety, S., and Jalinot, P. (2002) Association of the mammalian proto-oncoprotein Int-6 with the three protein complexes eIF3, COP9 signalosome and 26S proteasome. *FEBS Lett* **527**: 15-21.

Hochstrasser, M. (2000) Evolution and function of ubiquitin-like protein-conjugation systems. *Nat Cell Biol* **2**: E153-157.

Hoffmann, B., Mösch, H.U., Sattlegger, E., Barthelmess, I.B., Hinnebusch, A., and Braus, G.H. (1999) The WD protein Cpc2p is required for repression of Gcn4 protein activity in yeast in the absence of amino-acid starvation. *Mol Microbiol* **31**: 807-822.

Hoffmann, B., Wanke, C., Lapaglia, S.K., and Braus, G.H. (2000) c-Jun and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans*. *Mol Microbiol* **37**: 28-41.

Hoffmann, B., Valerius, O., Andermann, M., and Braus, G.H. (2001) Transcriptional autoregulation and inhibition of mRNA translation of amino acid regulator gene *cpcA* of filamentous fungus *Aspergillus nidulans*. *Mol Biol Cell* **12**: 2846-2857.

Hofmann, K., and Bucher, P. (1998) The PCI domain: a common theme in three multiprotein complexes. *Trends Biochem Sci* **23**: 204-205.

Holstege, F.C., Jennings, E.G., Wyrick, J.J., Lee, T.I., Hengartner, C.J., Green, M.R., Golub, T.R., Lander, E.S., and Young, R.A. (1998) Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* **95**: 717-728.

Hope, I.A., and Struhl, K. (1985) GCN4 protein, synthesized in vitro, binds HIS3 regulatory sequences: implications for general control of amino acid biosynthetic genes in yeast. *Cell* **43**: 177-188.

Hope, I.A., and Struhl, K. (1986) Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* **46**: 885-894.

Irniger, S., and Braus, G.H. (2003) Controlling transcription by destruction: the regulation of yeast Gcn4p stability. *Curr Genet* **44**: 8-18.

Isono, E., Saeki, Y., Yokosawa, H., and Toh-e, A. (2004) Rpn7 Is required for the structural integrity of the 26 S proteasome of *Saccharomyces cerevisiae*. *J Biol Chem* **279**: 27168-27176.

Ito, T. (2003) Nucleosome assembly and remodeling. *Curr Top Microbiol Immunol* **274**: 1-22.

Jia, M.H., Larossa, R.A., Lee, J.M., Rafalski, A., Derose, E., Gonye, G., and Xue, Z. (2000) Global expression profiling of yeast treated with an inhibitor of amino acid biosynthesis, sulfometuron methyl. *Physiol Genomics* **3**: 83-92.

John, M., Leppik, R., Busch, S.J., Granger-Schnarr, M., and Schnarr, M. (1996) DNA binding of Jun and Fos bZip domains: homodimers and heterodimers induce a DNA conformational change in solution. *Nucleic Acids Res* **24**: 4487-4494.

Kabe, Y., Goto, M., Shima, D., Imai, T., Wada, T., Morohashi, K., Shirakawa, M., Hirose, S., and Handa, H. (1999) The role of human MBF1 as a transcriptional coactivator. *J Biol Chem* **274**: 34196-34202.

Käfer, E. (1977) The anthranilate synthetase enzyme complex and the trifunctional *trpC* gene of *Aspergillus*. *Can J Genet Cytol* **19**: 723-738.

Kapelari, B., Bech-Otschir, D., Hegerl, R., Schade, R., Dumdey, R., and Dubiel, W. (2000) Electron microscopy and subunit-subunit interaction studies reveal a first architecture of COP9 signalosome. *J Mol Biol* **300**: 1169-1178.

Karniol, B., Yahalom, A., Kwok, S., Tsuge, T., Matsui, M., Deng, X.W., and Chamovitz, D.A. (1998) The *Arabidopsis* homologue of an eIF3 complex subunit associates with the COP9 complex. *FEBS Lett* **439**: 173-179.

Karniol, B., Malec, P., and Chamovitz, D.A. (1999) *Arabidopsis* FUSCA5 encodes a novel phosphoprotein that is a component of the COP9 complex. *Plant Cell* **11**: 839-848.

Khan, A.U., and Krishnamurthy, S. (2005) Histone modifications as key regulators of transcription. *Front Biosci* **10**: 866-872.

Khochbin, S., and Kao, H.Y. (2001) Histone deacetylase complexes: functional entities or molecular reservoirs. *FEBS Lett* **494**: 141-144.

Kim, T., Hofmann, K., von Arnim, A.G., and Chamovitz, D.A. (2001) PCI complexes: pretty complex interactions in diverse signaling pathways. *Trends Plant Sci* **6**: 379-386.

Kim, Y.J., Bjorklund, S., Li, Y., Sayre, M.H., and Kornberg, R.D. (1994) A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**: 599-608.

Kleemann, R., Hausser, A., Geiger, G., Mischke, R., Burger-Kentischer, A., Flieger, O., Johannes, F.J., Roger, T., Calandra, T., Kapurniotu, A., Grell, M., Finkelmeier, D., Brunner, H., and Bernhagen, J. (2000) Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. *Nature* **408**: 211-216.

Kleinschmidt, M., Grundmann, O., Bluthgen, N., Mösch, H.U., and Braus, G.H. (2005) Transcriptional profiling of *Saccharomyces cerevisiae* cells under adhesion-inducing conditions. *Mol Genet Genomics* **273**: 382-393.

Kominami, K., Ochotorena, I., and Toda, T. (1998) Two F-box/WD-repeat proteins Pop1 and Pop2 form hetero- and homo-complexes together with cullin-1 in the fission yeast SCF (Skp1-Cullin-1-F-box) ubiquitin ligase. *Genes Cells* **3**: 721-735.

Kornberg, R.D., and Lorch, Y. (1991) Irresistible force meets immovable object: transcription and the nucleosome. *Cell* **67**: 833-836.

Kornitzer, D., Raboy, B., Kulka, R.G., and Fink, G.R. (1994) Regulated degradation of the transcription factor Gcn4. *EMBO J* **13**: 6021-6030.

Kouzarides, T., and Ziff, E. (1989) Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding. *Nature* **340**: 568-571.

Kubota, H., Sakaki, Y., and Ito, T. (2000) GI domain-mediated association of the eukaryotic initiation factor 2alpha kinase GCN2 with its activator GCN1 is required for general amino acid control in budding yeast. *J Biol Chem* **275**: 20243-20246.

Kubota, H., Ota, K., Sakaki, Y., and Ito, T. (2001) Budding yeast GCN1 binds the GI domain to activate the eIF2alpha kinase GCN2. *J Biol Chem* **276**: 17591-17596.

Kwok, S.F., Staub, J.M., and Deng, X.W. (1999) Characterization of two subunits of *Arabidopsis* 19S proteasome regulatory complex and its possible interaction with the COP9 complex. *J Mol Biol* **285**: 85-95.

Laplaza, J.M., Bostick, M., Scholes, D.T., Curcio, M.J., and Callis, J. (2004) *Saccharomyces cerevisiae* ubiquitin-like protein Rub1 conjugates to cullin proteins Rtt101 and Cul3 in vivo. *Biochem J* **377**: 459-467.

Lier, S., and Paululat, A. (2002) The proteasome regulatory particle subunit Rpn6 is required for *Drosophila* development and interacts physically with signalosome subunit Alien/CSN2. *Gene* **298**: 109-119.

Liu, C., Powell, K.A., Mundt, K., Wu, L., Carr, A.M., and Caspari, T. (2003) Cop9/signalosome subunits and Pcu4 regulate ribonucleotide reductase by both checkpoint-dependent and -independent mechanisms. *Genes Dev* **17**: 1130-1140.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**: 251-260.

Lusk, C.P., Makhnevych, T., and Wozniak, R.W. (2004) New ways to skin a kap: mechanisms for controlling nuclear transport. *Biochem Cell Biol* **82**: 618-625.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., and Deshaies, R.J. (2001) Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**: 1382-1385.

Lykke-Andersen, K., Schaefer, L., Menon, S., Deng, X.W., Miller, J.B., and Wei, N. (2003) Disruption of the COP9 signalosome Csn2 subunit in mice causes deficient cell proliferation, accumulation of p53 and cyclin E, and early embryonic death. *Mol Cell Biol* **23**: 6790-6797.

Ma, L., Zhao, H., and Deng, X.W. (2003) Analysis of the mutational effects of the COP/DET/FUS loci on genome expression profiles reveals their overlapping yet not identical roles in regulating *Arabidopsis* seedling development. *Development* **130**: 969-981.

Matangkasombut, O., Auty, R., and Buratowski, S. (2004) Structure and function of the TFIID complex. *Adv Protein Chem* **67**: 67-92.

Maytal-Kivity, V., Piran, R., Pick, E., Hofmann, K., and Glickman, M.H. (2002a) COP9 signalosome components play a role in the mating pheromone response of *S. cerevisiae*. *EMBO Rep* **3**: 1215-1221.

Maytal-Kivity, V., Reis, N., Hofmann, K., and Glickman, M.H. (2002b) MPN+, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. *BMC Biochem* **3**: 28.

Meimoun, A., Holtzman, T., Weissman, Z., McBride, H.J., Stillman, D.J., Fink, G.R., and Kornitzer, D. (2000) Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. *Mol Biol Cell* **11**: 915-927.

Michel, J.J., McCarville, J.F., and Xiong, Y. (2003) A role for *Saccharomyces cerevisiae* Cul8 ubiquitin ligase in proper anaphase progression. *J Biol Chem* **278**: 22828-22837.

Misera, S., Muller, A.J., Weiland-Heidecker, U., and Jurgens, G. (1994) The FUSCA genes of *Arabidopsis*: negative regulators of light responses. *Mol Gen Genet* **244**: 242-252.

Mundt, K.E., Porte, J., Murray, J.M., Brikos, C., Christensen, P.U., Caspari, T., Hagan, I.M., Millar, J.B., Simanis, V., Hofmann, K., and Carr, A.M. (1999) The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. *Curr Biol* **9**: 1427-1430.

Mundt, K.E., Liu, C., and Carr, A.M. (2002) Deletion mutants in COP9/signalosome subunits in fission yeast *Schizosaccharomyces pombe* display distinct phenotypes. *Mol Biol Cell* **13**: 493-502.

Natarajan, K., Jackson, B.M., Zhou, H., Winston, F., and Hinnebusch, A.G. (1999) Transcriptional activation by Gcn4p involves independent interactions with the SWI/SNF complex and the SRB/mediator. *Mol Cell* **4**: 657-664.

Natarajan, K., Meyer, M.R., Jackson, B.M., Slade, D., Roberts, C., Hinnebusch, A.G., and Marton, M.J. (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* **21**: 4347-4368.

Neely, K.E., Hassan, A.H., Brown, C.E., Howe, L., and Workman, J.L. (2002) Transcription activator interactions with multiple SWI/SNF subunits. *Mol Cell Biol* **22**: 1615-1625.

Noll, M., and Kornberg, R.D. (1977) Action of micrococcal nuclease on chromatin and the location of histone H1. *J Mol Biol* **109**: 393-404.

Ohta, T., Michel, J.J., Schottelius, A.J., and Xiong, Y. (1999) ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol Cell* **3**: 535-541.

Ohta, T., and Xiong, Y. (2001) Phosphorylation- and Skp1-independent in vitro ubiquitination of E2F1 by multiple ROC-cullin ligases. *Cancer Res* **61**: 1347-1353.

Oliphant, A.R., Brandl, C.J., and Struhl, K. (1989) Defining the sequence specificity of DNA-binding proteins by selecting binding sites from random-sequence oligonucleotides: analysis of yeast GCN4 protein. *Mol Cell Biol* **9**: 2944-2949.

Oron, E., Mannervik, M., Rencus, S., Harari-Steinberg, O., Neuman-Silberberg, S., Segal, D., and Chamovitz, D.A. (2002) COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in *Drosophila melanogaster*. *Development* **129**: 4399-4409.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E.A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2000) Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. *EMBO J* **19**: 3475-3484.

Pan, Z.Q., Kentsis, A., Dias, D.C., Yamoah, K., and Wu, K. (2004) Nedd8 on cullin: building an expressway to protein destruction. *Oncogene* **23**: 1985-1997.

Peng, Z., Serino, G., and Deng, X.W. (2001a) Molecular characterization of subunit 6 of the COP9 signalosome and its role in multifaceted developmental processes in *Arabidopsis*. *Plant Cell* **13**: 2393-2407.

Peng, Z., Serino, G., and Deng, X.W. (2001b) A role of *Arabidopsis* COP9 signalosome in multifaceted developmental processes revealed by the characterization of its subunit 3. *Development* **128**: 4277-4288.

Peng, Z., Staub, J.M., Serino, G., Kwok, S.F., Kurepa, J., Bruce, B.D., Vierstra, R.D., Wei, N., and Deng, X.W. (2001c) The cellular level of PR500, a protein complex related to the 19S regulatory particle of the proteasome, is regulated in response to stresses in plants. *Mol Biol Cell* **12**: 383-392.

Peng, Z., Shen, Y., Feng, S., Wang, X., Chitteti, B.N., Vierstra, R.D., and Deng, X.W. (2003) Evidence for a physical association of the COP9 signalosome, the proteasome, and specific SCF E3 ligases in vivo. *Curr Biol* **13**: R504-505.

Piotrowska, M., Kruszewska, A., and Paszewski, A. (1980) Effect of regulatory mutations of sulphur metabolism on the levels of cysteine- and homocysteine-synthesizing enzymes in *Neurospora crassa*. *Acta Biochim Pol* **27**: 395-403.

Potocki, L., Chen, K.S., and Lupski, J.R. (1999) Subunit 3 of the COP9 signal transduction complex is conserved from plants to humans and maps within the smith-magenis syndrome critical region in 17p11.2. *Genomics* **57**: 180-182.

Potocki, L., Glaze, D., Tan, D.X., Park, S.S., Kashork, C.D., Shaffer, L.G., Reiter, R.J., and Lupski, J.R. (2000) Circadian rhythm abnormalities of melatonin in Smith-Magenis syndrome. *J Med Genet* **37**: 428-433.

Pries, R., Bömeke, K., Irniger, S., Grundmann, O., and Braus, G.H. (2002) Amino acid-dependent Gcn4p stability regulation occurs exclusively in the yeast nucleus. *Eukaryot Cell* **1**: 663-672.

Pries, R., Bömeke, K., Draht, O., Künzler, M., and Braus, G.H. (2004) Nuclear import of yeast Gcn4p requires karyopherins Srp1p and Kap95p. *Mol Genet Genomics* **271**: 257-266.

Qiu, H., Dong, J., Hu, C., Francklyn, C.S., and Hinnebusch, A.G. (2001) The tRNA-binding moiety in GCN2 contains a dimerization domain that interacts with the kinase domain and is required for tRNA binding and kinase activation. *EMBO J* **20**: 1425-1438.

Qiu, H., Hu, C., Yoon, S., Natarajan, K., Swanson, M.J., and Hinnebusch, A.G. (2004) An array of coactivators is required for optimal recruitment of TATA binding protein and RNA polymerase II by promoter-bound Gcn4p. *Mol Cell Biol* **24**: 4104-4117.

Quinn, J., Fyrberg, A.M., Ganster, R.W., Schmidt, M.C., and Peterson, C.L. (1996) DNA-binding properties of the yeast SWI/SNF complex. *Nature* **379**: 844-847.

Rechsteiner, M., and Rogers, S.W. (1996) PEST sequences and regulation by proteolysis. *Trends Biochem Sci* **21**: 267-271.

Roberts, S.M., and Winston, F. (1997) Essential functional interactions of SAGA, a *Saccharomyces cerevisiae* complex of Spt, Ada, and Gcn5 proteins, with the Snf/Swi and Srb/mediator complexes. *Genetics* **147**: 451-465.

Rolfes, R.J., and Hinnebusch, A.G. (1993) Translation of the yeast transcriptional activator GCN4 is stimulated by purine limitation: implications for activation of the protein kinase GCN2. *Mol Cell Biol* **13**: 5099-5111.

Rotenberg, S.A., and Sun, X.G. (1998) Photoinduced inactivation of protein kinase C by dequalinium identifies the RACK-1-binding domain as a recognition site. *J Biol Chem* **273**: 2390-2395.

Sachs, M.S. (1996) General and cross-pathway control of amino acid biosynthesis. In *The Mykota*. Esser, K. and Lemke, P.A. (eds). Berlin, Heidelberg, New York: Springer-Verlag, pp. 315-345.

Sattlegger, E., and Hinnebusch, A.G. (2000) Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells. *EMBO J* **19**: 6622-6633.

Scheel, H., and Hofmann, K. (2005) Prediction of a common structural scaffold for proteasome lid, COP9-signalosome and eIF3 complexes. *BMC Bioinformatics* **6**: 71.

Schwechheimer, C., and Deng, X.W. (2001) COP9 signalosome revisited: a novel mediator of protein degradation. *Trends Cell Biol* **11**: 420-426.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., and Deng, X.W. (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. *Science* **292**: 1379-1382.

Schwechheimer, C., Serino, G., and Deng, X.W. (2002) Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. *Plant Cell* **14**: 2553-2563.

Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (1998) A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. *Faseb J* **12**: 469-478.

Seeger, M., Gordon, C., and Dubiel, W. (2001) Protein stability: the COP9 signalosome gets in on the act. *Curr Biol* **11**: R643-646.

Serino, G., Tsuge, T., Kwok, S., Matsui, M., Wei, N., and Deng, X.W. (1999) *Arabidopsis* cop8 and fus4 mutations define the same gene that encodes subunit 4 of the COP9 signalosome. *Plant Cell* **11**: 1967-1980.

Serino, G., and Deng, X.W. (2003) THE COP9 SIGNALOSOME: Regulating Plant Development Through the Control of Proteolysis. *Annu Rev Plant Physiol Plant Mol Biol* **54**: 165-182.

Serino, G., Su, H., Peng, Z., Tsuge, T., Wei, N., Gu, H., and Deng, X.W. (2003) Characterization of the last subunit of the *Arabidopsis* COP9 signalosome: implications for the overall structure and origin of the complex. *Plant Cell* **15**: 719-731.

Serlupi-Crescenzi, O., Kurtz, M.B., and Champe, S.P. (1983) Developmental defects resulting from arginine auxotrophy in *Aspergillus nidulans*. *J Gen Microbiol* **129**: 3535-3544.

Shalev, A., Valasek, L., Pise-Masison, C.A., Radonovich, M., Phan, L., Clayton, J., He, H., Brady, J.N., Hinnebusch, A.G., and Asano, K. (2001) *Saccharomyces cerevisiae* protein Pci8p and human protein eIF3e/Int-6 interact with the eIF3 core complex by binding to cognate eIF3b subunits. *J Biol Chem* **276**: 34948-34957.

Sharpless, N.E., and DePinho, R.A. (2002) p53: good cop/bad cop. *Cell* **110**: 9-12.

Shaw, B.R., Herman, T.M., Kovacic, R.T., Beaudreau, G.S., and Van Holde, K.E. (1976) Analysis of subunit organization in chicken erythrocyte chromatin. *Proc Natl Acad Sci U S A* **73**: 505-509.

Shemer, R., Meimoun, A., Holtzman, T., and Kornitzer, D. (2002) Regulation of the transcription factor Gcn4 by Pho85 cyclin PCL5. *Mol Cell Biol* **22**: 5395-5404.

Sherr, C.J., and Roberts, J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13**: 1501-1512.

Slingerland, J., and Pagano, M. (2000) Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* **183**: 10-17.

Smith, P., Leung-Chiu, W.M., Montgomery, R., Orsborn, A., Kuznicki, K., Gressman-Coberly, E., Mutapcic, L., and Bennett, K. (2002) The GLH proteins, Caenorhabditis elegans P granule components, associate with CSN-5 and KGB-1, proteins necessary for fertility, and with ZYX-1, a predicted cytoskeletal protein. *Dev Biol* **251**: 333-347.

Suh, G.S., Poeck, B., Chouard, T., Oron, E., Segal, D., Chamovitz, D.A., and Zipursky, S.L. (2002) *Drosophila* JAB1/CSN5 acts in photoreceptor cells to induce glial cells. *Neuron* **33**: 35-46.

Sun, Y., Wilson, M.P., and Majerus, P.W. (2002) Inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome by binding to CSN1. *J Biol Chem* **277**: 45759-45764.

Takemaru, K., Harashima, S., Ueda, H., and Hirose, S. (1998) Yeast coactivator MBF1 mediates GCN4-dependent transcriptional activation. *Mol Cell Biol* **18**: 4971-4976.

Tang, Z., Li, B., Bharadwaj, R., Zhu, H., Ozkan, E., Hakala, K., Deisenhofer, J., and Yu, H. (2001) APC2 Cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex. *Mol Biol Cell* **12**: 3839-3851.

Tateishi, K., Omata, M., Tanaka, K., and Chiba, T. (2001) The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. *J Cell Biol* **155**: 571-579.

Timmers, H.T., and Tora, L. (2005) SAGA unveiled. *Trends Biochem Sci* **30**: 7-10.

Tomoda, K., Kubota, Y., Arata, Y., Mori, S., Maeda, M., Tanaka, T., Yoshida, M., Yoneda-Kato, N., and Kato, J.Y. (2002) The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex. *J Biol Chem* **277**: 2302-2310.

Tomoda, K., Yoneda-Kato, N., Fukumoto, A., Yamanaka, S., and Kato, J.Y. (2004) Multiple functions of Jab1 are required for early embryonic development and growth potential in mice. *J Biol Chem* **279**: 43013-43018.

Tsuge, T., Matsui, M., and Wei, N. (2001) The subunit 1 of the COP9 signalosome suppresses gene expression through its N-terminal domain and incorporates into the complex through the PCI domain. *J Mol Biol* **305**: 1-9.

Turner, R., and Tjian, R. (1989) Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. *Science* **243**: 1689-1694.

Tyers, M., and Jorgensen, P. (2000) Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr Opin Genet Dev* **10**: 54-64.

Uhle, S., Medalia, O., Waldron, R., Dumdey, R., Henklein, P., Bech-Otschir, D., Huang, X., Berse, M., Sperling, J., Schade, R., and Dubiel, W. (2003) Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. *EMBO J* **22**: 1302-1312.

Valasek, L., Phan, L., Schoenfeld, L.W., Valaskova, V., and Hinnebusch, A.G. (2001) Related eIF3 subunits TIF32 and HCR1 interact with an RNA recognition motif in PRT1 required for eIF3 integrity and ribosome binding. *EMBO J* **20**: 891-904.

Valenzuela, L., Aranda, C., and Gonzalez, A. (2001) TOR modulates GCN4-dependent expression of genes turned on by nitrogen limitation. *J Bacteriol* **183**: 2331-2334.

Valerius, O., Draht, O., Kübler, E., Adler, K., Hoffmann, B., and Braus, G.H. (2001) Regulation of *hisHF* transcription of *Aspergillus nidulans* by adenine and amino acid limitation. *Fungal Genet Biol* **32**: 21-31.

Verger, A., and Crossley, M. (2004) Chromatin modifiers in transcription and DNA repair. *Cell Mol Life Sci* **61**: 2154-2162.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R., 3rd, Koonin, E.V., and Deshaies, R.J. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298**: 611-615.

Wang, X., Kang, D., Feng, S., Serino, G., Schwechheimer, C., and Wei, N. (2002) CSN1 N-terminal-dependent activity is required for *Arabidopsis* development but not for Rub1/Nedd8 deconjugation of cullins: a structure-function study of CSN1 subunit of COP9 signalosome. *Mol Biol Cell* **13**: 646-655.

Wang, X., Feng, S., Nakayama, N., Crosby, W.L., Irish, V., Deng, X.W., and Wei, N. (2003) The COP9 signalosome interacts with SCF UFO and participates in *Arabidopsis* flower development. *Plant Cell* **15**: 1071-1082.

Wang, Y., Penfold, S., Tang, X., Hattori, N., Riley, P., Harper, J.W., Cross, J.C., and Tyers, M. (1999) Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. *Curr Biol* **9**: 1191-1194.

Wee, S., Hetfeld, B., Dubiel, W., and Wolf, D.A. (2002) Conservation of the COP9/signalosome in budding yeast. *BMC Genet* **3**: 15.

Wee, S., Geyer, R.K., Toda, T., and Wolf, D.A. (2005) CSN facilitates Cullin-RING ubiquitin ligase function by counteracting autocatalytic adapter instability. *Nat Cell Biol* **7**: 387-391.

Wei, N., and Deng, X.W. (1992) COP9: a new genetic locus involved in light-regulated development and gene expression in *Arabidopsis*. *Plant Cell* **4**: 1507-1518.

Wei, N., Chamovitz, D.A., and Deng, X.W. (1994) *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. *Cell* **78**: 117-124.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M., and Deng, X.W. (1998) The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. *Curr Biol* **8**: 919-922.

Wei, N., and Deng, X.W. (2003) The COP9 signalosome. *Annu Rev Cell Dev Biol* **19**: 261-286.

Wek, S.A., Zhu, S., and Wek, R.C. (1995) The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. *Mol Cell Biol* **15**: 4497-4506.

Wilson, M.P., Sun, Y., Cao, L., and Majerus, P.W. (2001) Inositol 1,3,4-trisphosphate 5/6-kinase is a protein kinase that phosphorylates the transcription factors c-Jun and ATF-2. *J Biol Chem* **276**: 40998-41004.

Wolf, D.A., Zhou, C., and Wee, S. (2003) The COP9 signalosome: an assembly and maintenance platform for cullin ubiquitin ligases? *Nat Cell Biol* **5**: 1029-1033.

Yahalom, A., Kim, T.H., Winter, E., Karniol, B., von Arnim, A.G., and Chamovitz, D.A. (2001) *Arabidopsis* eIF3e (INT-6) associates with both eIF3c and the COP9 signalosome subunit CSN7. *J Biol Chem* **276**: 334-340.

Yan, J., Walz, K., Nakamura, H., Carattini-Rivera, S., Zhao, Q., Vogel, H., Wei, N., Justice, M.J., Bradley, A., and Lupski, J.R. (2003) COP9 signalosome subunit 3 is essential for maintenance of cell proliferation in the mouse embryonic epiblast. *Mol Cell Biol* **23**: 6798-6808.

Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Di, X., Wang, X., Rodriguez-Suarez, R.J., Zhang, H., and Wei, N. (2002) The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via deneddylation of SCF Cul1. *Curr Biol* **12**: 667-672.

Yao, T., and Cohen, R.E. (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* **419**: 403-407.

Yelton, M.M., Hamer, J.E., de Souza, E.R., Mullaney, E.J., and Timberlake, W.E. (1983) Developmental regulation of the *Aspergillus nidulans trpC* gene. *Proc Natl Acad Sci U S A* **80**: 7576-7580.

Zheng, J., Yang, X., Harrell, J.M., Ryzhikov, S., Shim, E.H., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (2002) CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. *Mol Cell* **10**: 1519-1526.

Zhou, C., Seibert, V., Geyer, R., Rhee, E., Lyapina, S., Cope, G., Deshaies, R.J., and Wolf, D.A. (2001) The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. *BMC Biochem* **2**: 7.

Zhou, C., Wee, S., Rhee, E., Naumann, M., Dubiel, W., and Wolf, D.A. (2003) Fission yeast COP9/signalosome suppresses cullin activity through recruitment of the deubiquitylating enzyme Ubp12p. *Mol Cell* **11**: 927-938.

Zhu, S., Sobolev, A.Y., and Wek, R.C. (1996) Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate in vitro phosphorylation of eIF-2. *J Biol Chem* **271**: 24989-24994.

Chapter 2

Aspergillus nidulans sexual development is dependent on COP9 deneddylation activity

2.1 Abstract

The COP9 signalosome (CSN) is a multifunctional protein complex essential for the development of many eukaryotic organisms. Besides its regulatory activity on SCF ubiquitination activity and some associated kinase activities little is known about single subunit functions. Here we present the characterization of the first and largest subunit of the COP9 signalosome, CsnA, in *A. nidulans*. A *csnA* deletion mutant displays a similar phenotype as described earlier for *csnD* and *csnE* deletions – the abundance of formation of mature sexual fruitbodies and red pigmentation of the mycelium on solid medium (Busch *et al.*, 2003). We were able to show that the mutant phenotypes of red pigmentation and inability to form cleistothecia are linked. Furthermore by expressing truncated CsnA proteins we were able to show that the N-terminal and the PCI domain of the protein have seperatable functions in the cells, the C-terminal part of the protein is essential for COP9 subunit interaction and sexual development in *A. nidulans* and the N-terminus for regulation of hyphal growth. Sexual fruitbody formation is directly or indirectly effected by a loss of deneddylation activity of the COP9 signalosome.

2.2 Introduction

The COP9 signalosome is a conserved protein complex found in eukaryotes, though it could be shown, that not in every eukaryote all eight subunits are present. The COP9 signalosome was found in *Arabidopsis thaliana* as a regulator of light response (Wei *et al.*, 1994). The mammalian COP9 complex was originally isolated as a co-purifying byproduct of the 26S proteasome (Seeger *et al.*, 1998). The complete isolation of subnunits of the COP9 complex from animals and plants revealed the evolutionary conservation of the protein complex. The COP9 signalosome resembles the regulatory 19S subunit of the proteasome and the eIF3. The complex of higher eukaryotes consists of eight subunits CSN1 to CSN8 with decreasing molecular weight (Deng *et al.*, 2000). Whereas *S. pombe* was only known to show six of the eight subunits and *S. cerevisiae* only a COP9 like complex with CSN5 being the only highly similar protein amongst the potentially identified subunits (Mundt *et al.*, 1999; Mundt *et al.*, 2002; Wee *et al.*, 2002; Zhou *et al.*, 2001). Six of the subunits form the core complex and harbor so-called PCI domains, which are found in the proteasome, CSN and eIF3, in their C-termini. Two subunits harbor so-called MPN domains (Mpr1p/Pad1 N-terminal) (Aravind and Ponting, 1998; Hofmann and Bucher, 1998; Ponting *et al.*, 1999). In general the three complexes are referred to as PCI complexes.

The best studied intrinsic function of the COP9 signalosome is its deneddylation activity. Rub1 or Nedd8 is a small ubiquitin-like protein which is used by the cell to control the ubiquitin-dependent degradation of proteins through the SCF complex (Lyapina *et al.*, 2001). Similar to ubiquitin ligation the conjugation of Nedd8 requires the Nedd8 activating enzymes APP-BP1 and Uba3 (E1) and the conjugating enzyme Ubc12 (E2) (Amir *et al.*, 2002). The neddylation pathway is essential in *S. pombe*, *C. elegans* and mice and plays a significant role in plant auxin responses (del Pozo *et al.*, 2002; Osaka *et al.*, 2000; Tateishi *et al.*, 2001). The SCF represents a E3 type ubiquitin ligase that catalyzes the third step in the substrate specific ubiquitin conjugation of target proteins. The COP9 signalosome complex binds to the SCF complex through CSN2, CSN6 and CSN1´s N-terminus and deconjugates Nedd8 from the cullin subunit of the SCF through a metalloprotease activity assigned to CSN5 (Lyapina *et al.*, 2001; Schwechheimer *et al.*, 2001; Wang *et al.*, 2002; Yang *et al.*, 2002). CSN5 contains a metalloprotease motif called a JAMM (Jab1/MPN domain-associated metalloisopeptdase) (Ambroggio *et al.*, 2004). The highly dynamic neddylation/deneddylation status of the cullin

regulates the activity of ubiquitin conjugation to target proteins of the SCF complex (Yang *et al.*, 2002; Zheng *et al.*, 2002).

Recent investigations showed a common structural scaffold for PCI domains, which display relatively high interspecies divergence and vary highly in their degree in conservation between single PCI domain proteins (Scheel and Hofmann, 2005). Especially the N-terminal boundary of PCI domains is often difficult to determine. *In silico* investigations revealed that a PCI domain actually consists of two subdomains. The C-terminal half-domain is a globular α/β structure with a "αβααββ" arrangement and can be classified as a winged helix fold or WH domain. The N-terminal half of the PCI domain is entirely helical and consists of three antiparallel hairpins that connect six regularly spaced helices. This half-domain resembles in a superhelix which is also found in HEAT and Armadillo (ARM) repeats and is thus called a HAM domain. ARM and HEAT motifs are tandemly repeated sequences of approximately 50 amino acid residues that occur in a wide variety of eukaryotic proteins (Andrade *et al.*, 2001). In some PCI proteins a TPR-like repeat can be found to extend the HAM domain towards the N-terminus of the protein (Scheel and Hofmann, 2005).

The largest CSN subunit CSN1 has an essential role in complex assembly. A complete loss of this subunit in *A. thaliana* leads to accumulation of CSN8 and a decrease in CSN4 and CSN7 protein level, furtheron the fifth subunit dissociates from the complex, resulting in the inability to deneddylate its substrates (Wang *et al.*, 2002). The N-terminal part of CSN1 is insufficient to incorporate into the complex, whereas the central domain and the C-terminus which harbors the PCI domain is sufficient to incorporate (Tsuge *et al.*, 2001; Wang *et al.*, 2002). The restoration of the complex by complementing the *fus6* mutant in *A. thaliana* with either a truncated protein harbouring parts of the PCI domain or the full PCI domain restores the Rub1 deconjugation or deneddylation activity of the complex (Wang *et al.*, 2002). Recent studies revealed that one of at least three associated kinase activities directly interacts with CSN1 (Sun *et al.*, 2002). The kinase is a inositol 1,3,4-triphosphate 5/6 kinase with a relatively wide substrate spectrum. It is able to phosphorylate c-Jun, IkBa, ATF2 and p53 and is inhibited by curcumin. This leads to the hypothesis that CNS regulates p53 in a highly subtle manner. CSN-mediated phosphorylation leads to p53 degradation in the 26S proteasome but CSN-mediated deneddylation may stabilize c-Jun and thus indirectly activate AP-1 (Sun *et al.*, 2002; Wilson *et al.*, 2001).

For the *A. nidulans* genome we could show that all eight subunits of the COP9 signalosome are represented by homologous genes (unpublished results). Deletion of the fourth and fifth subunit *csnD* and *csnE* lead to pleiotrophic phenotypes in *A. nidulans*. Fungal strains deleted

for either of the subunit encoding genes are in contrast to higher eukaryotes, which are embryonal lethal, viable but produce shortened cells harboring a so far unknown red pigment after reaching developmental competence. Additionally, *csn* deletions result in a block in sexual fruitbody formation at the stage of primordia (Busch, *et. al*., 2003).

In this work we focus on the first and largest subunit of the COP9 signalosome, CsnA, in *A. nidulans* development. CsnA corresponds to CSN1 and follows the gene/protein nomenclature of *A. nidulans* proposed by the *Aspergillus* genome annotation consortium (Galagan *et al.*, (submitted)). We analyzed the phenotypes generated by a loss of this single subunit and hence whole complex functions and parts of it in *A. nidulans* development.

2.3 Material and methods

2.3.1 Strains, media and growth conditions

Strains of *A.nidulans* were cultivated at 37°C with minimal medium supplemented as described earlier (Bennett and Lasure, 1991; Käfer, 1977). Developmental cultures were synchronized by obtaining vegetative mycelium from submerged liquid culture after 18 hours to reach sexual competence and then transfer to solid medium. Sexual development was induced by cultivating strains in the dark under oxygen limitation, asexual development was induced by continous white light. Developmental cultures were synchronized by cultivating them in liquid culture for 18 hours to reach sexual competence and then transfer to solid medium. Expression from the a*lcA* promoter was induced by adding 2% ethanol and 2% glycerol instead of glucose to the medium (Felenbok, 1991). In *csnA* deletion strain AGB223 the *csnA* gene was replaced by a *pyrG* cassette with flanking zeocin-resistance repeats. Counterselecting against the *pyrG* markergene the marker was rescued due to mitotic recombination (Krappmann and Braus, 2003). Microscopy was performed by differential interface contrast (DIC).

2.3.2 Molecular methods

E. coli (Inoue *et al.*, 1990) and *A. nidulans* (Eckert *et al.*, 2000) transformations were carried out as described. Genomic DNAs of *A. nidulans* were isolated as described by Lee and Taylor, 1990. Standard techniques were applied for Southern analysis (Rave *et al.*, 1979; Southern, 1975). DNAs for probes were obtained by digesting pME2937 with *Bam*HI producing an approximately 1.5kb fragment. DNA sequencing was carried out with an ABI310 genetic analyzer. Further sequence analysis was carried out with the Lasergene software from DNASTAR (DNASTAR, Madison, WI.). The nucleotide sequence of *csnA* was deposited in the GenBank database under accession Number AY574249.

2.3.3 Isolation of genomic and cDNA of *A. nidulans*

The *csnA* gene was isolated by colony hybridisation of a genomic *Sal*I sublibrary of *A. nidulans* and subcloned into pBluescript SK(+) (Stratagene) revealing plasmid pME2499. For colony hybridisation (Moseley *et al.*, 1980) a probe obtained with primers SB104 and SB105 was used producing a 750bp DNA fragment. cDNA was obtained by colony hybridisation of a cDNA library and revealed plasmid pME2498. For the Southern experiment (Southern, 1975) revealing which chromosome carries the *csnA* gene the BAC library of *A. nidulans* obtained from Clemson University was used.

2.3.4 Construction of plasmids for *A. nidulans* manipulation

Deletion constructs were obtained according to Krappman *et al.*, 2002. The 5´-flanking region was amplified by primers Elke19 5`-ATC CCC GAA TAT TCC ACG CTG-3` and Elke20 5`-CGT CTA TGC TGG ACA GCT CG-3`. The 3´-flanking region was obtained by PCR with primers *csnA*-KO3 5`-CCG TCG GAT ACT ACG TTT GGC-3` and *csnA*-KO4 5`-AAC GAT CTC TCC ACC GGG ATG-3`. The 5´-region and the 3´-region were integrated into pME2409 and *in vivo* recombination was carried out with the excised deletion cassette and pME2499 producing the deletion construct pME2505.
The constructs for paritial complementation of the *csnA* deletion mutant were obtained by linearizing plasmid pME1565 with *Sma*I and inserting the truncated versions of the *csnA* ORF into the vector. The truncated versions of the ORF were produced by amplification with KOD proofreading polymerase using the following primer pairs ODOL-csnA1 5´-AAA GGT ACC ATG GAG CCC ATG TTA CCA GAAGCG-3´and ODOL-csnA2 5´-AAA CCC GGG TTA TTG CTT CAT CCC CGT CGC CC –3´to produce the full-length ORF resulting in pME2937, ODOL-csnA1 and ODOL-csnA3 5´- AAA CCC GGG TTA CAT GGA GGC TAG GGC ACA GAG –3´to amplify a truncated ORF encoding only amino acids 1 to 270 producing pME2934, ODOL-csnA1 and ODOL-csnA4 5´- AAA CCC GGG TTA TTG TTC TTC CGG CTT CCC CCC –3´ to amplify a truncated ORF encoding for amino acids 1 to 228 producing pME2936 and ODOL-csnA5 5´- AAA GGT AAC ATG CAA GCG AAA CAC CAG CCT AAG –3´ and ODOL-csn2 to amplify a truncated ORF encoding for amino acids 229 to the UAA STOP codon to produce pME2937. START and STOP codons have been integrated into the primers where needed.

2.4 Results

2.4.1 The *A. nidulans csnA* gene encodes a PCI domain protein similar to subunit I of the COP9 signalosome

The COP9 signalosome (CSN) is a multiprotein complex found in higher eukaryotes (Wei and Deng, 2003). Two subunits of the *A. nidulans* CSN have been characterized and deleted in *A. nidulans* (Busch *et al.*, 2003). Deletions of the genes for subunit 4 (*csnD*) and 5 (*csnE*) lead to multiple pleiotropic phenotypes. A third subunit of the proposed AnCSN, *csnA*, was found in the CEREON database (http://microbial.cereon.com) by comparison to the genes for CSN1 subunits of other eukaryotes. According to the unified COP9 signalosome nomenclature the gene was named *csnA*, though another gene of this name was already described to be a chitosanase by Rodriguez *et al*., 2004 (Accession number AAR85471). A 750bp fragment of genomic *A. nidulans* DNA was amplified and used as probe to isolate a cosmid from the ordered cosmid library of *A. nidulans* and a cDNA from a cDNA library. Southern hybridisations with genomic *A. nidulans* DNA revealed a single copy of the *csnA* gene on chromosome 7. The 750bp PCR fragment served to produce a genomic 8kb *Sal*I fragment containing an open reading frame (ORF) of 1497bp (Figure 5). A corresponding cDNA of 1923bp was isolated from a cDNA library containing a 298bp 5´ flanking region and a 128bp 3´ flanking region. Comparisons between the isolated cDNA and the genomic DNA revealed that the open reading frame is disrupted by three introns of 48bp, 59bp and 52bp respectively at positions +142, +235 and +576 relative to the A of the START codon set at position +1 (Figure 5). The deduced peptide sequence had a length of 498 amino acids and a calculated protein mass of 55,7kDa. The peptide sequence contains a putative PCI domain (proteasome, COP9, eIF) and displayed identities up to 36,2% (calculated over the full protein) to various other proteins described as first subunits of the COP9 signalosome and even up to 44,4% if only the respective PCI domains were compared (Figure 6). Notably the sequence identities to higher eukaryotes were higher than those to its fungal relative *S. pombe*.

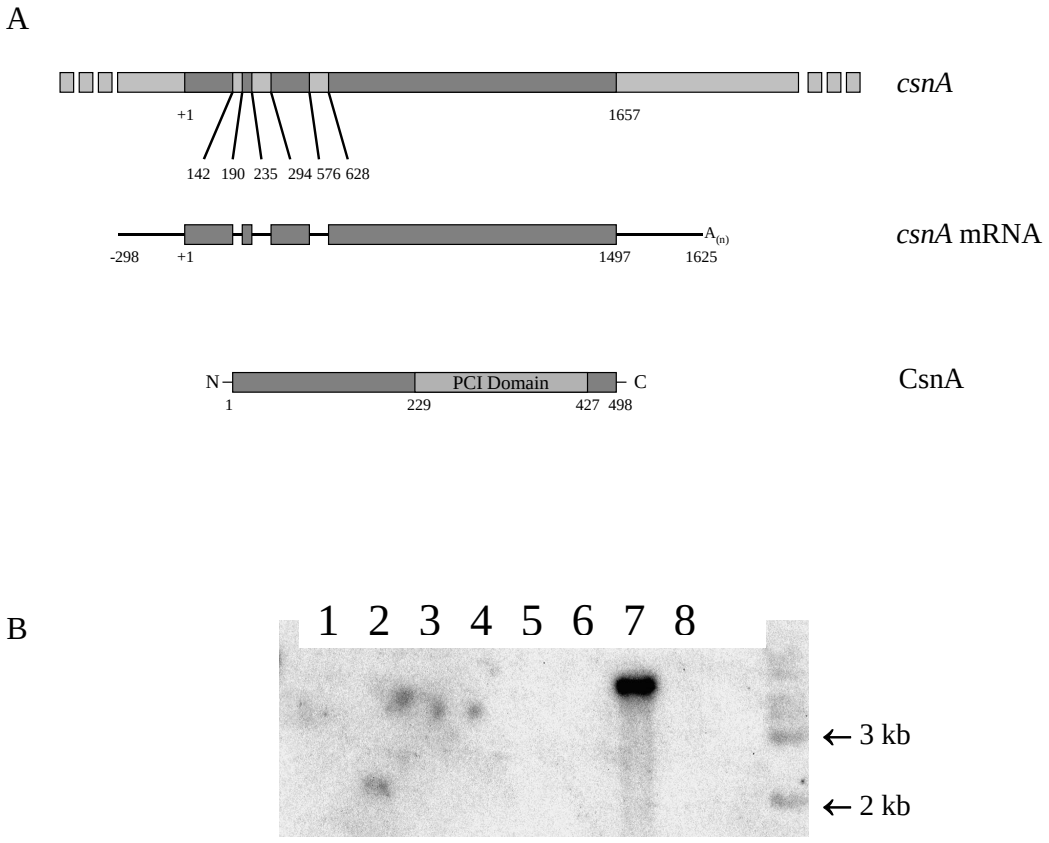


Fig. 5: The *csnA* gene of *A. nidulans* is located on chromosome 7
(A) The *csnA* gene locus (top), the resulting gene products (middle) and protein (bottom) are schematically displayed. The open reading frame starts at the A of the AUG start codon as position +1 and consists of 1497 bps. The ORF is interrupted by three introns at positions 142-190, 235-294 and 576-629 respectively. The isolated cDNA is shown as white bar and includes a 5´ and 3´ untranslated region of 298 bp and 128 bp respectively. The deduced CsnA protein consists of 498 amino acids with a calculated mass of 55,6 kDa. The predicted PCI domain presumably necessary for protein-protein interaction is shown in grey.
(B) Southern hybridisations with genomic *A. nidulans* DNA revealed a single copy of the *csnA* gene on chromosome 7.

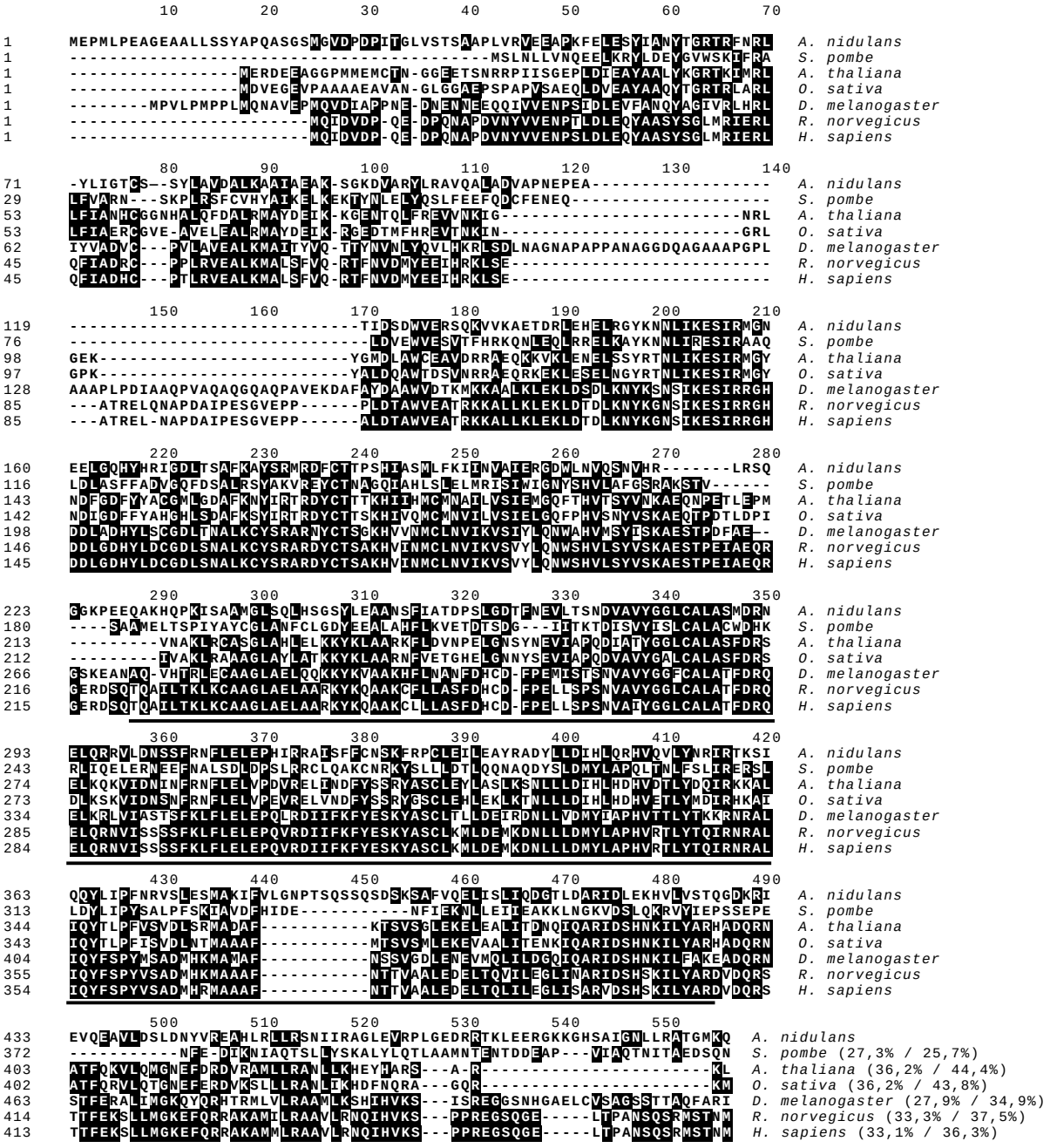


Fig. 6: CsnA of *A. nidulans* shows high amino acid identities to Csn1 proteins of higher eukaryotes
The multialignment of deduced amino acid sequences of CsnA (AY574249) to the corresponding sequences of *S. pombe* (O94308), *A. thaliana* (P45432), *O. sativa* (AAF40112), *D. melanogaster* (AAD28605), *R. norvegicus* (P97834) and *H. sapiens* (Q13098) is shown. Residues identical for all species are highlighted. The predicted conserved PCI domain presumably needed for interaction with other CSN subunits is underlined. Amino acid identities between CsnA and the Csn1 subunits of other organisms are displayed in brackets in the bottom row of the alignment. The first and second percentage numbers represent the identities of the full length protein, and the PCI domain respectively.

2.4.2 *Aspergillus nidulans* strains deleted for *csnA* are blocked in sexual development and produce altered secondary metabolites

In *A. nidulans* strain AGB152 (a derivative of wild type strain A4 with the relevant genotype *pyrG*89) a *csnA* deletion was introduced, resulting in strain AGB223 (Δ*csnA*/*csnA*::(*zeoR:pyrG:zeo*)) to compare the phenotype with Δ*csnD* and Δ*csnE* deletion strains which are blocked in sexual development (Busch *et al.*, 2003). An overview of all *A. nidulans* strains with their COP9 related genotypes and associated phenotypes is presented in Figure 7. The *csnA* deletion strain AGB223 ($pyrG^{+}$) was treated with 5-fluorotic acid (5-FOA) to recycle the *pyrG*-marker of the deletion cassette (Krappmann and Braus, 2003) for further experiments resulting in strain AGB234 ($pyrG^{-}$). *A. nidulans* strain AGB234 shows a similar phenotype to AGB223 on minimal medium containing uridine (Figure 8). When grown in liquid selective minimal medium no distinction from the wild type could be made regarding the growth behaviour. However, when grown on solid minimal medium for 48 hours, phenotypic anomalities become apparent. The Δ*csnA* strain shows reduced cell size and red colouring (Figure 8). Equal to the other *csn* deletions of *A. nidulans*, the hyphae penetrate the agar and show a red pigmentation which is not observable in wild type strains. Sexual development in the Δ*csnA* strain is arrested at the state of Hülle cells and primordia, but does not progress to the formation of fertile, mature sexual fruitbodies (see Figure 7). The growth behaviour of strain AGB223 and AGB234 respectively changes over time compared to wildtype strain AGB152. After approximately 10 passages of conidia harvest and growing of the newly harvested conidia on solid medium the strains continually produce less conidia and stop producing sexual structures at all (see Figure 8).

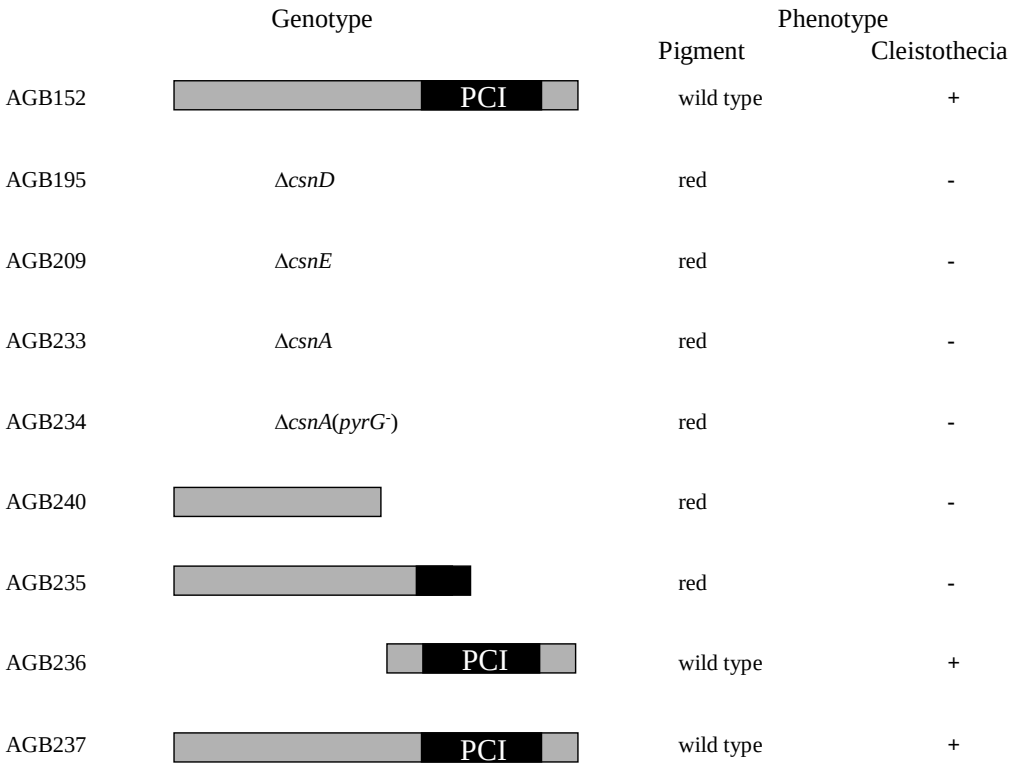


Fig. 7: Nomenclature and associated phenotypes of the investigated *A. nidulans* strains impaired in the COP9 signalosome in comparison to wildtype

This figure gives an overview of the investigated strains in this study and summarizes the phenotypes. Pigmentation of the hyphae is indicated as red for red pigmentation and as wild type for wild type pigmentation. The ability to form cleistothecia is indicated by plus, if the strains are not able to form fruitbodies, this is indicated by minus.

2.4.3 Expression of truncated CsnA peptides can restore wild type secondary metabolism and sexual development in *A. nidulans csnA* deletion strains

Aspergillus nidulans strains that lack either the CSN subunits CsnA, CsnD or CsnE exhibit pleiotropic mutant phenotypes of red pigmented hyphae during secondary metabolism and the disability to form fertile sexual fruitbodies. To assign a function to CsnA as part of the COP9 signalosome, we had to distinguish between phenotypes due to loss of assembly of the CSN complex caused by missing subunits and phenotypes that are caused by a loss of CsnA itself. To distinguish between these phenotypes and to investigate the role and function of CsnA in the multiprotein complex, truncated CsnA peptides were expressed in a *csnA* deletion strain. The truncated peptides were expressed from an *alcA* promoter (Felenbok, 1991) under

repressive conditions to simulate low expression of the gene product and to avoid titration of CsnA complexing subunits, though overexpression of CSN1 subunits was not found to produce any effect in higher eukaryotes (Wang *et al.*, 2002).

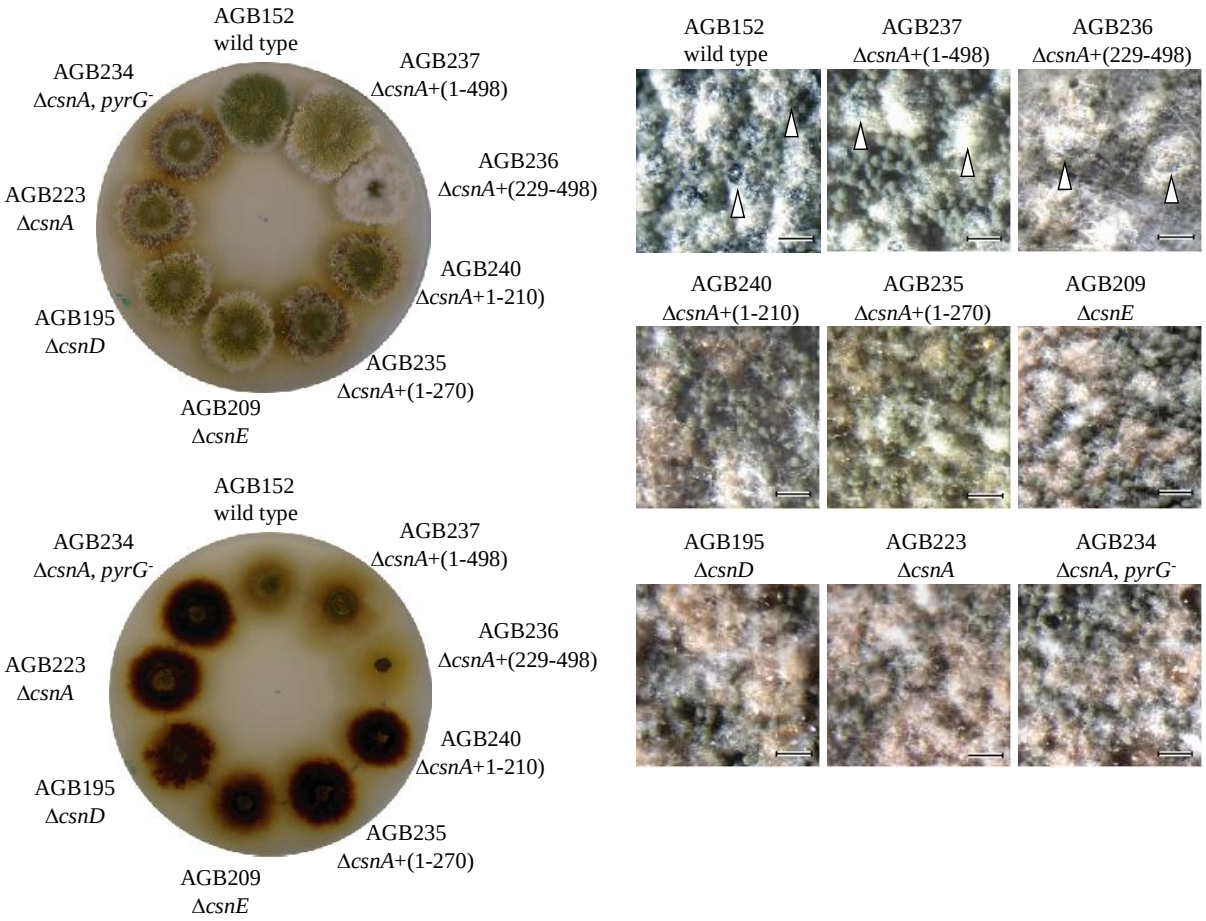


Fig. 8: Phenotypes of *A. nidulans csnA* mutant strains
Secondary metabolite and fruitbody (cleistothecia) formation of CSN deletion strains and partial complementations of *csnA* deletion mutant are shown. *A. nidulans* strains that lack CSN subunits exhibit the pleiotropic mutant phenotypes of red pigmented hyphae and the disablility to form fertile sexual fruitbodies after 96 hours of growth. *A. nidulans* strains AGB152 (wild-type), AGB223 (Δ*csnA*), AGB234 (Δ*csnA* with restored *pyrG*-marker), AGB195 (Δ*csnD*) and AGB209 (Δ*csnE*), were grown on solid air-medium interface to allow development as control strains for AGB235 (Δ*csnA* + (1-289)), AGB240 (Δ*csnA* + (1-228)), AGB236 (Δ*csnA* + (229-498)) and AGB237 (Δ*csnA* + (1-498)). The left hand pictures show the overall colony phenotype of colonies grown for 96 hours on solid medium (top) to allow development and production of secondary metabolites, in this case the red pigment of the hyphae which becomes clearly visible after thorough washing off of the non-agar-penetrating parts of the colonies (bottom). The right hand pictures show a magnified view of the same colonies indicating cleistothecia when present by a white arrow. The black bar indicates a relative length of 200µm. AGB223 and AGB243, the *csn* deletion strains, strain AGB235 and AGB240 display the above mentioned pleiotropic phenotypes. In the remaining strains the expression of parts of CsnA complements the pleiotropic phenotypes.

As control a strain was constructed that expresses the full length protein from the *alcA* promoter to show differences between the complementation strain and the wild type. *A. nidulans* strains AGB152 (wild type), AGB195 (Δ*csnD*), AGB209 (Δ*csnE*), AGB223 (Δ*csnA*) and AGB234 (AGB223 with restored pyrG-marker) were grown on solid air-medium interface to allow development in comparison to the strains AGB240 (Δ*csnA* + (1-228)), AGB235 (Δ*csnA* + (1-289)), AGB236 (Δ*csnA* + (229-498)) and AGB237 (Δ*csnA* + (1-498)) expressing truncated versions of CsnA. In Figure 8 the overall colony phenotype of the strains is displayed after 96 hours of growth, allowing sexual differentiation and induction of secondary metabolism.

To show the altered pigmentation of the mutant strains, the plates were thoroughly washed after incubation and excess mycelium was removed from the agar surface to depict pigmented hyphae which have penetrated the agar. The wild type forms mature cleistothecia and the underground of the colony is not colored after 96 hours of growth. The complementation strain AGB237 expressing the *csnA*-cDNA behind an *alcA* promoter in strain AGB234 restores all pleiotropic phenotypes typical for *csn* deletion mutants. The *csnA*, *csnD* and *csnE* deletion strains of *A. nidulans* are unable to form cleistothecia and stop sexual development at the stage of primordia. The underground of the colonies is reddish-brown due to pigmentation of the agar penetrating hyphae. In strain AGB240 only the N-terminus of CsnA is expressed lacking the central domain and the PCI domain of the protein. This strain displays the same phenotype as the *csnA* deletion strain AGB234. Strain AGB235 expresses the N-terminus and parts of the PCI domain of CsnA, its phenotype is similar to the investigated *csn* deletion strains. In the right column all investigated strains are displayed in a magnified view. In strain AGB236 the PCI domain of the protein and the central domain are expressed. The strain is able to form mature cleistothecia and lacks the typical red pigmentation of the deletion strain. The top of the colony is covered with a thick layer of aerial hyphe that covers the mass of conidia and nests with mature cleistothecia. These results suggest a correlation of a defect in sexual development and formation of red pigmented hyphae in *csn* mutant strains. On the other hand the PCI domain of CsnA alone is sufficient to restore the pleiotropic effects seen in a *csnA* null mutant.

2.4.4 COP9 mutants display a growth defect in vegetative mycelium on solid medium at 37°C

The radial growth of all investigated strains was measured at 30°C and 37°C and compared to the wild type to investigate the growth rate of the *csn* mutant strains and complemented strains. We could show that the growth rate of all investigated mutants is similar to the growth rate of the wild type strain at 30°C. Interestingly when grown at 37°C the growth rate of the wild type strain increased, whereas the growth rate of the mutant strains and of strain AGB237 expressing the full-length csnA-cDNA and AGB236 encoding the PCI domain of CsnA does not increase (Figure 9). We could show that the red hyphae phenotype and the acleistothecial phenotype are linked and can be complemented by CsnA in a csnA deletion strain. The results of the growth tests indicate that the exact amount of CsnA seems to be important to restore a wild type growth phenotype, since the expression of the *csnA*-cDNA from the *alcA* promoter does not complement the growth phenotype at 37°C but only the formation of cleistothecia and abolishes formation of red hyphae.

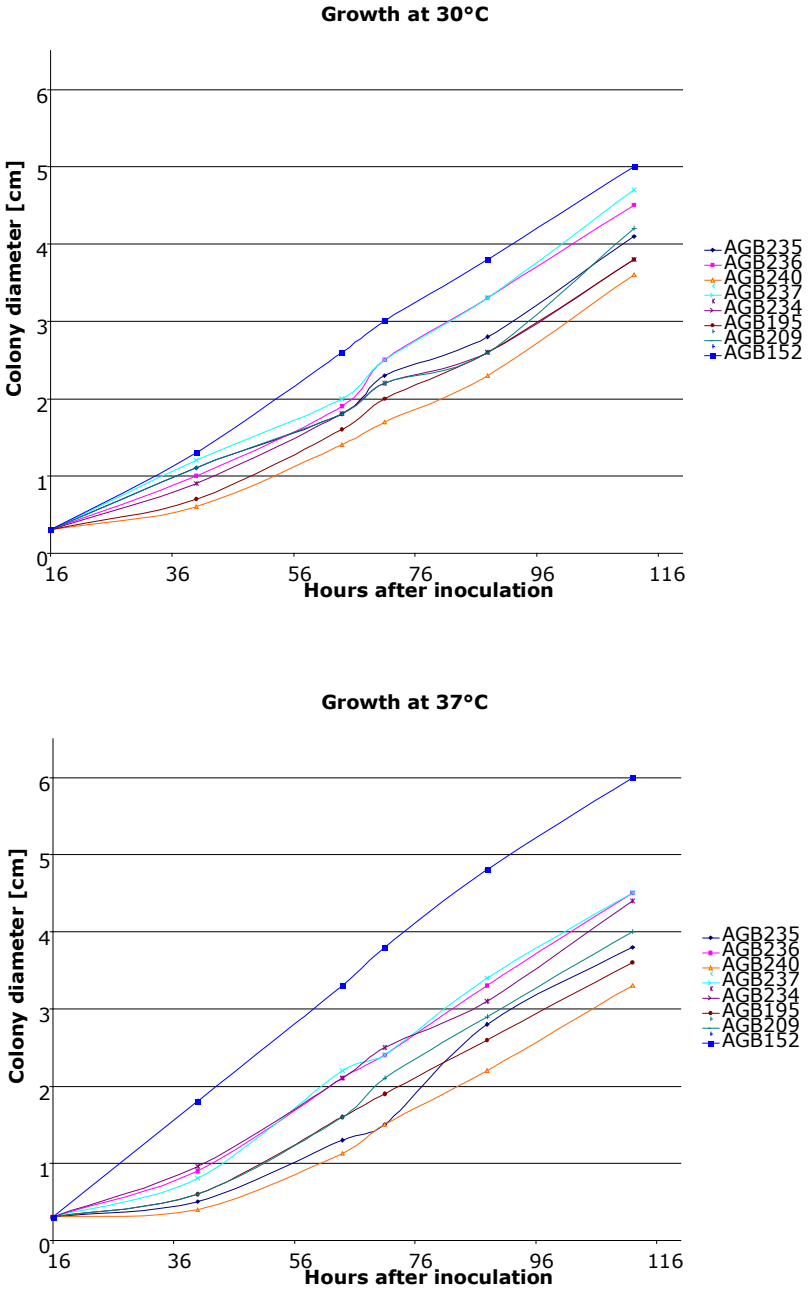


Fig. 9: Growth curves of the *A. nidulans* strains expressing full or truncated *csnA* genes at 30°C and 37°C

The colony diameter at given time points after inoculation was determined by measuring 3 independent colonies of the respective strains. The growth rate at 30°C and 37°C is shown. Whereas at 30°C all investigated strains grow with approximately the same growth rate at 37°C only the wild type strain AGB152 is able to increase its growth rate. The full and partially complemented strains, as well as the *csn* deletion strains, are unable to grow faster at 37°C.

2.4.5 Structural *in silico* analysis of the investigated truncated CsnA proteins of *A. nidulans*

In eukaryotic organisms at least three distinct multi-protein complexes exist, that are referred to as PCI complexes which display a similar subunit architecture despite their different function: the 26S proteasome lid, the eukaryotic translation initiation factor eIF3 and the COP9 signalosome. These three multi-protein complexes have subunits harboring PCI domains and MPN domains in common. The degree of conservation between PCI subunits is highly variable. Apart from the similarities between proteasome subunits and subunits of the COP9 signalosome, which are relatively easy to identify, the similarities between other paralogous PCI subunits are hard to detect by simple sequence comparison. A bioinformatic approach (Scheel and Hofmann, 2005) predicts that the PCI domain is not necessarily a single domain in a structural sense but rather consists of two subdomains: a C-terminal winged helix domain WH domain with a key role in PCI:PCI interaction and a preceeding helical repeat region consisting of HEAT or Armadillo-like repeats (HAM domain). A TPR-like repeat (tetratrico-peptide repeat) region N-terminal of the HAM domain seems most likely to form an uninterrupted extension of the HAM domain. Interestingly this prediction was made on the base of secondary structure prediction and not on sequence similarity and seems to be the case for all the investigated PCI domain proteins. We adapted the structure prediction of Scheel *et al*. to our experiments in expressing truncated CsnA proteins in *A. nidulans* by aligning CsnA to the investigated highly similar hsCSN1 and transferring the secondary structure to CsnA to investigate which regions of the CsnA protein are still present in our truncated proteins. We deduced that in strain AGB240 only the N-terminal TPR-like repeats are expressed, whereas in strain AGB235 the TPR-like repeats and three of six helices of the HAM domain are expressed. In strain AGB236 the HAM domain and the WH domain are expressed and the protein lacks the TPR-like repeats of the full protein (Figure 10). We conclude that the WH and HAM domain are essential for complexation of the CSN, since our results show that these domains are sufficient to complement the acleistothecial and red hyphae phenotype.

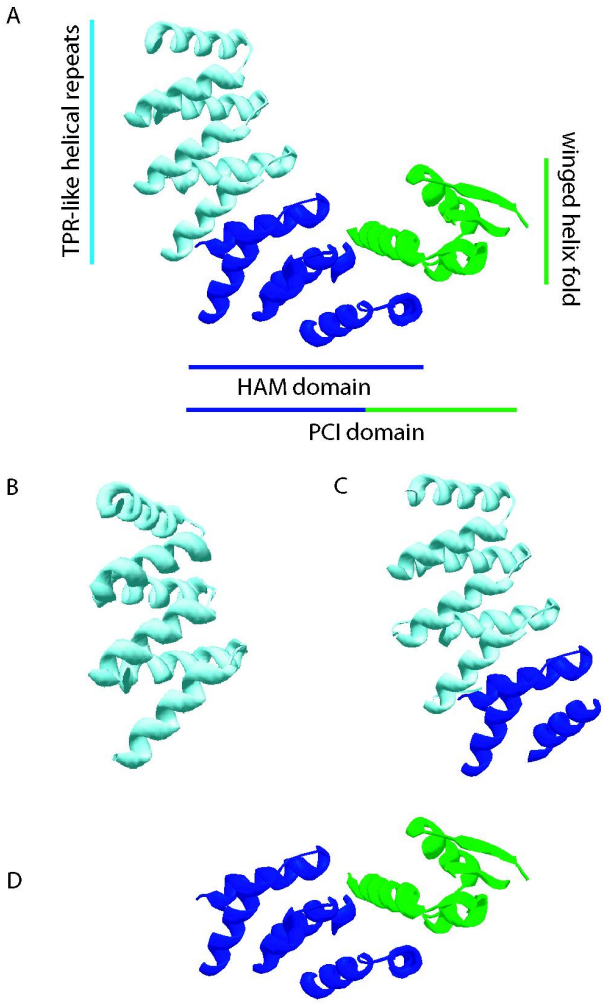


Fig. 10: Structure prediction of truncated versions of the CsnA protein according to Scheel and Hofmann

A predicted protein structure of the CsnA protein and its truncated versions are shown. The structural modelling was done according to Scheel *et. al.*, 2005. (**A)** the bipartite PCI domain is indicated as a WH-fold (winged helix fold, green) and a HAM (HEAT/Armadillo repeats, dark blue) domain. The WH domain consists of three short β-strands which form an antiparallel sheet and three α-helices in an "αβααββ" arrangement, whereas the HAM-domain consists of 3 α-hairpins. The N-terminal TPR-repeat part (light blue) was assumed by Scheel and Hofmann as a general part of PCI domain proteins. (**B)** The truncated protein expressed in strain AGB240 is shown. It contains only the TPR-like repeats, but lacks all of the bipartite PCI domain. (**C**) The truncated protein expressed in strain AGB235 is shown, only the first and half of the second α-hairpin of the HAM-domain remains. (**D**) The truncated CsnA protein is shown that is expressed in AGB236. The full PCI domain is expressed, but it lacks the TPR-like repeats.

2.5 Discussion

We provide evidence that the *csnA* gene codes for the first and largest of eight subunits of the COP9 complex in *A. nidulans*. The protein, especially the PCI domain of the protein, shows high similarities to PCI domains of homologues of higher eukaryotes and slightly lower amino acid similarities to its counterpart of *S. pombe*. Comparing only the PCI domain of the protein to other CsnA homologues we find that the bipartite domain is significantly higher conserved than the rest of the protein. The fact that the CsnA subunit of the *A. nidulans* COP9 complex is higher conserved to higher eukaryotes like *A. thaliana* and *H. sapiens* in combination to the fact that other fungi like *S. pombe* contain only six of the eight subunits of the signalosome (Mundt *et al.*, 1999) and *S. cerevisiae* forms only a COP9-like complex (Maytal-Kivity *et al.*, 2002; Wee *et al.*, 2002), with only subunit five subunits beeing conserved, leads us to the assumption that the complex plays a more significant role in the life and development of the filamentous fungus *A. nidulans* and serves as a more suitable model organism to investigate overall COP9 functions than the above named unicellular fungi.

The deletion of the *csnA* gene leads to a similar pleiotropic phenotype that was formerly reported for deletions in the genes of subunits CsnD and CsnE (Busch *et al.*, 2003). All so far characterized *csn* deletion mutants form distinct red hyphae that deeply penetrate the medium and are blocked in sexual development at the stage of primordia formation. Growth tests at 30°C and 37°C showed that *csn* deletion mutants display a slow growth phenotype at 37°C in contrast to the wild type. Where the wild type grows faster at 37°C than at 30°C the increase in growth rate can not be observed in *csn* deletion mutants. Partial or even full complementation of the *csnA* gene expressed under the *alcA* promoter did not restore this phenotype, though other observed phenotypes can be restored by partial or full complementation of the the *csnA* gene. We assume that expression of the gene under control of the *alcA* promoter may have an effect on the protein level of CsnA which leads to stochiometric discrepancies to other subunits of the COP9 signalosome and thus can not fully restore the wild type growth but is sufficient to restore the effects leading to the observed pleiotropic phenotypes.

CSN1 has an essential role in complex assembly. Complete loss of CSN1 in *Arabidopsis* abolishes accumulation of CSN8, CSN5 dissociates from the complex, and leads to a significant reduction in the levels of CSN4 and CSN7 (Wang *et al.*, 2002). Plants and other higher eukaryotes as mammals and fly that lack the functionality of any subunit of the signalosome die during early embryogenesis (Freilich *et al.*, 1999; Wei and Deng, 1992). In

contrast to this the deletion of *csn* subunits in the filamentous fungus *A. nidulans* is not lethal for the whole organism but leads to the formation of a red pigment and a block in sexual development. The block of sexual development at the stage of primordia though, may well be regarded as embryonal lethality. So far we were unable to separate these two phenotypes. We assume that the loss of single subunits leads to either the complete loss of the complex in *A. nidulans* and to the loss of COP9 signalosome specific functions which are necessary to abolish the production of the red metabolite and drive the sexual development past the point of primordia formation. Interestingly the introduction of loss of function point mutations into the conserved JAMM domain of CsnE leads to similar phenotypes as reported for deletion mutants in any of the subunits (K. Nahlik, unpublished results). This hypothesis is strengthened by the fact that the expression of the C-terminal domain containing the PCI domain of CsnA, which is important for complex formation in plants (Tsuge *et al.*, 2001; Wang *et al.*, 2002), in strain AGB236 is sufficient to complete sexual development and abolish the production of the red hyphae phenotype. We deduce that the expression of the PCI domain alone and thus the proper formation of the CSN complex is essential for sexual development in *A. nidulans*. The HAM part of the PCI domain and the N-terminal domain of the CsnA protein are insufficient to restore sexual development and abolish red pigment formation, as proven in strain AGB235. Thus we could show that both parts of the bipartite PCI domain, the N-terminal HAM domain and the C-terminal WH domain are essential for formation of the complex and to restore specific complex functions.

It could be shown in plant that the inability to form the COP9 complex abolishes the deneddlyation activity of the COP9 complex due to dissociation of the metalloprotease subunit CSN5 (Wang *et al.*, 2002). The inability to deneddylate and thus regulate the SCF complex activity leads to early embryonic death in higher eukaryotes which we can compare to an early embryonic death of *A. nidulans* progeny at the stage of primordia. Again, this is strengthened by the fact that mutants defective in deneddylation exhibit a similar phenotype (K. Nahlik, unpublished results). On the other hand the expression of the C-terminus including the PCI domain of CsnA is insufficient to fully restore a wild type phenotype. The lack of the N-terminus of the CsnA protein leads to the formation of excessive aerial hyphae in the filamentous fungus *A. nidulans*, whereas the expression of the N-terminus alone or the expression of the N-terminus and the HAM domain seem to suppress this phenotype. In *Arabidopsis* mutants that lack the N-terminus of CSN1 are not viable despite the fact that the COP9 complex and deneddylation activity are restored (Wang *et al.*, 2002), whereas Δ*csnA* mutants of *A. nidualns* expressing the C-terminus only are able to produce mature sexual

fruitbodies. This leads us to the conclusion that the inability to deneddylate due to a loss of the COP9 complex has a direct or indirect effect on sexual development in *A. nidulans*. So far one can only speculate if the N-termius is additionally required for the deneddylation activity of the complex or if a direct interaction of the N-terminus with the Rbx1 subunit of the SCF complex affects other aspects of SCF activity (Wang *et al.*, 2003) and thus viability or in this case the repression of production of excessive aerial hyphae.

In summary, we have shown that the loss of the largest subunit of the *A. nidulans* CSN leads to either the loss of the whole complex or at least to the loss of specific CSN functions. Most likely the loss of the deneddylation activity results in acleistothecial strains producing red hyphae. Additionally the exact amount of CsnA protein seems to be important for growth at higher temperatures. Separating CSN complex function and activities from CsnA activities, our results indicate a direct or associated activity in the N-terminal domain of CsnA.

2.6 References

Ambroggio, X.I., Rees, D.C., and Deshaies, R.J. (2004) JAMM: A Metalloprotease-Like Zinc Site in the Proteasome and Signalosome. *PLoS Biol* **2**: E2.

Amir, R.E., Iwai, K., and Ciechanover, A. (2002) The NEDD8 pathway is essential for SCF(beta -TrCP)-mediated ubiquitination and processing of the NF-kappa B precursor p105. *J Biol Chem* **277**: 23253-23259.

Andrade, M.A., Petosa, C., O'Donoghue, S.I., Muller, C.W., and Bork, P. (2001) Comparison of ARM and HEAT protein repeats. *J Mol Biol* **309**: 1-18.

Aravind, L., and Ponting, C.P. (1998) Homologues of 26S proteasome subunits are regulators of transcription and translation. *Protein Sci* **7**: 1250-1254.

Bennett, J.W., and Lasure, L.L. (1991) Growth media. In *More gene manipulation in fungi*. Bennett, J.W. and Lasure, L.L. (eds). San Diego: Academic Press, pp. 441-457.

Busch, S., Eckert, S.E., Krappmann, S., and Braus, G.H. (2003) The COP9 signalosome is an essential regulator of development in the filamentous fungus *Aspergillus nidulans*. *Mol Microbiol* **49**: 717-730.

del Pozo, J.C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W.M., and Estelle, M. (2002) AXR1-ECR1-dependent conjugation of RUB1 to the *Arabidopsis* Cullin AtCUL1 is required for auxin response. *Plant Cell* **14**: 421-433.

Deng, X.W., Dubiel, W., Wei, N., Hofmann, K., and Mundt, K. (2000) Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. *Trends Genet* **16**: 289.

Eckert, S.E., Kübler, E., Hoffmann, B., and Braus, G.H. (2000) The tryptophan synthase-encoding *trpB* gene of *Aspergillus nidulans* is regulated by the cross-pathway control system. *Mol Gen Genet* **263**: 867-876.

Felenbok, B. (1991) The ethanol utilization regulon of *Aspergillus nidulans*: the *alcA-alcR* system as a tool for the expression of recombinant proteins. *J Biotechnol* **17**: 11-17.

Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D., and Chamovitz, D.A. (1999) The COP9 signalosome is essential for development of *Drosophila melanogaster*. *Curr Biol* **9**: 1187-1190.

Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L., Wortman, J., Batzoglou, S., Lee, S., Brudno, M., Bastürkmen, M., Spevak, C.C., Clutterbuck, J., Kapitonov, V., J., J., Scazzocchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G.H., Draht, O., Busch, S., D´Enfert, C., Bouchier, C., Goldman, G.H., Griffiths-Jones, S., Vienken, K., Pain, A., Selker, E.U., Archer, D., Penalva, M.Á., Oakley, B.R., Momany, M., Sano, M., Tanaka, T., Kumagai, T., Machida, M., Nierman, W.C., Denning, D.W., Caddick, M., Hynes, M., Paoletti, M., Fischer, E.R., Miller, J.B., Dyer, P., Sachs, M.S., Osmani, S.A., and Birren, B. (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Manuscript submitted.

Hofmann, K., and Bucher, P. (1998) The PCI domain: a common theme in three multiprotein complexes. *Trends Biochem Sci* **23**: 204-205.

Inoue, H., Nojima, H., and Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**: 23-28.

Käfer, E. (1977) The anthranilate synthetase enzyme complex and the trifunctional *trpC* gene of *Aspergillus*. *Can J Genet Cytol* **19**: 723-738.

Krappmann, S., and Braus, G.H. (2003) Deletion of *Aspergillus nidulans aroC* using a novel blaster module that combines ET cloning and marker rescue. *Mol Genet Genomics* **268**: 675-683.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., and Deshaies, R.J. (2001) Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**: 1382-1385.

Maytal-Kivity, V., Piran, R., Pick, E., Hofmann, K., and Glickman, M.H. (2002) COP9 signalosome components play a role in the mating pheromone response of *S. cerevisiae*. *EMBO Rep* **3**: 1215-1221.

Moseley, S.L., Huq, I., Alim, A.R., So, M., Samadpour-Motalebi, M., and Falkow, S. (1980) Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. *J Infect Dis* **142**: 892-898.

Mundt, K.E., Porte, J., Murray, J.M., Brikos, C., Christensen, P.U., Caspari, T., Hagan, I.M., Millar, J.B., Simanis, V., Hofmann, K., and Carr, A.M. (1999) The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. *Curr Biol* **9**: 1427-1430.

Mundt, K.E., Liu, C., and Carr, A.M. (2002) Deletion mutants in COP9/signalosome subunits in fission yeast *Schizosaccharomyces pombe* display distinct phenotypes. *Mol Biol Cell* **13**: 493-502.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E.A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2000) Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. *Embo J* **19**: 3475-3484.

Ponting, C.P., Aravind, L., Schultz, J., Bork, P., and Koonin, E.V. (1999) Eukaryotic signalling domain homologues in archaea and bacteria. Ancient ancestry and horizontal gene transfer. *J Mol Biol* **289**: 729-745.

Rave, N., Crkvenjakov, R., and Boedtker, H. (1979) Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. *Nucleic Acids Res* **6**: 3559-3567.

Scheel, H., and Hofmann, K. (2005) Prediction of a common structural scaffold for proteasome lid, COP9-signalosome and eIF3 complexes. *BMC Bioinformatics* **6**: 71.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., and Deng, X.W. (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. *Science* **292**: 1379-1382.

Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (1998) A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. *Faseb J* **12**: 469-478.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**: 503-517.

Sun, Y., Wilson, M.P., and Majerus, P.W. (2002) Inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome by binding to CSN1. *J Biol Chem* **277**: 45759-45764.

Tateishi, K., Omata, M., Tanaka, K., and Chiba, T. (2001) The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. *J Cell Biol* **155**: 571-579.

Tsuge, T., Matsui, M., and Wei, N. (2001) The subunit 1 of the COP9 signalosome suppresses gene expression through its N-terminal domain and incorporates into the complex through the PCI domain. *J Mol Biol* **305**: 1-9.

Wang, X., Kang, D., Feng, S., Serino, G., Schwechheimer, C., and Wei, N. (2002) CSN1 N-terminal-dependent activity is required for *Arabidopsis* development but not for Rub1/Nedd8 deconjugation of cullins: a structure-function study of CSN1 subunit of COP9 signalosome. *Mol Biol Cell* **13**: 646-655.

Wang, X., Feng, S., Nakayama, N., Crosby, W.L., Irish, V., Deng, X.W., and Wei, N. (2003) The COP9 signalosome interacts with SCF UFO and participates in *Arabidopsis* flower development. *Plant Cell* **15**: 1071-1082.

Wee, S., Hetfeld, B., Dubiel, W., and Wolf, D.A. (2002) Conservation of the COP9/signalosome in budding yeast. *BMC Genet* **3**: 15.

Wei, N., and Deng, X.W. (1992) COP9: a new genetic locus involved in light-regulated development and gene expression in *Arabidopsis*. *Plant Cell* **4**: 1507-1518.

Wei, N., Chamovitz, D.A., and Deng, X.W. (1994) *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. *Cell* **78**: 117-124.

Wei, N., and Deng, X.W. (2003) The COP9 signalosome. *Annu Rev Cell Dev Biol* **19**: 261-286.

Wilson, M.P., Sun, Y., Cao, L., and Majerus, P.W. (2001) Inositol 1,3,4-trisphosphate 5/6-kinase is a protein kinase that phosphorylates the transcription factors c-Jun and ATF-2. *J Biol Chem* **276**: 40998-41004.

Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Di, X., Wang, X., Rodriguez-Suarez, R.J., Zhang, H., and Wei, N. (2002) The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via deneddylation of SCF Cul1. *Curr Biol* **12**: 667-672.

Zheng, J., Yang, X., Harrell, J.M., Ryzhikov, S., Shim, E.H., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (2002) CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. *Mol Cell* **10**: 1519-1526.

Zhou, C., Seibert, V., Geyer, R., Rhee, E., Lyapina, S., Cope, G., Deshaies, R.J., and Wolf, D.A. (2001) The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. *BMC Biochem* **2**: 7.

Chapter 3

Annotation of cross-pathway control related genes in the genome of *Aspergillus nidulans*, *Aspergillus fumigatus* and *Aspergillus oryzae*

3.1 Abstract

Amino acids are essential building blocks of protein biosynthesis. The regulatory network dealing with the uptake of amino acids, the synthesis of amino acids from precursors and their channeling into protein production is complex. A comparative genome analysis of the model organism *Aspergillus nidulans*, the biotechnologically important fungus *Aspergillus oryzae* and the opportunistic human pathogen *Aspergillus fumigatus* was performed with focus on the general control/cross-pathway control (gc/cpc) of amino acid biosynthesis. The extracted data were compared to other lower and higher eukaryotes as the bakers yeast *Saccharomyces cerevisiae*. Central part of the investigation is the gc/cpc but our results also encompass neighbouring pathways like the sensing of amino acids, their transport into the cells and regulation of protein biosynthesis from transcription to degradation of proteins. We found that the general control system is present in the investigated *Aspergilli*, but neighbouring systems partly differ from well characterized *S. cerevisiae* systems mainly in respect of amino acid sensing and transport. We also found that basic compounds of the translation machinery in *Aspergilli* rather resemble those of higher eukaryotes than of yeast.

3.2 Introduction

The *Aspergilli* comprise a diverge group of filamentous fungi (Galagan *et al.*, 2005). Among the over 185 *Aspergilli* are several species with impact on human health, including 20 human pathogens. In addition several economically, medically and agriculturally important fungal species are part of the *Aspergillus* family (Galagan *et al.*, 2005).

Bioactive molecules like β-lactam antibiotics and aflatoxins are secreted by *A. fumigatus* and *A. flavus* (Brakhage *et al.*, 2004; Gugnani, 2003; Sales and Yoshizawa, 2005). Additionally, *A. fumigatus* is an important human pathogen causing invasive aspergillosis in immunocompromised patients (Gagnadoux, 2005).

A. oryzae is of high importance in asian countries, where it is used to produce sake, miso and soy sauce in industrial standards (Thammarongtham *et al.*, 2001).

A. nidulans constitutes a representative of this fungal genus that is capable of diverse and complex biosynthesis and differentiation processes like the well characterized sexual differentiation process where after mating with a compatible partner or "selfing" cleistothecia are formed which contain octades of ascospores (Brüggeman *et al.*, 2003; Hoffmann *et al.*, 2001a). During the last century the eukaryotic model organism was found to be easily investigated and manipulated by molecular methods. This makes *Aspergillus* species particularly suited for in depth studies on regulatory networks and cross-connections between environmental stimuli, metabolism and development and has steadily advanced our understanding of eukaryote physiology (Figure 11).

Amino acids are the building blocks for protein synthesis. The preferred nitrogen sources of *Aspergilli* are ammonium, glutamine or glutamate. Under energy limiting conditions amino acids can also serve as nitrogen and carbon sources in metabolism. All nitrogen related processes pass at some stage through the amino acids glutamine and glutamate where ammonium as nitrogen donor is easily transferred between these two amino acids and other keto-group containing molecules (Davis *et al.*, 2005; Minehart and Magasanik, 1992). Fungi are able to use a variety of nitrogen sources like proteins, amino acids, nitrate, purines and even acetamide and specific permeases for different more or less preferred nitrogen sources (Davis *et al.*, 2005; Marzluf, 1997). The biosynthesis of these permeases and enzymes needed for the related catabolism often have to be induced according to the availability in the environment. Nitrogen supply is carefully regulated in fungi and specific GATA transcription factors like AreA, Gln3p in yeast or NIT-2 in *N. crassa* regulate the synthesis of catabolic enzymes related to nitrogen (Davis *et al.*, 2005; Marzluf, 1997).

Amino acids are as well essential precursors for ribosomal biosynthesis of proteins as for non-ribosomal products of secondary metabolism like β-lactam antibiotics synthesis (Brakhage, 1998; Brakhage *et al.*, 2005). Most fungi prefer to take up amino acids from their diet since the synthesis includes several energy consuming steps. Amino acid uptake depends on the nutritional conditions in their surroundings and relies on sensors and uptake systems for these precursors. Most fungi are also able to secrete proteases into the medium to break down proteins in their medium and take up the remaining amino acids, though these actions are carefully regulated and mainly happen under stress conditions like nitrogen starvation and the presence of extra-cellular protein (Kredics *et al.*, 2005; Ogrydziak, 1993; Pavlukova *et al.*, 1998).

When no amino acids are available in the environment, these essential building blocks have to be either taken up from nutrient sources or *de novo* synthesized, whereas fungi, plants and prokaryotes are able to synthesize their amino acids, the uptake of amino acids from their diet is essential for mammals which are unable to produce their own amino acids. Fungal cells have to adjust rapidly to changing environmental conditions and therefore need to be able to build up or degrade proteins or other macromolecules and secondary metabolites in their surroundings.

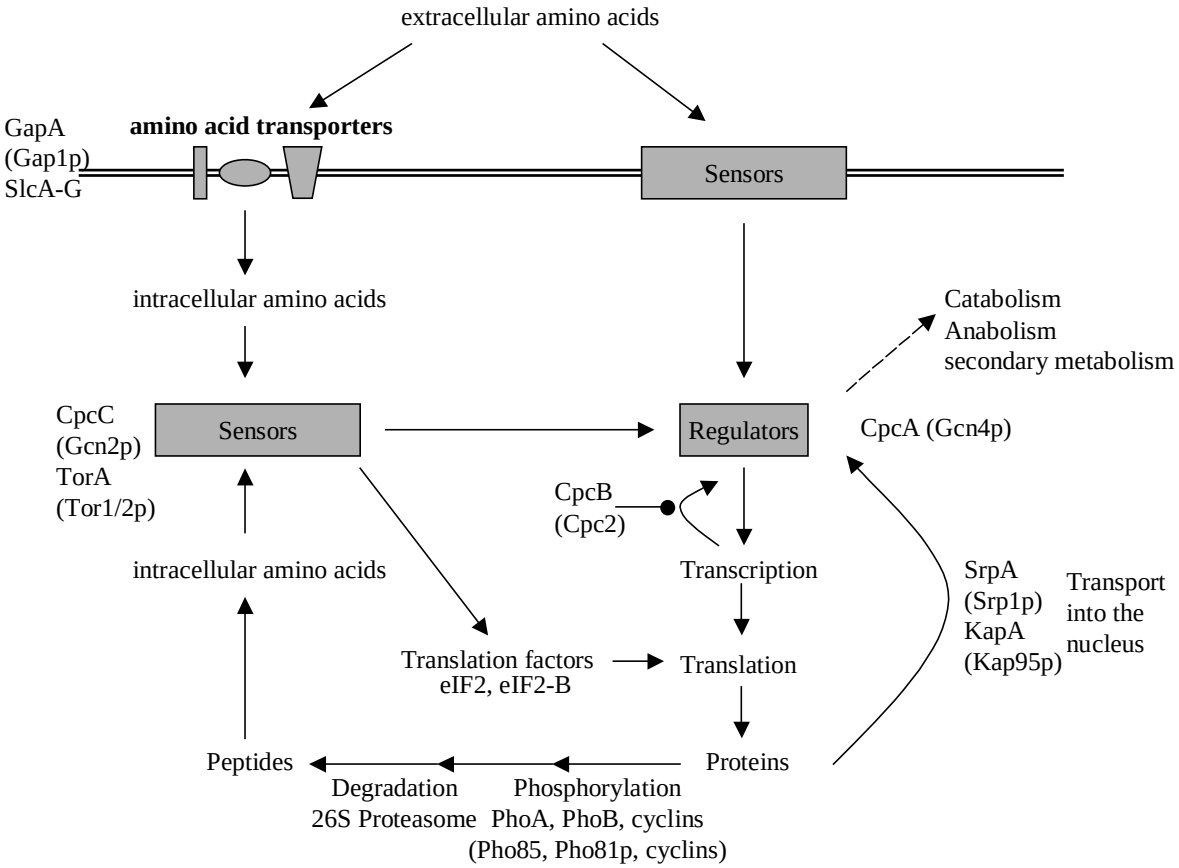


Fig. 11: Overview of the general control/cross-pathway control (gc/cpc) and neighbouring pathways of *Aspergilli* and *S. cerevisiae*
This figure shows a roadmap of cellular processes around the cross-pathway control and indicates related proteins for the respective processes. Extracellular amino acids are sensed by sensors in the cell wall and transported into the cells by a variety of transporters. Inside the cell amino acids are sensed by different sensors and signals are passed on to key regulators of the gc/cpc. According to amino acid availability the main transcription regulator enhances or decreases transcription of metabolic genes, translation can be increased or decreased. In times of nutritional hardship un-needed proteins can be degraded for their compounds or the central transcription factor can be degraded under non-limiting conditions in a regulatory manner (Klionsky, 2005). Under non-starvation conditions factors negatively regulate transcription of transcription factors (Schneper *et al.*, 2004).

Under very limiting conditions or if proteins are no longer required proteins can be broken down and their building blocks recycled to put to further use (Omura *et al.*, 2001). Not only single proteins can be broken down in serious starvation or stress conditions, though. It is possible for the cell to do bulk degradations of large compartments of cells by autophagy in the vacuole (Klionsky, 2005). A controlled timely expression and destruction of proteins is a major regulatory mechanism in cellular processes (Hilt, 2004; Wittenberg and Reed, 2005). The main eukaryotic degradation machinery, the 26S proteasome, specifically degrades ubiquitinylated proteins (Miller and Gordon, 2005). Proteolysis is performed by the ATP-independent peptidase activity of the 20S core of the proteasome, whereas substrate specificity is mediated by the 19S regulatory particle consisting of a base complex which confers ATPase and chaperon activity and a 26S lid complex of so far unknown function (Takeuchi and Tamura, 2004). Proteins are targeted for degradation by poly-conjugation of the small ubiquitin protein. The first covalent binding is engaged between glycin 76 of ubiquitin and an epsilon-amino group of an internal lysin of the target protein and further isopeptidic elongation of the ubiquitin chain through lysine 48 of the first ubiquitin (Krappmann and Scheidereit, 2005; van Nocker and Vierstra, 1993). Newer insights to ubiquitin conjugation indicate that the alternative formation of ubiquitin chains through the lysine 63 residue do not mark a protein for degradation. This type of linkage, as opposed to the more typical Lys-48-linked chains, serves as a non-proteolytic marker of protein targets involved in error-free post-replicative DNA repair and NF-kappa B signal transduction (Krappmann and Scheidereit, 2005; McKenna *et al.*, 2003). The corresponding ubiquitinylation cascade has been studied in-depth (Hershko and Ciechanover, 1998; Jackson and Eldridge, 2002). An ubiquitin activating enzyme (E1) activates the ubiquitin by a thioester linkage prior to transfer to a lysine residue of a specific protein substrate by a ubiquitin conjugating enzyme (E2). An ubiquitin ligase (E3) catalyzes substrate recognition and assists in ubiquitinylation. Several E3 ubiquitin ligase complexes are known, most prominent is the cullin-RING-H2-family that includes SCF (Skp1p/Cdc53p/F-box) (Deshaies, 1999; He *et al.*, 2005; Tyers and Jorgensen, 2000) which plays an important role in the regulated destruction of the central transcription factor of the general control of amino acid biosynthesis Gcn4p of *S. cerevisiae* (Irniger and Braus, 2003).

The uptake of amino acids has to be carefully controlled and the reason for uptake has to be distinguished from another. One reason might be the uptake of amino acids as poor carbon or nitrogen sources in times of nitrogen or carbon starvation (Davis *et al.*, 2005; Magasanik and Kaiser, 2002; Valenzuela *et al.*, 2001), another the usual uptake of amino acids as building

blocks for amino acid biosynthesis from their diet (Andreasson *et al.*, 2004). If a fungus senses only poor nitrogen sources like proline in the diet, the nitrogen discrimination pathway or nitrogen catabolite repression (NDP or NCP) is required for their utilisation. GATA transcription factors like Gln3p, AreA or NIT-2 are activated to increase the production of enzymes needed for uptake and utilization (Abdel-Sater *et al.*, 2004; Davis *et al.*, 2005; Mo and Marzluf, 2003). The NDP is repressed if rich nitrogen sources are present (Boczko *et al.*, 2005; Sosa *et al.*, 2003). The first step needs to be in any case the sensing of amino acids outside of the cell (Forsberg *et al.*, 2001). A subsequent signal cascade is needed to pass the signal for availability on into the cell and channel the signal into appropriate actions - to take up the amino acids as nitrogen or carbon sources or for anabolism and pass the signal on so that no longer required proteins can be degraded and recycled (Forsberg *et al.*, 2001).

Most knowledge on the coordination network of amino acid uptake and metabolism was aquired in the bakers yeast *Saccharomyces cerevisiae* (Braus, 1991; Hinnebusch, 1986), but a lot is known in filamentous fungi like *A. nidulans* or *N. crassa*, as well (Busch *et al.*, 2003; Ebbole *et al.*, 1991; Eckert *et al.*, 2000; Hoffmann *et al.*, 2001b; Luo *et al.*, 1995; Paluh and Yanofsky, 1991). A prominent example of such regulatory networks is the cross-pathway control of amino acid biosynthesis (cpc) or the general control of amino acid biosynthesis (gc). Originally the general control defined the increase of amino acid biosynthesis enzymes upon starvation on one amino acid (Carsiotis and Jones, 1974; Carsiotis *et al.*, 1974), later followed by the discovery of global activators like Gcn2p and Gcn4p and repressors of the general control (Hinnebusch, 1985). This well studied complex regulatory network ensures increased transcription of a variety of amino acid biosynthetic genes when supply of a single amino acid is limited. This system is common in several filamentous fungi like *Neurospora crassa, A. nidulans, A. fumigatus* and *A. niger* (Hoffmann *et al.*, 2001b; Krappmann *et al.*, 2004; Paluh *et al.*, 1988; Wanke *et al.*, 1997). It is also found in the budding yeast *Saccharomyces cerevisiae* (Hinnebusch, 1984). The central transcription factor Gcn4p of *S. cerevisiae* is activated under amino acid limiting conditions. A transcription factor of a general control of amino acid biosynthesis itself can not be found in mammals, though c-jun, a human oncoprotein, shares high homologies to Gcn4p and CpcA. Activation of the general control is regulated via a complex regulatory cascade. The Gcn2p sensor kinase is attached to the ribosome in the cytoplasm. It monitors whether tRNAs are charged with amino acids. The protein controls the Gcn4p synthesis, but also controls the overall translation rate. Gcn2p-like kinases seem to be typical for eukaryotic cells. Interestingly there is a mammalian GCN2 which is induced by amino acid or serum starvation (Berlanga *et al.*, 1999; Sood *et al.*, 2000).

If Gcn2p is replaced by its human counterparts, yeast Gcn4p is synthesized independently of the presence or absence of amino acids (Dever *et al.*, 1993). Gcn4p binds to *cis*-acting palindromic GCREs (general control response elements) 5´-ATGA (G/C) TCAT-3´ in the promoter regions of amino acid biosynthetic target genes and thus enhances the transcription of these genes. For the filamentous fungi *N. crassa* and *A. niger*, homologues of this transcription factor have been described to bind on similar motifs called CPREs (cross-pathway control response elements) (Hinnebusch, 1997; Hoffmann *et al.*, 2001b). The homologue transcription factors in *Aspergilli* are known as CpcA or CPC-1 in *N. crassa* (Hoffmann *et al.*, 2001b; Krappmann *et al.*, 2004; Paluh *et al.*, 1988). The regulation of the gc/cpc takes place on translational, transcriptional and protein stability level with various input signals and related pathways. The synthesis of these proteins is controlled in the cytoplasm. During translation the sensing of the on-site amino acid availability decides if the transcription factors are translated or not. The transcription factor needs to be transported into the nucleus after translation in the cytosol. In the nucleus the half-life of these transcription factors is regulated depending on amino acid availability, each of these checks ensuring a rapid response to many environmental stress conditions.

The general control seems to be not fully conserved in higher eukaryotes due to the inability of mammals to *de novo* synthesize all required amino acids. Nonetheless an amino acid sensing system similar to that of lower eukaryotes was found recently in the mammal piriform cortex, a region of the brain located in the rhinencephalon and part of the telencephalon related to olfaction. Certain nutrients, among others amino acids, possibly act through sensing mechanisms and have the capability to initiate cell-signalling events and regulate gene expression in the absence of hormonal influence (Hyde *et al.*, 2003). Studies have shown that elevated amino acid levels sustain anabolism and inhibit catabolism in eukaryotic cells (Meijer and Dubbelhuis, 2004), meaning an increase in global mRNA levels and protein synthesis (Averous *et al.*, 2003). Overall the intracellular amino acid limitation sensing and resulting processes in mammals seems similar to those in yeast, if a little more complex (Harding *et al.*, 2000). It was shown that a lack of indespensable amino acids in mice leads to mGCN2 dependent phosphorylation of eIF2α in mouse brains to restore indespensable amino acid homeostasis (Hao *et al.*, 2005). The sensor kinase mGCN2 senses uncharged tRNAs and phosphorylates eIF2α (Harding *et al.*, 2000). The general downregulation of translation leads to expression of ATF-4 due to an upstream open reading frame in the mRNA similar to the mechanism known from yeast Gcn4p (Averous *et al.*, 2003; Mielnicki *et al.*, 1996). To induce expression of target genes, like CHOP (CCAAT/enhancer binding protein (C/EBP)

homologous protein) (Bruhat *et al.*, 1997) or asparagine synthethase AS (Hutson and Kilberg, 1994; Hutson *et al.*, 1997) and the cationic amino acid transporter (Cat-1) (Fernandez *et al.*, 2003) gene, ATF-2 needs to be phosphorylated as well (Averous *et al.*, 2004).

Different mechanisms have been proposed where amino acid transporters may impact on mammalian signal transduction. The first one would be that an amino acid transporter acts as a substrate specific receptor and passes on a signal during transport of its substrate (Hyde *et al.*, 2003). A second impact amino acid transporters could have is that during import of the amino acid substrate other molecules are symported or antiported impacting on intracellular changes of the cell physiology (pH, change in membrane potential, cellular volume change) (Hyde *et al.*, 2003). A third impact amino acid transporters could have is the import of substrates and sensing through intracellular sensors like mGNC2 (Averous *et al.*, 2003). Mammalian cells have a broad range of mechanisms for the transmembrane transport of amino acids (Hyde *et al.*, 2003). The transporters have been classified into distinct "systems" defining substrate specificity, transport mechanism and regulatory properties (Hyde *et al.*, 2003). The system can be divided up into layers. The first layer discriminates between sodium dependent and independent transporters. The second layer defines the charge of the substrate: neutral, anionic or cationic. The third layer defines the actual "system", which groups the transporters due to their substrate specificity (Hyde *et al.*, 2003).

System BETA transporters belong to the SLC6 family and are mainly localized in the neuronal gaps. They transport the neurostransmitter amino acid GABA, but are also able to transport glycine and glutamate. The flux of GABA transporters is regulated by binding of diverse proteins to their N-terminus (Chen *et al.*, 2004). SLC7 family transporters transport cationic, neutral and both amino acids (H, M, L, I, V, F, Y, W, Q) (Hyde *et al.*, 2003; Wipf *et al.*, 2002). Along with the Ssy1p protein of the yeast SPS system they belong to the family of APC (amino acid polyamine-choline) transporters (Verrey *et al.*, 1999). Many of the transporters of the SLC7 family require the presence of glycoproteins to form a functional holotransporter in mammalian cells (Padbury *et al.*, 2004). One of its members of this family, the LAT1 transporter, is suggested to act as an environmental sensor of amino acid availability (Padbury *et al.*, 2004; Verrey *et al.*, 1999). The SLC1 family of amino acid transporters transports glutamic acid and aspartic acid in astrocytes (Hyde *et al.*, 2003). Astrocytes grow like filamentous fungi star shaped and form the brain-blood barrier in the brain (Volterra and Meldolesi, 2005). They also support the exchange of nutrients from blood to the nervous system. The glial glutamic acid transporter EAAT1 not only regulates glial signal transduction but is also important for development of the astrocytes as was shown for

diseases like lissencephaly (Furuta *et al.*, 2005; Zagami *et al.*, 2005). The imino or SLC36 family of mammalian amino acid transporters is essential for uptake of degradation products from the lysosome and uptake of nutrients from the gut (Boll *et al.*, 2004). The transporters were shown to effect or modulate growth through the TOR pathway in *Drosophila* in a yet not well characterized way (Goberdhan *et al.*, 2005).

In this work we present the manual annotation and subsequent in depth genome analysis regarding the gc/cpc of the genomes of *A. nidulans*, *A. oryzae* and *A. fumigatus*. We analyzed the conservation of a general control-like system in these filamentous fungi, where not much more than the central transcription factor of the cross-pathway control was known (Hoffmann *et al.*, 2001b; Krappmann *et al.*, 2004; Wanke *et al.*, 1997) and decided to broaden the analysis to the search for components both known in yeast, as well as in higher eukaryotes. The respective gene products were compared to those of *Homo sapiens*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Neurospora crassa* (in some cases to *Magnaporthe grisea*), *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* and gene names according to the rules of the genome project were assigned if necessary.

3.3 Methods

3.3.1 *A. nidulans* annotation and analysis

The *A. nidulans* genome was annotated using the Calhoun annotation system (Galagan *et al.*, 2002; Galagan *et al.*, 2003; Galagan *et al.*, 2005). The genome sequence was searched against the public protein databases using BLASTX (Altschul *et al.*, 1997) with threshold E≤1e-5. Genes were predicted using a combination of FGENESH, FGENESH+ (www.softberry.com) and GENEWISE (www.psc.edu/general/software/packages/genewise/genewise.html). Predicted genes were validated against ESTs aligned to the genome using BLAST (Altschul *et al.*, 1997). All predicted proteins were searched against the PFAM set of hidden Markov models using the HMMER (http://hmmer.wustl.edu/) program and the public protein databases using BLASTP (Altschul *et al.*, 1997).

Repeat sequences were detected by searching the genome sequence against itself using CrossMatch (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm), filtering for alignments longer than 200bp in length and clustering pairs based on region overlap.

Repeats were characterized using RepeatMasker (ftp.genome.washington.edu/RM/RepeatMasker.html) and RepBase (Jurka, 1998) followed by manual inspection. The *A. fumigatus* and *A. oryzae* genomes were annotated as described in separated reports (Nierman, W.C. *et al.*, manuscript submitted, Machida, M. *et al.*, manuscript submitted). For manual annotation the ERGO bioinformatics suite (Integrated Genomics, Chicago, USA) was used.

3.4 Results

85 cross-pathway control related genes, or their proteins respectively, were investigated in all three *Aspergilli* (Table 1). In *A. nidulans* 77 homologues of these genes were found, 75 in *A. oryzae* and 72 in *A. fumigatus* indicating that processes may work differently in *Aspergillus spec*. than in the well studied general control of *S. cerevisiae*. The respective deduced protein sequences of *A. nidulans* were again blasted (tblastx) against a non-redundant database to confirm the given annotations and the returned hits against the above mentioned organisms were protocolled. 48 genes of the originally primarily annotated open reading frames were found for *Homo sapiens*, 44 genes for *Arabidopsis thaliana*, 49 genes for *Drosophila melanogaster*, 77 genes for *Neurospora crassa*, 64 genes for *Schizosaccharomyces pombe* and 69 genes for *Saccharomyces cerevisiae*. Twenty-eight of those genes were found to be the intersection and were used to generally compare the different organisms with *A. nidulans* on similarity level, showing an decreasing order of averaged homology from *N. crassa* to *S. cerevisiae* to *S. pombe* to *A. thaliana* to *H. sapiens* to *D. melanogaster* (Table 2).

3.4.1 Sensing of extracellular and intracellular amino acids

3.4.1.1 The SPS amino acid sensing system

The search for a system similar to the yeast SPS (Ssy1p – Ptr3p – Ssy5p) (Forsberg *et al.*, 2001) amino acid sensor does not seem to exist in these three *Aspergilli* (nor can it be found in the genome of *N. crassa*). Ssy1p is a protein consisting of 12 transmembrane spanning domains and strongly resembles an amino acid transporter but in contrast to these has an unusually elongated N-terminus which is required for activity. Similar transmembrane proteins can be found throughout the three *Aspergillus* genomes but none exhibits the elongated N-terminus indicating that a Ssy1p homologue is not present. In addition a homologous region of the enlongated N-terminus can be found in neither of the three genomes. Neither Ptr3p nor Ssy5p could not be found in any of the *Aspergilli* (Forsberg *et al.*, 2001). The SPS cleaves an NLS-masking domain from the heterodimeric transcription factor Stp1/2p when amino acids are present in the medium, sending it to the nucleus to enhance transcription of amino acid transporters (Andreasson and Ljungdahl, 2002).

3.4.1.2 Amino acid sensing through the TOR pathway

The TOR pathway (in this specific context) is known to be another sensor of amino acid availability. Whereas in *S. cerevisiae* the two redundant TOR kinases Tor1p and Tor2p can be found, in each of the investigated *Aspergilli* only one gene for one protein similar to both TOR kinases of *S. cerevisiae* TorA could be found in the database with 48% similarity to the yeast TOR proteins, 58% similarity to *N. crassa* TOR, 54% similarities to the TOR proteins of *S. pombe* and *D. melanogaster*, 44% similarity to human TOR and 42% similarity to the *A. thaliana* protein. The TOR kinase is known to interfere with gc/cpc in different ways. On the one hand inactivation of the TOR pathway in yeast results (among other processes) in a global translation repression and on the other hand during nitrogen starvation negatively affects the stability of high affinity amino acid transporters in the cell membrane (Cherkasova and Hinnebusch, 2003). If nitrogen is plentiful a kinase of the TOR-pathway phosphorylates Gcn2p and thus inhibits the protein kinase domain and binding to tRNA of Gcn2p (Cherkasova and Hinnebusch, 2003). Tap42p, a mediator of the TOR pathway can also be found in each of the *Aspergilli*. Tap42p is known to dephosphorylate Gcn2p and induce Gcn4p transcription when the TOR kinase is inactive (Rohde *et al.*, 2004; Wang *et al.*, 2003). The Tap42p ortholog TapA can be found in all three *Aspergilli*, remarkably the similarity to the orthologs of higher eukaryotes lies under 30% whereas the fungal orthologs display similarities of 43,5% for *N. crassa*, 37% for *S. pombe* and 34% for *S. cerevisiae*.

3.4.1.3 The gc/cpc sensor kinase of *Aspergilli*

During starvation on amino acids the amount of uncharged tRNAs increases, which in yeast is sensed by the sensor kinase Gcn2p/CpcC and results in CpcC phosphorylating eIF-2α thus lowering overall translation (Figure 12). The sensor kinase CpcC is present in the investigated *Aspergillus* species and the protein sequence is highly similar in all organisms (Zhu *et al.*, 1996). The above effect is Gcn1p/Gcn20p dependent (Vazquez de Aldana *et al.*, 1995), both of which can be found in all three genomes. Their orthologs were named CpcD for Gcn1p and CpcE for Gcn20p. Interestingly no protein similar to CpcE can be found in *A. thaliana*, whereas the orthologs of CpcD are almost identical and so are the orthologs of the CpcE protein in the other compared organisms.

3.4.2 Uptake of amino acids in the filamentous fungus *A. nidulans*

3.4.2.1 Amino acid uptake systems of *Aspergillus* species

A prominent example of well characterized amino acid transporters in yeast can be found in all three *Aspergillus* species. Gap1p (general amino acid permease) of *S. cerevisiae* is a general high capacity amino acid permease which is known to transport all naturally occuring L-amino acids and also various D-amino acids (Jorgensen *et al.*, 1998). Equally well known are the Trp/Tyr transporters Tat1p and Tat2p (Schmidt *et al.*, 1994). All three *Aspergillus species* display an amino acid transport system GapA which is homologous to both the Gap1p and Tat1/2p and shows similarities over 50% to its fungal counterparts (Table 3). If these transporters found in the *Aspergilli* are compared to higher eukaryotes no equivalents can be found. A homologue of the proline-specific permease Put4p called PutD can also be found in the *Aspergilli* with similarities of approximately 40% to the yeasts and *Neurospora*, but can not be found in higher eukaryotes.

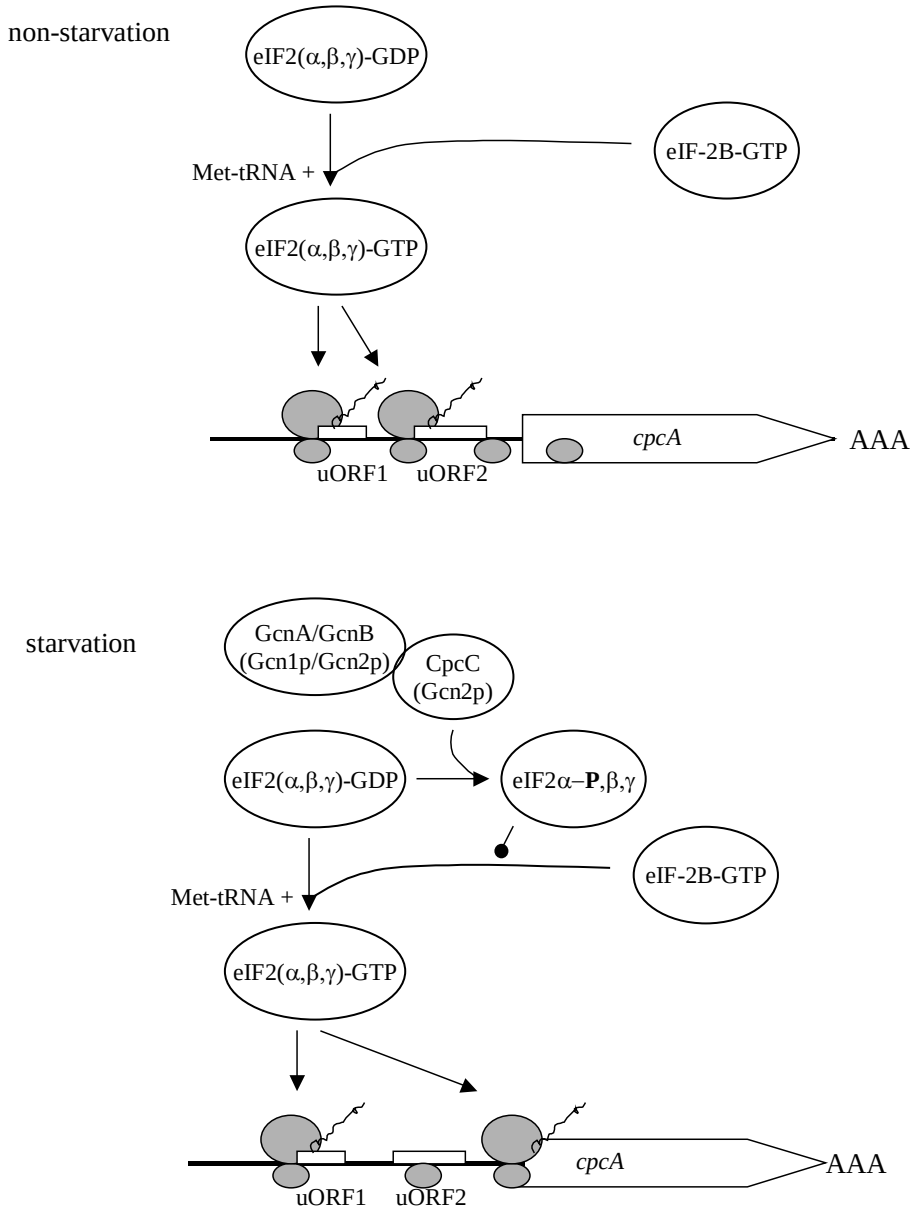


Fig. 12: Translational control of the gc/cpc regulator mRNA CpcA
Amino acids are either available or fungal cells starve for amino acids. Under non-starvation conditions CpcC is not active. When cells endure amino acid limitation, uncharged tRNA molecules are sensed by CpcC in cooperation with GcnA/GcnB. CpcC then phosphorylates the α subunit of the eukaryotic initiation factor eIF2. Phosphorylated eIF2a inhibits the eIF-2B complex, which acts as a guanine nucleotide exchange factor for eIF2. Inhibition of eIF-2B results in a global downregulation of translation due to lower amounts of charged tRNAs and GTP. The low amount of ternary complexes under starvation conditions leads to delayed reinitiation of the reassembled ribosome. This in turn leads to increased translation of the CpcA ORF instead of the uORFs under non-starvation conditions. The respective *S. cerevisiae* homologues to the *Aspergillus* proteins are shown in brackets (Hinnebusch *et al.*, 2004).

3.4.2.2 *Aspergillus* amino acid uptake systems in comparison to mammalian counterparts

In mammalian systems nutrients, especially amino acids, play an important role in the regulation of endocrine processes since mammalian systems are unable to *de novo* synthesize essential amino acids. In mammalian cells an SPS-like system is not present and it is not entirely clear how external signals are processed to the nucleus to react. Since there is no SPS system the sensing of amino acids in *Aspergillus*, sensing needs to work in a manner different from yeast. It might be possible that sensing of amino acids and propagation of the resulting signals takes place in the same manner as in cells of higher eukaryotes. It was shown that amino acid signals have a major impact on growth and development next to sensing the nutritional status and regulating transport of amino acids into the cell. This implies that amino acid transport might be similar to that in cells of higher eukaryotes, as well.

The *Aspergillus* genomes were searched for different mammalian amino acid transporter systems. We were able to find *Aspergillus* proteins that resemble different transporter families. In general similar proteins for each characterized amino acid transporter SlcA-F could be found in the genome of *N. crassa* or *M. grisea* (see table 1) with identities over 60%.

Blast searches in the genomes carried out with members of the human SLC7 or L* family revealed proteins in all three *Aspergillus* genomes we named SlcA. They display a 30% similarity to the human LAT1 transporter and 22% identity to a so far uncharacterized amino acid transporter of *Drosophila*. Similarities to any yeast protein are below 20%. LAT1 is supposed to be an ubiquitously expressed transporter acting as an environmental amino acid sensor (Padbury *et al.*, 2004; Verrey *et al.*, 1999). It is characterized to transport mainly large hydrophobic amino acids like H, M, L, I, V, F, Y, W and Q (Hyde *et al.*, 2003).

The SlcB protein was only found in *A. nidulans*. No gene coding for a similar protein was found throughout the genomes of the other *Aspergilli*, nor could any similar proteins be found in the investigated yeasts and the identity to *Drosophila* proteins was below 20%. The SlcB protein shows 33% identity to the human EAA1 protein of the human system X^-_{AG} (SLC1 family) (Hyde *et al.*, 2003). Members of the SLC1 family K+ antiport glutamic acid and aspartic acid into the cells (Hyde *et al.*, 2003). They are present in mammalian astrocytes (Gonzalez-Gonzalez *et al.*, 2005). EAA1 plays an important role in astrocyte development, as was shown for diseases like lissencephaly (Furuta *et al.*, 2005).

The search for proteins similar to mammalian system imino transporters (SLC36) three proteins for each *Aspergillus* were found named SlcC, SlcE and SlcF, with relatively low homologies to the mammalian imino system transporters. SlcC is 30% similar to the so far uncharacterized hypothetical transporter FLJ39822, but shows 45% identity to yeast Avt2p (Russnak *et al.*, 2001). SlcE and SlcF each show approximately 33% identities to human proteins of the imino group and 50%/45% identities to the yeast Avt3 proteins (Russnak *et al.*, 2001). The yeast proteins Avt2/3 and system imino transporters are involved in the transport of small neutral amino acids (Q, N, I, L and Y) from the lysosome/vacuole after bulk degradation of proteins (Russnak *et al.*, 2001). So far known members of the imino family were shown to be involved in growth regulation by influencing the TOR pathway in *Drosophila* (Goberdhan *et al.*, 2005). These findings indicate at least a role for these three *Aspergillus* transporters in a similar function to the Avt2/3p of yeast.

A search for System BETA transporter revealed homologues only in human (32% identity), fly (29% identity) and *N. crassa*. An *A. fumigatus* homologue of these systems could not be found. The proteins found were named SlcD. System BETA transporters of the SLC6 family are known to transport GABA, glycine and glutamate (Hyde *et al.*, 2003; Wipf *et al.*, 2002).

In summary, we found six different amino acid transporters, which are all present in *A. nidulans*, whereas *A. oryzae* lacks SlcB and *A. fumigatus* SlcB and SlcD. Phylogenetic analyses of the three *Aspergilli* and *Neurospora* show that these fungi originate from a common ancestor and that *A. nidulans* and *N. crassa* separated first from the ancestor, the other two *Aspergilli* separated later from each other (Galagan *et al.*, 2005). The transporters SlcC/E/F resemble transporters that are known to transport amino acids to or from the lysosome, which indicates the cellular function of these proteins (Boll *et al.*, 2004; Russnak *et al.*, 2001). SlcA is similar to the human LAT1 APC transporter, indicating a possible role in extracellular amino acid sensing (Padbury *et al.*, 2004). SlcB is only present in *A. nidulans* and *N. crassa* indicate that the other *Aspergilli* lost the gene encoding SlcB after separation from *A. nidulans*. Regarding its relationship with EAA1 it might be possible that one function of this protein might be involvement in (polar)growth in *A. nidulans* besides amino acid uptake (Furuta *et al.*, 2005; Zagami *et al.*, 2005). The gene coding for SlcD is not present in *A. fumigatus*, which indicates a loss of the gene after separation from *A. oryzae*.

3.4.3 Regulation of fungal protein biosynthesis in response to intracellular amino acid availability

3.4.3.1 Regulation of expression of the central transcription factor of gc/cpc in *Aspergillus* species

Due to the special structure of the promoter of the transcription factor CpcA of the gc/cpc of *Aspergillus* the translation of the *cpcA*-mRNA is increased under starvation conditions resulting in more CpcA protein (Hoffmann *et al.*, 2001b). CpcA is able to positively autoregulate its own transcription, as well as the transcription of amino acid biosynthesis genes through CpcA recognition elements (CPREs) under starvation conditions (Hoffmann *et al.*, 2001b). The orthologs of CpcA are hard to find at a first glance. Similarities are mainly found in the C-terminal leucine zipper region of CpcA which is responsible for DNA-binding. The leucine zipper motif is conserved throughout all organisms from fungus to man. The leucine zipper can also be found in the human c-Jun and Fos-family of proteins, thus these proteins are counted to the c-Jun like family. The leucine zippers though consisting of 1 to 5 leucine residues are fully interchangeable and restore functionality in complementation experiments (Kouzarides and Ziff, 1989).

CpcB of *A. nidulans* is a Gβ-like protein homologous to the mammalian RACK1 repressing the transcription of CpcA under non-starvation conditions (Hoffmann *et al.*, 1999). The protein is constitutively expressed in *A. nidulans* (Hoffmann *et al.*, 1999). The yeast homologue Cpc2 was shown to interact directly with the 40S subunit of the ribosome providing a platform for other ribosome bound proteins during translation with their propeller-like WD40 repeats at the mRNA exit site of the ribosome (Nilsson *et al.*, 2004; Sengupta *et al.*, 2004; Shor *et al.*, 2003). RACK1 and its homologues are found to be highly conserved in all organisms investigated.

Interestingly the third intron of *A. nidulans cpcB* is conserved in the *S. cerevisiae CPC2* and *N. crassa cpc-2* genes and harbors the U24 small nucleolar RNA (snoRNA) coding region (Hoffmann *et al.*, 1999; Qu *et al.*, 1995). The U24 snoRNA is required for site specific 2´-o-methylation of 25S rRNA (Nazar, 2004). Though the coding region can be found in the ascomycete *A. nidulans* its deuteromycete family members *A. fumigatus* and *A. oryzae* do not harbor the coding region of the U24 snoRNA anywhere near CpcB.

3.4.3.2 The elongation initiation factor eIF-2 of *Aspergillus*

For initiation of translation the eukaryontic ribosomal pre-initiation complex scans mature mRNA towards the 3´-end. On arrival at the AUG Start codon the 80s ribosomes finally assemble (Preiss and Hentze, 2003). The translation machinery needs phosphorylated eIF-2γ, which is subsequently dephosphorylated during translation and it needs to be recycled by the guanine nucleotide exchange factor GEF (eIF-2B) to maintain steady translation (Preiss and Hentze, 2003). By phosphorylation to further activate eIF-2γ the translation is repressed due to stoichiometrically decreasing GEF and a subsequent lack of the initiation factor (Clemens, 1994; Kapp and Lorsch, 2004; Preiss and Hentze, 2003). Each of the components of the initiation factor eIF-2 share high similarities to the components of the initiation factors of other organisms. Among the *Aspergilli* the components of the involved factors can be found easily, indicating a similar mechanism of translation control as in yeast. The alpha, beta and delta subunits of the GEF eIF-2B are well conserved between all compared organisms. The epsilon subunit is better conserved in the investigated fungi than in man, fly and plant, with similarities to its fungal counterparts of >42% and to the other eukaryotes <30%. The gamma subunit of eIF-2B is not too well conserved at all, though orthologs can be found in all compared organisms, the highest homology is shared with *N. crassa* followed by *H. sapiens*. The eukaryotic translation initiation factor eIF3 functions by interacting with eIF2 and stabilizing the interaction between the ternary complex (composed of eIF2·GTP·Met-tRNA$_i$) and the 40 S ribosomal subunit, thereby forming the 43 S ribosomal complex (Chaudhuri *et al.*, 1999). Twelve well conserved subunits of eIF3, eIF-C(a-l) can be found in the three *Aspergilli*, whereas only six are present in the yeast *S. cerevisiae* and only nine in *S. pombe*. Nearly no conservation at all can be found for subunit eIF3j to the higher eukaryotes, but it is found highly conserved to its counterpart of *N. crassa*. The eIF3, the lid of the proteasome and the Cop9 signalosome are regulatory multiprotein complexes whose components can be characterized through the specific PCI (proteasome, Cop9, eIF3) or MPN (Mpr1p, Pad1 N-terminal) protein domains (see table 1, see chapter 2)(Glickman *et al.*, 1998; Kapelari *et al.*, 2000; Kim *et al.*, 2001).

3.4.3.3 Transport of the transcription factor into the nucleus

Nuclear import is essential for Gcn4p to induce transcription of numerous genes whose products are involved in many different biosynthetic pathways in *S. cerevisiae* during response to amino acid starvation, glucose starvation and other stresses (Engelberg *et al.*, 1994; Hinnebusch and Natarajan, 2002; Mösch *et al.*, 1990; Natarajan *et al.*, 2001). CpcA as counterpart of Gcn4p in *A. nidulans* needs to be transported to the nucleus, as well, to play its role as a transcription factor during amino acid starvation response (Pries *et al.*, 2002). It was shown by Pries *et al.*, 2004 that the α-importin Srp1p and the β-importin Kap95p act as a heterodimer to channel Gcn4p into the yeast nucleus via the nuclear pores. So far no experimental procedures have been carried out to find whether similar proteins take over this task in *A. nidulans* or any other fungal species. Blast searches in the now available *Aspergillus* and *N. crassa* genomes revealed that proteins with high identities to Srp1p (over 58%) and Kap95p (over 39%) can be found to be encoded in the available *Aspergillus* and *Neurospora crassa* genomes. The assigned ORF for the Srp1p homologue SrpA was annotated as AN2142.1 and the homologue to the Kap95p homologue KapA was annotated as AN0906.1 during the automated and manual annotation of the *A. nidulans* genome. Blast searches in the available *Aspergillus nidulans* genome revealed that there is only one α-importin like protein and twelve members of the importin beta superfamily present (see chapter 4), which means that the *Aspergillus* genomes harbor one less beta importin member than yeast.

3.4.3.4 Regulation of the protein level of the central transcription factor of the gc/cpc

The yeast transcription factor Gcn4p is a highly unstable protein with a half-life of apr. 5 minutes under non-starvation conditions. A well conserved PEST region (Kornitzer *et al.*, 1994) and ubiquitinylation sites are responsible for the instability of this protein and its subsequent ubiquination by the SCF complex and degradation in the 26s proteasome (Irniger and Braus, 2003; Meimoun *et al.*, 2000). Nothing is known about the half-life of its fungal counterparts so far. The cyclin dependent kinases Pho85p and Srb10p are responsible for destabilisation of the transcription factor (Irniger and Braus, 2003; Qiu *et al.*, 2004). The homologues PhoA and SrbA were found to be highly conserved in all investigated organisms, still a homologue of SrbA can not be found in *A. thaliana*. PhoA shows 81% identity to the

N. crassa protein and 72% and 67% to the proteins of *S. pombe* and *S. cerevisiae* respectively. The identity compared to the proteins of higher eukaryotes is >55%. SrbA was found to be 51% identical to *N. crassa* and 45% and 49% identical to the respective proteins of *S. pombe* and *S. cerevisiae*. The respective proteins of *Drosophila* and human showed 36% and 42% identity respectively. We could find a corresponding cyclin Srb11p, named SrbB in the *Aspergilli*, but like in all investigated cyclins the identity to other proteins was generally low (<40%) only the proteins of *N. crassa* and *S. cerevisiae* showed identities of 47% and 42% respectively. Under non-starvation conditions Pcl5p is required for Pho85p mediated Gcn4p degradation in yeast (Shemer *et al.*, 2002). The cyclin dependent kinase Pho85p was shown to phosphorylate Gcn4p at T165 and thus mark it for ubiquitination and further degradation at the 26s proteasome (Meimoun *et al.*, 2000). Ten different cyclins are known to interact with the cyclin dependent kinase Pho85p. A search throughout the genome of the *Aspergilli* revealed relatively low homologies for a cyclin further called PclA with identities of 53% and 35% to yeast the proteins Pcl1p and Pcl2p respectively. PclL displayed 46% identity to Pho80p and 71% identities to Nuc-1 of *N. crassa*. PclE displayed a low identity of only 34% to Pcl5p and even lower identities of 34% and 28% to the proteins of *Arabidopsis* and *S. pombe*. The identity to the *Neurospora* protein is 45%. PclF resembles Pcl6p and Pcl7p with higher identity to Pcl6p (38%) and high identity to the *Neurospora* protein (60%). PclH shows less than 20% identity to Pcl10p, but 28% identity to Pcl8p. The *Neurospora* protein is 60% identical to PclH. PclI is 49% identical to Pcl9p and the respective *Neurospora* protein, whereas PclK shows only 26% identity to Clg1p and 36% identity to its other fungal counterparts. Generally it has to be remarked that the cyclins are relatively weakly conserved among the investigated organisms. Comparing the *Aspergillus* CpcAs and Gcn4p of *S. cerevisiae* one finds the phosphorylation site T165 well conserved through the *Aspergilli*. It is notable that the phosphorylation site is exchanged from threonine to serine in the *Aspergilli* and that the site is not conserved in *N. crassa* (Figure 13).

```
132  V K A E P T V S S P T V K P V S S P A R S    CpcA  A. nidulans
143  A K E I S V P P S P A V G K S A S P A P S    CpcA  A. fumigatus
160  V K A D V T P A S P M I R T T S S R A T S    CpcA  A. oryzae
157  V S T T S F L P T P V L E D A K L T Q T R    Gcn4p S. cerevisiae
140  V Q S V Q P T V Q P T V E Q T V H S V E A    CPC1  N. crassa
```

Fig. 13: The degradation-related phosphorylation site of CpcA-like proteins is highly conserved
In yeast it was shown that phosphorylation of Gcn4p at Thr165 leads to rapid degradation of the protein (Meimoun *et al*., 2000). Multialignments of the phosphorylation site Thr165 of *S. cerevisiae* with their homologues of *Aspergillus species* and *Neurospora crassa* show that the phosphorylation site is highly conserved from yeast to *Aspergillus*, though instead of threonine the amino acid serine is used as a phosphorylation site in *Aspergilli.* The respective amino acids are depicted in bold. In *N. crassa* no conservation of this site can not be found, though neighbouring threonines (*italic*) might play a role in phosphorylation and further degradation of CPC1.

Phosphorylated Gcn4p is subsequently ubiquitinylated by the SCF^{Cdc4} ligase which targets it to degradation by the 26S proteasome, whereas the SCF complex is constitutive and Gcn4p stability is subject to the phosphorylation state of Gcn4p mediated by Pho85p (Kornitzer *et al.*, 1994; Meimoun *et al.*, 2000). The E3 ubiquitin ligase specific for Gcn4p consists of the cullin Cdc53p, Skp1p, binding the specific F-box protein, the RING-H2 protein Hrt1p and the specificity protein Cdc4p (Irniger and Braus, 2003)(Figure 14). We were able to identify homologues of these proteins in all three *Aspergilli* under the names CulA, SkpA (also known as SconC (Piotrowska *et al.*, 2000)), HrtA and CdcD respectively. SkpA or SconC was earlier characterized as a Skp1p-like protein interacting with the F-box protein SconB as negative regulators of the sulphur-metabolism in *A. nidulans* (Piotrowska *et al.*, 2000). The cullin and the specificity protein CdcD are highly conserved among all organisms with overall identities of >56% for the cullin and >40% identities among compared F-box proteins. We were unable to find a homologue of CdcD in plant. The *Aspergillus* genomes also revealed Rub1p homologues with identities >50% to the proteins of the other compared organisms (S. Busch, personal communication).

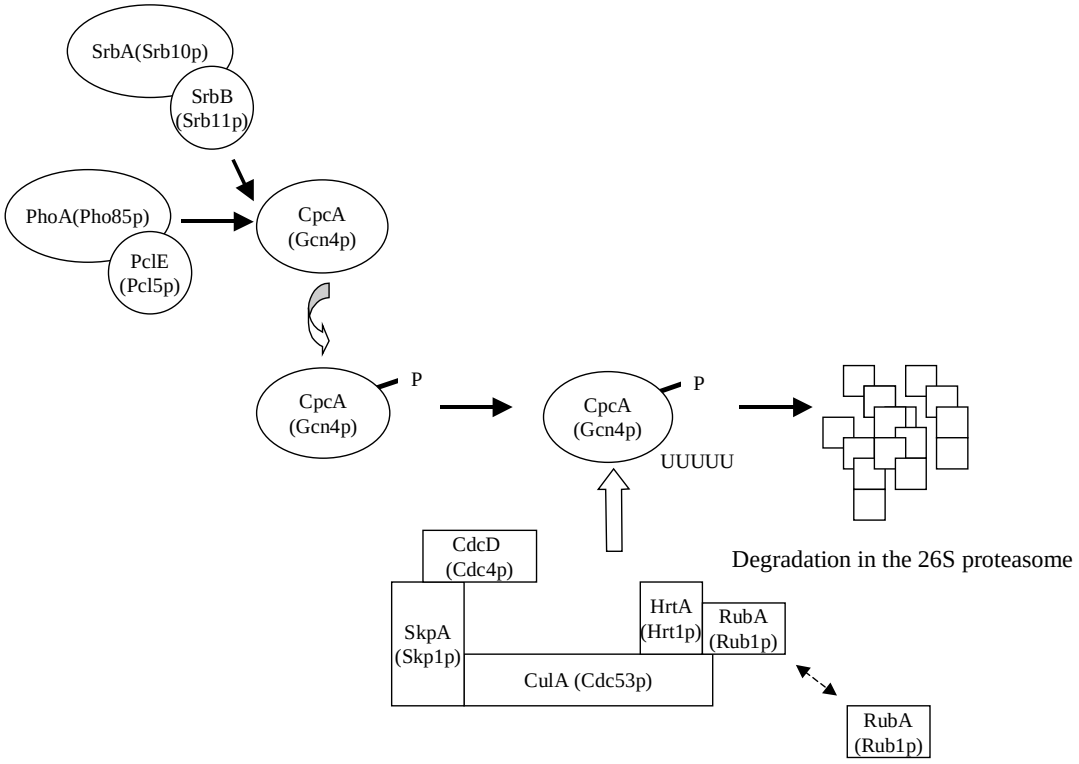


Fig. 14: Degradation of the transcription factor CpcA
Degradation of CpcA is initiated by phosphorylation of specific threonine residues. The CpcA degradation is triggered by two cyclin dependent kinases SrbA and PhoA. The second step of the degradation is the poly-ubiquitinylation of CpcA mediated by the E3 ubiquitin ligase complex SCF^{CdcD}, targeting the protein for degradation at the 26S proteasome. The SCF^{CdcD} activity is regulated by dynamic neddylation/deneddylation (see chapter 2).

3.4.4 Fungal amino acid biosynthesis investigated on selected examples

Since all three *Aspergilli* are able to grow without amino acid supplementation they should be able to synthesize all amino acids. We had a closer look at five biosynthetic pathways – the biosynthesis of histidine and lysine accompanied by the putative production of the branching secondary metabolism – the penicillin biosynthesis – and the formation of tryptophan, tyrosine and phenylalanine. For the ease of reading the respective names of the *S. cerevisiae* proteins have been added in brackets to their respective *Aspergillus* homologues.

3.4.4.1 Histidine biosynthesis

Histidine is produced by six biosynthetic enzymes (EC numbers are added to the attached table, suggested names were also added) HisA (His1p), HisB (His3p), HisC (His4p), HisD (His2p), HisE (His5p) and HisHF (His7p) starting from PRPP (Figure 15). HisA is an ATP phosphoribosyltransferase forming phosphoribosyl–ATP. The next two steps are accomplished by HisC (trifunctional histidinol dehydrogenase) forming first phosphoribosyl–AMP, then phosphoribosylformimino AICAR-P, the branchpoint to the purine metabolism. HisHF forms imidazolglycerole-3-phosphate (Künzler *et al.*, 1993; Valerius *et al.*, 2001). The next step is taken over by HisB forming imidazoleacetol-phosphate (Busch *et al.*, 2001). HisE then forms L-histidinol-phosphate which is converted to L-histidinol by HisD. The last two steps are accomplished by HisC forming first L-histidinal, then L-histidine. The genes for histidine biosynthesis were found to be highly conserved amongst the three *Aspergillus* species.

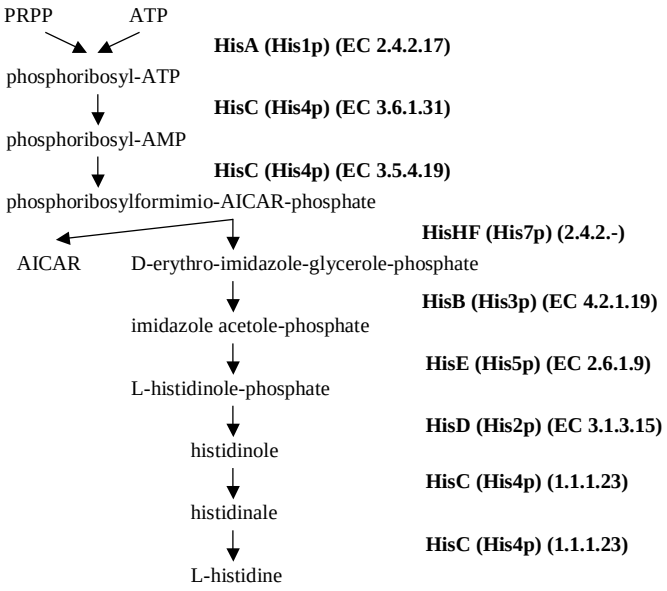


Fig. 15: Histidine biosynthesis and associated enzymes
The catalytic steps of the histidine biosynthesis are described. The enzymes catalysing the enzymatic reactions are depicted in bold. The respective *S. cerevisiae* homomlogues to the *Aspergillus* proteins are shown in brackets.

3.4.4.2 Lysine and penicillin biosynthesis of *Aspergilli*

The amino acid lysine is produced from aspartate through the diaminopimelate (DAP) pathway in most bacteria and higher plants. In fungi, in the thermophilic bacterium *Thermus thermophilus*, and in several archaeal species, lysine is synthesized by a completely different pathway called the α-aminoadipate pathway (Nishida *et al.*, 1999). In *A. nidulans* the penicillin biosynthesis pathway branches from the lysine pathway. It was suggested that upon amino acid starvation the cross-pathway control overrules penicillin biosynthesis and favors lysine production (Busch *et al.*, 2003) (Figure 16).

Lysine biosynthesis starts with homocitrate which is converted by LysF (Lys4p) to Homo-Isocitrate, which is then turned over by LysE (Lys12p) to 2-Oxoadipate. LysD then forms L-2-Aminoadipate from 2-Oxoadiapate. L-2-Aminoadipate-6-semialdehyde is then formed by LysB (Lys2p) from L-2-Aminoadipate. The Saccharopine dehydrogenase LysG (Lys9p) then forms L-Saccharopine, which is then made to L-Lysine by LysA (Lys1p). All necessary enzymes can be expressed in all three *Aspergilli*. One can observe that the proteins of the penicillin metabolism as a secondary metabolism starting from L-2-Aminoadipate are only present in *A. nidulans* and *A. oryzae*. Penicillin is synthesized in a three step reaction starting from L-2-Aminoadipate mediated by the enzymes AcvA, IpnA and AatA, leading to the assumption that *A. oryzae* is able to produce penicillin, as well as *A. nidulans*, whereas *A. fumigatus* lacks the necessary enzymes.

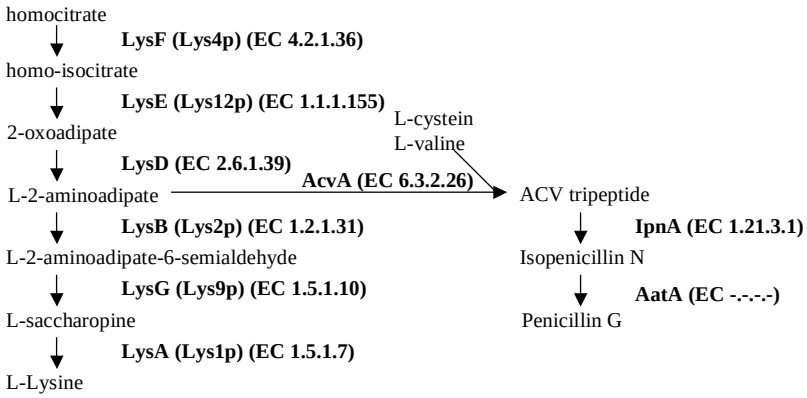


Fig. 16.: The α-aminoadipate pathway of lysine biosynthesis and penicillin biosynthesis branch of *A. nidulans* and *A. oryzae*
The catalytic steps of the lysine and penicillin biosynthesis are described. The enzymes catalyzing the enzymatic reactions are depicted in bold. At the α-aminoadipate step the cells are able to choose between two biosynthetic ways. Penicillin synthesis could either be reduced to favor lysine biosynthesis or increased to kill food sources and use them as amino acid or nitrogen sources. The respective *S. cerevisiae* homologues to the *Aspergillus* proteins are shown in brackets.

3.4.4.3 Aromatic amino acid biosynthesis

The biosynthetic cascade resulting in the aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan links carbohydrate metabolism to biosynthesis of aromatic compounds. Whereas animals are only able to form tyrosine by hydroxylation of phenylalanine and therefore require this amino acid together with tryptophan in their diet, bacteria, plants and fungi are competent to synthesize all three aromatic amino acids *de novo* (Sirtori *et al.*, 2005). The shikimate pathway leads to the formation of chorismate, the last common intermediate of the three pathways (Quevillon-Cheruel *et al.*, 2004). After that the three pathways divide into the tryptophan biosynthetic branch and the biosynthesis of prephenate, which then divides up into the biosynthetic branch of phenylalanine and tyrosine production (Figure 17).

3.4.4.3.1 Tryptophan biosynthesis

The intermediate chorismate is transformed in a two step reaction to anthranilate by the anthranilate synthase TrpA (Trp2p) and the trifunctional glutamine amidotransferase/N-(5´-phosphoribosyl)anthranilate isomerase/indole-3-glycerol transferase (Hütter *et al.*, 1986). TrpD (Trp4p), the phosphoribosyl transferase then forms N-(5-phospho-b-D-ribosyl)-anthranilate, which is then subsequently transformed to 1-(2-carboxyphenylamino)-1-deoxy-D-ribulose-5-phosphate and (3-indolyl)-glycerol phosphate by TrpC (Trp3p) (Yelton *et al.*, 1983). The final step, the formation of tryptophan, is taken over by TrpB (Trp5p) the phosphoribosyl transferase (Hütter and DeMoss, 1967).

3.4.4.3.2 Tyrosine and phenylalanine biosynthesis

After the formation of prephenate from chorismate by AroC (Aro7p) (Andrews *et al.*, 1973), the chorismate mutase, the phenylalanine/tyrosine specific branch of the aromatic amino acid biosynthesis branches in two alternative routes to form the end products (Krappmann *et al.*, 1999). One proceeds via the formation of phenylpyrovate by PhaA (Pha1p), a dehydratase followed by transamination by AroH (Aro8/9p) to phenylalanine (Jensen and Fischer, 1987). On the other hand 4-hydroxyphenylpyrovate is formed by TyrA (Tyr1p), which is subsequently transaminated by AroH to tyrosine (Jensen and Fischer, 1987).

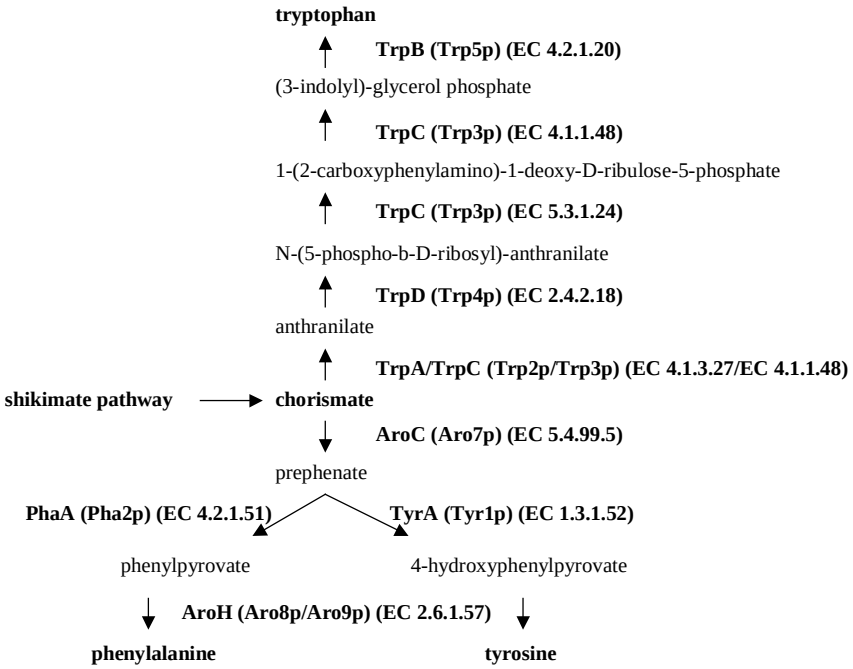


Fig. 17.: The aromatic amino acid biosynthesis of *Aspergillus* species
The catalytic steps of the tryptophan, phenylalanine and tyrosine biosynthesis are described. The enzymes catalyzing the enzymatic reactions are depicted in bold. The shikimate pathway leads to the last common intermediate of the aromatic amino acid biosynthesis, chorismate, where the pathways divide into the tryptophan branch and the tyrosine/phenylalanine branch. The respective *S. cerevisiae* homologues to the *Aspergillus* proteins are shown in brackets.

3.5 Discussion

The *A. nidulans* genome sequence and our comparative analysis with the genome sequences of *A. fumigatus* and *A. oryzae* was shown in comparison to higher eukaryotes and other fungi. The genomic annotation in context with general control and cross-pathway control opens new perspectives and broadens our field of view on this field of research. A complex overview of the gc/cpc related genes and proteins is given in this comparative paper. The regulation of transcription, translation and post-translational regulation of the central transcription factor CpcA and in addition the uptake and sensing of amino acids were being addressed.

We were able to *in silico* identify the central components of a gc/cpc that are yet only known from *S. cerevisiae*. On the other hand we found that other systems accompanying the gc/cpc partly differ from yeast, mainly on behalf of the transport and sensing of extracellular amino acids. Interestingly we found that the elements of a basic system like the translation machinery much more resemble their orthologs of higher eukaryotes than the compared yeasts. It was shown by Hoffman *et al*., 2001 that CpcA is capable of autoregulating its own transcription under amino acid starvation conditions. Under non-starvation conditions this autoregulatory effect is inhibited by CpcB in a yet unclear mechanism (Hoffmann *et al.*, 2000). It could be shown, that necessary genes and their respective proteins needed for translational regulation of the expression of CpcA are available in all three *Aspergilli*. We were able to identify the sensor kinase CpcC which is presumably able to sense the availability of intracellular amino acids and phosphorylate eIF-2α, a part of the translation machinery, under amino acid starvation conditions which in turn represses translation in general, though the translation of CpcA increases. The known parts of the elongation initiation factor 2 known to be involved in gc/cpc were identified in the *Aspergilli*. Generally we found the genes for the subunits of the elongation initiation factor well conserved towards the other compared fungi, whereas the guanine nucleotide exchange factor eIF-2Bε subunit is far more similar to the orthologs of higher eukaryotes. Interestingly the epsilon subunit is not only the largest but also the catalytic subunit of the complex (Gomez *et al.*, 2002). It was shown that mutations in the gene for eIF-2Bε can exhibit a decrease in complex formation following decreased GTP/GDP exchange rate resulting altered mRNA transcription and lead to leukoencephalopathy, the vanishing of white matter (VWM), which is a severe inherited human neurodegenerative disorder in man (Li *et al.*, 2004). Due to the high similarity of the respective proteins in *Aspergillus*, an easily genetically manipulated organism, genetic and

biochemical research on the effect of mutations in the respective genes may help understand the manifestation of this wasting desease.

All twelve subunits of the elongation factor eIF3 were found to be present in *Aspergillus*, in contrast to *S. cerevisiae* where only 6 subunits can be found and to *S. pombe*, where only nine of the subunits can be found. The subunits eIF3j and eIF3k were only very weakly conserved to those of higher eukaryotes and one can only speculate if the genome harbors genes encoding for these proteins due to the lack of physical evidence.

According to our data the transport into the nucleus of the central transcription factor of the gc/cpc might be similar to the mechanisms taking place in yeast, at least the necessary factors are present in the *Aspergilli*. Nothing is so far known about the half-life of the protein in the nucleus or in the cytosol. The presence of putative proteins involved in yeast in Gcn4p targeting and degradation in the 26S proteasome in *Aspergillus* indicates similar mechanisms for CpcA degradation. An additional hint is the conservation of the phosphorylatable Thr165 residue, though in all investigated *Aspergilli* this residue was found to be exchanged for a serine residue. Interestingly an alignment of this protein region shows that this phosphorylatable residue is not conserved in *N. crassa*, though phosphorylatable threonine residues can be found in the direct vicinity of the expected spot (Figure 13).

Several mechanisms have been described and proposed for different amino acid uptake systems in mammalian cells. These amino acid uptake systems seem in general not only regulatable, some of them seem to transmit signals of amino acid abundance to directly or indirectly regulate corresponding cellular responses. These mechanisms are so far not well understood. We were able to identify at least four proteins SlcB and SlcC/E/F *in silico* that may have a similar effect on development and growth as their heigher eukaryotic relatives. SlcB resembles transporters of the SLC1 family. The function of EAA1, a member of the SLC1 family, was recently shown to have a direct effect on the morphology of astrocysts, star-like glial cells. Dysregulation of this glutamate transporter expression leads to disorganized cortex formation and altered astrocytic phenotypes, as was shown for type II lissencephaly patients and cell lines (Furuta *et al.*, 2005; Zagami *et al.*, 2005). On the other hand do imino amino acid transporters directly or indirectly influence cellular growth in fly through the TOR pathway (Goberdhan *et al.*, 2005). *Aspergillus* has so far proven to be a good model for amino acid dependent growth and regulation, since a dysregulation of intracellular amino acid biosynthesis leads to an arrest in fruitbody formation (Busch *et al.*, 2001; Eckert *et al.*, 1999; Hoffmann *et al.*, 2000; Valerius *et al.*, 2001). In this context it would be interesting to find new mechanisms reacting to amino acid starvation conditions that

have an influence on development and growth. The mechanisms of amnio acid regulated growth and development regulation are of great therapeutic interest since there are a lot of pathological circumstances associated with dysregulation of amino acid metabolism (anthropomorphic lateral sclerosis, altered amino acid availability/transport in tumor cells and tissue response to insulin). Nutritional or pharmaceutical intervention through such mechanisms would be of great benefit. Thus the findings of amino acid transporters similar to those of mammals in filamentous fungi (but not in other lower eukaryotes) may open the way for another field of research for these model organisms.

As examples of amino acid biosyntheses we were able to prove the existence of the necessary genes encoding for the proteins for histidine, lysine, tyrosine, tryptophan and phenylalanine biosynthesis, demonstrating that the investigated *Aspergilli* are able to produce these amino acids if no extracellular sources are available. In contrast, mammals are unable to produce all amino acids and have to rely on taking essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, valine) and semi-essential amino acids, that can substitute for essential amino acids under certain conditions (arginine, histidine, tyrosine, cysteine), up from their diet. In secondary metabolism *A. nidulans* is known to produce penicillin utilizing an intermediate of the lysine biosynthesis. Strikingly the genes encoding for the proteins conducting the enzymatic reactions to produce penicillin could be found in *A. oryzae*, but not in *A. fumigatus* rendering it unable to produce penicillin.

Concluding, we find that the basic regulatory cascade of regulation of amino acid biosynthesis is very similar to that of higher eukaryotes, if a little bit less complex regarding the activation of target genes. External (SPS-system) or internal (GCN2, TOR) sensors sense amino acid abundance and react to depletion by lowering the overall translation rate through eIF2. This leads to increased expression of transcription factors like Gcn4p, CpcA or CHOP and increased transcription of target genes. The transcription factor of the gc/cpc, the regulation of cellular expression and probably regulation of its stability seem rather to resemble those of yeast and other fungi, than higher eukaryotes. The sensing and uptake system of amino acids at least in part is more complex than in yeast. Amino acid transporters were found that are not present in yeast, but in higher eukaryotes like human and a yeast-like SPS amino acid sensing system is not present. This might indicate ways of uptake and sensing that are similar to those of higher eukaryotes and might have an impact on development and growth.

Table 1: Homology of *A. fumigatus* and *A. oryzae* to *A. nidulans* genes. Similarities with e-values < E-50 are shaded green, e-values < E-20 are shaded orange and e-values > E-20 are shaded yellow

	Protein	Coding sequence	*H. sapiens*	*A. thaliana*	*D. melanogaster*	*N. crassa*	*S. pombe*	*S. cerevisiae*
an	CpcA	AN3675.1				2,00E-09 CPC-1		3,00E-07 Gcn4p
af		58.m08917						
ao		20032.m00013						
an	CpcC	AN2246.1	0 eIF2a kinase	2,00E-58 Gcn2p homologue	2,00E-76 eIF2a kinase	0 Gcn2p	1,00E-154 CPC-3	0 Gcn2p
af		71.m15880						
ao		20163.m00313						
an	CpcB	AN4163.1	1,00E-139 GNB2L1	1,00E-118 AAL34190.1	1,00E-129 Rack1	1,00E-177 CPC-2	1,00E-136 Cpc2	3,00E-98 Cpc2p
af		58.m07362						
ao		20179.m00653						
an	eIF2b (Sui3p)	AN2992.1	3,00E-50 EIF2beta	2,00E-46 EIF2 beta	2,00E-47 EIF2 beta	2,00E-91 EIF2 beta	5,00E-63 EIF2 beta	3,00E-62 EIF2 beta Sui3p
af		59.m09022						
ao		20174.m00396						
an	eIF2α (Gcn3p)	AN3156.1	4,00E-71 EIF2 alpha	1,00E-72 EIF2 alpha	2,00E-65 EIF2 alpha	4,00E-118 EIF2 alpha	3,00E-97 EIF2 alpha	4,00E-95 EIF2 alpha Gcn3p
af		59.m08547						
ao		20093.m00085						
an	eIF2γ (Gcd11p)	AN4470.1	0 EIF2 gamma	4,00E-173 EIF2 gamma	0 EIF2 gamma	0 EIF2 gamma	0 EIF2 gamma	0 EIF2 gamma Gcd11p
af		58.m07860						
ao		20142.m00265						
an	eIF2-Bχ (Gcd1p)	AN0978.1	8,00E-20 EIF2B3	0,001	5,00E-08 AAG38016	4,00E-49 CAD21057	2,00E-10 Tif223	5,00E-10 Gcd1p
af		70.m15616						
ao		20178.m00545						
an	eIF2-Bδ (Gcd2p)	AN6864.1	5,00E-76 EIF2B4	1,00E-71 AAC23414	5,00E-62 EAL25620	2,00E-73 CAE76139	1,00E-89 CAA91965	1,00E-79 Gcd2p
af		71.m15275						
ao		20151.m00246						
an	eIF2-Bβ (Gcd7p)	AN1344.1	6,00E-53 EIF2B4	2,00E-58 AAF20216	2,00E-44 NP_570020	2,00E-66 CAD71011	1,00E-76 CAB52277	1,00E-56 Gcd7p
af		70.m14889						
ao		20084.m00045						
an	eIF2-Bα (Gcn3p)	AN0167.1	1,00E-46 EIF2B1	1,00E-46 AAF02861	6,00E-42 AAG38014	5,00E-52 XP_323697	2,00E-66 CAB57849	2,00E-62 Gcn3p
af		71.m15442|					
ao		20158.m00275						
an	eIF2-Bε (Gcd6p)	manual annotation	2,00E-91 eIF2-B epsilon	2,00E-99 CAB78832	1,00E-60 AAG38017	7,00E-123 XP_331190	5,00E-129 Tif225	6,00E-101 Gcd6p
af		69.m15713						
ao		20178.m00726						
an	CpcE	AN4315.1	4,00E-165 ABCF3		2,00E-164 NP_649129	0 CAE85618	0 NP_595837	0 Gcn20p
af		58.m08009						
ao		20067.m00019						
an	CpcD	AN5840.1	0 HsGCN1	0 NP_176659	0 EAA46127	0 XP_325658	0 CAA92385	0 Gcn1p
af		72.m18976						
ao		20177.m00380						
an	eIF3a (Tif32p)	10049.m00076	e-97 NP_003741 eIF3a S10	e-83 NP_192881 eIF3a	e-78 NP_649470 eIF3 S10	0.0 XP_322126	e-135 NP_596379 eIF3p110	e-65 NP_009635 Tif32p
af		54.m06688						
ao		20175.m00540						
an	eIF3b (Prt1p)	10006.m00160	e-130 NP_874371 eIF3b S9	e-117 NP_568498 eIF3b	e-112 NP_611228 eIF3 S9	0.0 XP_330984	e-169 NP_594528 3 beta	e-121 NP_015006 Prt1p
af		54.m06392						
ao		20155.m00213						

	Protein	Coding sequence	*H. sapiens*	*A. thaliana*	*D. melanogaster*	*N. crassa*	*S. pombe*	*S. cerevisiae*
an	eIF3c (Nip1p)	10119.m00059	e-75 AAC27674 eIF3c S8	e-72 AAC83464 eIF3c	e-65 NP_611242 eIF3 S8	e-121 XP_328537	e-143 NP_593828 eIF3 p98	e-104 NP_014040 Nip1p
af		89.m02023						
ao		20153.m00210						
an	eIF3d	10129.m00312	e-93 NP_003744 eIF3d S7	e-86 NP_193830 eIF3d	e-91 NP_524463 eIF3 p66	0.0 XP_327666	e-145 NP_594625 moe1	
af		72.m19557						
ao		20114.m00102						
an	eIF3e	10051.m00454	e-106 AAC51917 eIF3e S6	e-100 NP_567047 eIF3e	e-87 NP_477385 Int6	e-162 XP_325744	e-102 NP_595367 eIF3 p48	
af		59.m08758						
ao		20175.m00410						
an	eIF3f		e-38 XP_290345 eIF3f S5	e-36 NP_181528 eIF3f	e-30 NP_649489	e-96 XP_326514	e-85 CAA16829 (CSN6)	
af		70.m14877						
ao		20074.m00037						
an	eIF3g (Tif35p)	10103.m00031	e-36 NP_003746 eIF3g S4	e-31 AAG53636 eIF3g	e-30 NP_57001	e-61 XP_328752	e-52 595727 eIF3 p33	e-32 NP_010717 Tif35p
af		72.m19937						
ao		20177.m00635						
an	eIF3h	10017.m00197	e-42 NP_003747 eIF3h S3	e-51 NP_563880 eIF3h p38	e-29 NP_524834	e-109 XP_328635	e-54 NP_593158	
af		70.m14937						
ao		20169.m00238						
an	eIF3i (Tif34p)	10051.m00544	e-99 NP_003748 eIF3i S2	e-73 NP_182152 eIF3i	e-84 NP_523478 TRIP1	e-143 XP_323195	e-120 NP_594958 eIF3 p39	e-104 NP_013866 Tif34p
af		59.m09019						
ao		20174.m00390						
an	eIF3j (Hcr1p)	10098.m00332	0.0005 NP_003749 eIF3j S1	e-0,6 NP_850918 eIF3j	0.36 NP_610541 eIF3j	e-29 XP_328660		e-07 NP_013293 Hcr1p
af		69.m15631						
ao		20178.m00416						
an	eIF3k	10051.m00602	e-19 NP_037366 eIF3k	e-15 NP_195051 eIF3k	e-18 NP_611604	e-55 XP_330357		
af		59.m08960						
ao		20174.m00289						
an	eIF3l	10101.m00131	e-115 NP_057175 eIF3l	e-79 NP_680222 IF3l	e-92 NP_648553	0.0 XP_326134		
af		72.m19170						
ao		20177.m00684						
an	PclA	AN0453.1				2,00E-48 XM_324203		3,00E-31 Pcl1p
af		54.m06646						
ao		manual annotation						
an	PclA	AN0453.1				2,00E-48 XM_324203		3,00E-31 Pcl2p
af		54.m06646						
ao		manual annotation						
an	PclL	AN5156.1		1,00E-13 NM_130038		5,00E-50 XM_328176 Nuc-1	6,00E-11 AL021747	1,00E-26 Pho80p
af		54.m06862						
ao		20128.m00091						
an	PclE	AN9500.1		6,00E-05 NM_130072		6,00E-44 XM_331476	9,00E-20 Z98975	1,00E-07 Pcl5p
af		62.m03402						
ao		20043.m00015						
an	PclF	AN3755.1		3,00E-04 NM_130038		9,00E-63 XM_331979	1,00E-13 AL021747	3,00E-26 Pcl6p
af		65.m07369						
ao		20146.m00204						
an	PclF	AN3755.1		3,00E-04 NM_130038		9,00E-63 XM_331979	1,00E-13 AL021747	3,00E-26 Pcl7p
af		65.m07369						
ao		20146.m00204						

	Protein	Coding sequence	*H. sapiens*	*A. thaliana*	*D. melanogaster*	*N. crassa*	*S. pombe*	*S. cerevisiae*
an	PclH	AN5825.1			1,00E-15	3,00E-37		1,00E-15
af		72.m18950			NM_115919	BX284753		Pcl8p
ao		20097.m00074						
an	PclI	AN0453.1				2,00E-48		3,00E-31
af		54.m06646				XM_324203		Pcl9p
ao		manual annotation						
an	PclH	AN5825.1			1,00E-15	3,00E-37		1,00E-15
af		72.m18950			NM_115919	BX284753		Pcl10p
ao		20097.m00074						
an	PclK	AN4984.1			6,00E-06	5,00E-40	2,00E-22	2,00E-18
af		59.m08886			BT015746	XM_327578	Z98270	Clg1p
ao		20125.m00144						
an	PhoA	AN8261.1	1,00E-97	3,00E-93	1,00E-97	5,00E-143	4,00E-115	2,00E-109
af		53.m03696	BC003065	AAM61706	AY061049	XM_327865	AL031535	Pho85p
ao		20147.m00190						
an	PhoB	AN4310.1	6,00E-18		3,00E-18	0		8,00E-88
af		58.m09009	BX537917		NM_175928	NUC-2		Pho81p
ao		20147.m00190						
an	SrbA	AN2489.1	8,00E-56		5,00E-53	1,00E-113	0	6,00E-81
af		59.m08500	NM_015076		NM_080487	BX294028	Z98977	Srb10p
ao		20149.m00296	CDK8		CDK8			
an	SrbB	AN2172.1	3,00E-19	5,00E-15	6,00E-21	8,00E-61	1,00E-16	6,00E-43
af		72.m19668	NM_005190	NM_124239	AE003706	AL355930	AL035085	Srb11p
ao		20180.m00841	CCNC					
an	SkpA (SconC)	10038.m00099	e-41	e-37	e-34	e-49	e-48	e-36
af		71.m15951	NP_73377	AAK26104	NP_477390	XP331383	NP_595455	AAC49492
ao		20108.m00127	SKP1	ASK10			SKP1	Skp1p
an	CulA	10015.m00090	0	e-83	e-162	0	e-176	e-116
af		70.m15237	NP_003583	NP_567243	NP_523655	XP_324561	NP_594259	NP_010150
ao		20180.m01194	CUL1	CUL1	lin19		pcu1	Cdc53p
an	HrtA	10163.m00039	e-43	e-40	e-40	e-43	e-36	e-29
af		71.m15978	NP_055063	NP_189869	NP_569852	XP_326079	NP_593388	NP_014508
ao		20108.m00096	RBX1	ROC1	ROC1A		RBX1	Hrt1p
an	CdcD	AN5517.1	4,00E-66		1,00E-66	0	6,00E-104	1,00E-77
af		69.m15442	NM_033632		NM_168073	XM_325793	Z98602	Cdc4p
ao		20178.m00790	FBW7		ago			
an	SprA	AN2142.1	7,00E-145	7,00E-145		0	1,00E-175	2,00E-151
af		72.m19699	BT009843	Impa-4		XM_326741	AL031323	Spr1p
ao		AO070343000188		Y14616				
an	KapA	AN0906.1	1,00E-135	0	0	0	0	1,00E-149
af		70.m15515	BT009797	AK117217	Ketel	XM_329200	Z98532	Kap95p
ao		AO070320000082			AJ002729			
an	TapA	AN0120.1	3,00E-07	6,00E-09	6,00E-05	1,00E-52	4,00E-26	5,00E-23
af		71.m15400	NM_001551	BX831385	NM_165039	XM_328973	AL049522	Tap42p
ao		10004.m00070						
an	TorA	AN5982.1	0	0	0	0	0	0
af		72.m19942	FRAP1	TOR-1	Tor	XM_325462	AL049558	Tor1p
ao		20177.m00675	NM_004958	AF178967	NM_080152			

	Protein	Coding sequence	*H. sapiens*	*A. thaliana*	*D. melanogaster*	*N. crassa*	*S. pombe*	*S. cerevisiae*
an	GapA	AN5678.1				1,00E-171	2,00E-124	1,00E-137
af		65.m07404				XM_325684	AL353014	Gap1p
ao		20146.m00246						
an	PutD	20127.m00101				4,00E-94	7,00E-77	1,00E-73
af		56.m02346				XM_327414	AL132779	Put4p
ao		20127.m00101						
an	NprA	AN0115.1				1,00E-135	2,00E-97	2,00E-75
af		71.m15394				XM_323687	AL023518	Npr1p
ao		20148.m00245						
an	SlcA	AN6519.1	2,00E-62		1,00E-60	0		1-e29
af		62.m03107	SLC7		NP_730005	XM_323115		Mup1p
ao		20107.m00080	LAT1					
an	SlcB	AN6782.1	5,00E-24		2,00E-18	*M. grisea*		
af		-	EAA1		NP_477427	9,00E-80		
ao		-				XM_367728		
an	SlcC	AN4428.1	2,00E-31	2,00E-28	5,00E-57	1,00E-163	1,00E-25	4,00E-96
af		58.m09004	FLJ39822	AY149936	NM_136600	XM_367253	CAA20055	Avt2p
ao		20110.m00133	(SCL36?)					
an	SlcD	AN8966.1	9,00E-49		2,00E-47	*M. grisea*		
af		-	System Beta,		SerT			
ao		20170.m00328	P30531		NM_079122	0 EAA54090		
an	SlcE	AN4477.1	2,00E-30	1,00E-27	2,00E-32	0	1,00E-130	2,00E-112
af		58.m07843	SLC36	BX828060	AY119064	XM_323083	Z68144	Avt3p
ao		20142.m00243						
an	SlcF	AN7777.1	1,00E-30	5,00E-24	2,00E-28	1,00E-144	3,00E-95	1,00E-91
af		57.m05337	SLC36	BX827846	NM_206319	XM_325629	Z68144	Avt3p
ao		20123.m00162						
an	HisA	AN3748.1				1,00E-96	1,00E-79	1,00E-86
af	EC 2.4.2.17	65.m07383				XM_332011	his1	His1p
ao		20146.m00225						
an	HisB	AN6536.1				8,00E-55	5,00E-48	5,00E-51
af	EC 4..2.1.19	62.m03136				XM_326792	AL023286	His3p
ao		20107.m00111					His5	
an	HisC	AN0797.1		1,00E-106		0	4,00E-117	0
af	EC3.6.1.31,	70.m15398		BX830420		his-3	AL353012	His4p
	EC3.5.4.19,							
	EC1.1.1.23							
ao		20076.m00051						
an	HisD	AN7044.1				5,00E-76	2,00E-58	2,00E-38
af	EC 3.1.3.15	89.m02040				XM_327259	AL031324	His2p
ao		20119.m00140						
an	HisE	AN0717.1				4,00E-154	2,00E-112	3,00E-85
af	EC 2.6.1.9	70.m15350				XM_326214	his3	His5p
ao		20180.m01047						
an	HisHF	AN7430.1		7,00E-137		0	2,00E-176	0
af	EC 5.3.1.16,	20066.m00025		hisHF		XM_327441	AL033388	His7p
ao	EC 2.4.2.-	20066.m00025						
an	LysA	AN2873.1			2,00E-04	2,00E-109	1,00E-101	2,00E-98
af	EC 1.5.1.7	59.m08722			AAR82744	LYS-1	lys3	Lys1p
ao		20175.m00452						

	Protein	Coding sequence	*H. sapiens*	*A. thaliana*	*D. melanogaster*	*N. crassa*	*S. pombe*	*S. cerevisiae*
an	LysB	AN5610.1				0	0	0
af	EC 1.2.1.31	58.m07534				CAB97293	lys1	Lys2p
ao		20138.m00117						
an	LysD	AN8519.1	7,00E-51			9,00E-27	1,00E-39	1,00E-31
af	EC 2.6.1.39	manual annotation	AADAT			XP_331508	CAA21918	Aro8p?
ao		manual annotation						
an	LysE	AN5206.1	6,00E-40	2,00E-42	2,00E-40	5,00E-116	2,00E-89	3,00E-80
af	EC 1.1.1.155	69.m14903	NP_005521	NP_850549	Q9VWH4	CAE76248	NP_594004	Lys12p
ao		20037.m00016						
an	LysF	AN6521.1	1,00E-34	2,00E-37	1,00E-34	0	0	0
af	EC 4.2.1.36	71.m15675	AAD19351	NP_567405	CAB93521	CAD71225	NP_593437	Lys4p
ao		20107.m00082						
an	LysG	AN5601.1	4,00E-71	9,00E-97	2,00E-54	2,00E-148	7,00E-128	2,00E-133
af	EC 1.5.1.10	58.m07525	AAF03526	AAD00700	AAR82744	CAC28679	NP_596411	Lys9p
ao		20165.m00188						
an	AcvA	manual annotation						
af								
ao		20169.m00348						
an	IpnA	AN2622.1						
af								
ao		20169.m00349						
an	AatA	AN2623.1						
af								
ao		20169.m00350						
an	TrpA	AN3695.1				1,00E-116	1,00E-123	2,00E-134
af	EC 4.1.3.27	69.m15404				AL031966	BX284763	Trp2p
ao		20178.m00733						
an	TrpB	AN6231.1				0	0	0
af	EC 4.2.1.20	72.m19978				trp-3	Z98974	Trp5p
ao		20141.m00194						
an	TrpC	20180.m01178				0	0	2,00E-147
af	EC 4.1.1.48	70.m15251				trp-1	trp-1	Trp3p
ao		20180.m01178						
an	TrpD	AN3634.1				2,00E-97	2,00E-41	2,00E-37
af	EC 2.4.2.18	58.m07467				XM_323763	AL023554	Trp4p
ao		20165.m00263						
an	AroC	AN6866.1				6,00E-97	1,00E-51	7,00E-52
af	EC 5.4.99.5	71.m15266						Aro7p
ao		20151.m00232						
an	TyrA	AN5959.1				9,00E-96	7,00E-94	2,00E-87
af	EC 1.3.1.13	72.m19175				XM_322553	AL023776	Tyr1p
ao		20177.m00688						
an	AroH	AN6338.1	1,00E-54			1,00E-151	1,00E-105	8,00E-99
af	EC 2.6.1.57	72.m19971	BC031068			XM_331507	Z99261	Aro8p
ao		20073.m00044						
an	AroH	AN6338.1	1,00E-54			1,00E-151	1,00E-105	8,00E-99
af	EC 2.6.1.57	72.m19971	BC031069			XM_331508	Z99262	Aro8p
ao		20073.m00044						
an	PhaA	manual annotation			5,00E-31	1,00E-47	1,00E-31	9,00E-32
af		71.m15987			NM_202520	XM_322494	CAB10811	Pha2p
ao		20108.m00087						

Table 2.: Proteins found among all investigated organisms and average homology over all proteins to *A. nidulans*

Protein	Putative protein function	*N. crassa*	*S. cerevisiae*	*S. pombe*	*A. thaliana*	*H. sapiens*	*D. melanogaster*
CpcC	Sensor kinase of gc/cpc	0 Gcn2p	0 Gcn2p	1,00E-154 CPC-3	2,00E-58 Gcn2p	0 eIF2a kinase	2,00E-76 eIF2a kinase
CpcB	transcription repressor of CpcA	1,00E-177 CPC-2	3,00E-98 Cpc2p	1,00E-136 Cpc2	1,00E-118 AAL34190.1	1,00E-139 GNB2L1	1,00E-129 Rack1
eIF2b	Translation machinery	2,00E-91 EIF2 beta	3,00E-62 EIF2 beta	5,00E-63 EIF2 beta	2,00E-46 EIF2 beta	3,00E-50 EIF2beta	2,00E-47 EIF2 beta
eIF2α	Translation machinery	4,00E-118 EIF2 alpha	4,00E-95 EIF2 alpha Gcn3p	3,00E-97 EIF2 alpha	1,00E-72 EIF2 alpha	4,00E-71 EIF2 alpha	2,00E-65 EIF2 alpha
eIF2γ	Translation machinery	0 EIF2 gamma	0 Gcd11p	0 EIF2 gamma	4,00E-173 EIF2 gamma	0 EIF2 gamma	0 EIF2 gamma
eIF2-Bδ	Translation machinery	2,00E-73 CAE76139	1,00E-79 Gcd2p	1,00E-89 CAA91965	1,00E-71 AAC23414	5,00E-76 EIF2B4	5,00E-62 EAL25620
eIF2-Bβ	Translation machinery	2,00E-66 CAD71011	1,00E-56 Gcd7p	1,00E-76 CAB52277	2,00E-58 AAF20216	6,00E-53 EIF2B4	2,00E-44 NP_570020
eIF2-Bα	Translation machinery	5,00E-52 XP_323697	2,00E-62 Gcn3p	2,00E-66 CAB57849	1,00E-46 AAF02861	1,00E-46 EIF2B1	6,00E-42 AAG38014
eIF2-Bε	Translation machinery	7,00E-123 XP_331190	6,00E-101 Gcd6p	5,00E-129 Tif225	2,00E-99 CAB78832	2,00E-91 eIF2-B e	1,00E-60 AAG38017
GcnA	CpcC mediator	0 XP_325658	0 Gcn1p	0 CAA92385	0 NP_176659	0 HsGCN1	0 EAA46127
eIF3a	Translation machinery	0.0 XP_322126	E-65 NP_009635 Tif32p	E-135 NP_596379 eIF3p110	E-83 NP_192881 eIF3a	E-97 NP_003741 eIF3a S10	E-78 NP_649470 eIF3 S10
eIF3b	Translation machinery	0.0 XP_330984	E-121 NP_015006 Prt1p	E-169 NP_594528 3 beta	E-117 NP_568498 eIF3b	E-130 NP_874371 eIF3b S9	E-112 NP_611228 eIF3 S9
eIF3c	Translation machinery	E-121 XP_328537	E-104 NP_014040 Nip1p	E-143 NP_593828 eIF3 p98	E-72 AAC83464 eIF3c	E-75 AAC27674 eIF3c S8	E-65 NP_611242 eIF3 S8
eIF3i	Translation machinery	E-143 XP_323195	E-104 NP_013866 Tif34p	E-120 NP_594958 eIF3 p39	E-73 NP_182152 eIF3i	E-99 NP_003748 eIF3i S2	E-84 NP_523478 TRIP1
PhoA	CpcA degradation	5,00E-143 XM_327865	2,00E-109 Pho85p	4,00E-115 AL031535	3,00E-93 AAM61706	1,00E-97 BC003065	1,00E-97 AY061049
SrbB	CpcA degradation	8,00E-61 AL355930	6,00E-43 Srb11p	1,00E-16 AL035085	5,00E-15 NM_124239	3,00E-19 NM_005190 CCNC	6,00E-21 AE003706
SkpA (SconC)	SCF compound	E-49 XP331383	E-36 AAC49492 Skp1p	E-48 NP_595455 SKP1	E-37 AAK26104 ASK10	E-41 NP_73377 SKP1	E-34 NP_477390
CulA	SCF compound	0 XP_324561	E-116 NP_010150 Cdc53p	E-176 NP_594259 pcu1	E-83 NP_567243 CUL1	0 NP_003583 CUL1	E-162 NP_523655 lin19
HrtA	SCF compound	E-43 XP_326079	E-29 NP_014508 Hrt1p	E-36 NP_593388 RBX1	E-40 NP_189869 ROC1	E-43 NP_055063 RBX1	E-40 NP_569852 ROC1A
KapA	CpcA transport	0 XM_329200	1,00E-149 Kap95p	0 Z98532	0 AK117217	1,00E-135 BT009797	0 Ketel
TapA	TOR mediator	1,00E-52 XM_328973	5,00E-23 Tap42p	4,00E-26 AL049522	6,00E-09 BX831385	3,00E-07 NM_001551	6,00E-05 NM_165039
TorA	Sensor kinase of gc/cpc	0 XM_325462	0 Tor1p	0 AL049558	0 TOR-1 AF178967	0 FRAP1 NM_004958	0 Tor NM_080152
SlcF	Amino acid transport	0 XM_323083	2,00E-112 Avt3p	1,00E-130 Z68144	1,00E-27 BX828060	2,00E-30 SLC36A1	2,00E-32 AY119064
SlcG	Amino acid transport	1,00E-144 XM_325629	1,00E-91 Avt3p	3,00E-95 Z68144	5,00E-24 BX827846	1,00E-30 SLC36A2	2,00E-28 NM_206319
LysE 1.1.1.155	Lysine biosynthesis	5,00E-116 CAE76248	3,00E-80 Lys12p	2,00E-89 NP_594004	2,00E-42 NP_850549	6,00E-40 NP_005521	2,00E-40 Q9VWH4
LysF 4.2.1.36	Lysine biosynthesis	0 CAD71225	0 Lys4p	0 NP_593437	2,00E-37 NP_567405	1,00E-34 AAD19351	1,00E-34 CAB93521
LysG 1.5.1.10	Lysine biosynthesis	2,00E-148 CAC28679	2,00E-133 Lys9p	7,00E-128 NP_596411	9,00E-97 AAD00700	4,00E-71 AAF03526	2,00E-54 AAR82744
	Average homology	**2,14E-53**	**1,79E-24**	**3,57E-18**	**2,14E-10**	**1,07E-08**	**2,14E-06**

Table 3.: Overview of putative amino acid transporters in *A. nidulans*

Transporter	Putative function derived from ortholog function
GapA	General amino acid permease for L-amino acids, some D-amino acids Trp/Tyr transporter (Jauniaux and Grenson, 1990)
SlcA	Sodium independent neutral amino acid transporter (H, M, L, I, V, F, Y, W, Q), putative amino acid sensor (Padbury *et al.*, 2004; Verrey *et al.*, 1999)
SlcB	K^+ antiport of anionic amino acid transporter (E, D) (Furuta *et al.*, 2005; Gonzalez-Gonzalez *et al.*, 2005)
SlcC	Sodium independent neutral amino acid transporter (Q, N, I, L, Y), putative uptake of amino acids from lysosome (Goberdhan *et al.*, 2005; Russnak *et al.*, 2001)
SlcD	Cl^- dependent GABA, betaine and taurine transporter, Na^+ and Cl^- dependent high-affinity glycine transporter, Glutamate transporter (Hyde *et al.*, 2003)
SlcE	Sodium independent neutral amino acid transporter (Q, N, I, L, Y), putative uptake of amino acids from lysosome (Goberdhan *et al.*, 2005; Russnak *et al.*, 2001)
SlcF	Sodium independent neutral amino acid transporter (Q, N, I, L, Y), putative uptake of amino acids from lysosome (Goberdhan *et al.*, 2005; Russnak *et al.*, 2001)
PutD	Nitrogen regulated proline transporter (Andreasson *et al.*, 2004)

3.6 References

Abdel-Sater, F., Iraqui, I., Urrestarazu, A., and Andre, B. (2004) The external amino acid signaling pathway promotes activation of Stp1 and Uga35/Dal81 transcription factors for induction of the AGP1 gene in *Saccharomyces cerevisiae*. *Genetics* **166**: 1727-1739.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402.

Andreasson, C., and Ljungdahl, P.O. (2002) Receptor-mediated endoproteolytic activation of two transcription factors in yeast. *Genes Dev* **16**: 3158-3172.

Andreasson, C., Neve, E.P., and Ljungdahl, P.O. (2004) Four permeases import proline and the toxic proline analogue azetidine-2-carboxylate into yeast. *Yeast* **21**: 193-199.

Andrews, P.R., Smith, G.D., and Young, I.G. (1973) Transition-state stabilization and enzymic catalysis. Kinetic and molecular orbital studies of the rearrangement of chorismate to prephenate. *Biochemistry* **12**: 3492-3498.

Averous, J., Bruhat, A., Mordier, S., and Fafournoux, P. (2003) Recent advances in the understanding of amino acid regulation of gene expression. *J Nutr* **133**: 2040S-2045S.

Averous, J., Bruhat, A., Jousse, C., Carraro, V., Thiel, G., and Fafournoux, P. (2004) Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. *J Biol Chem* **279**: 5288-5297.

Berlanga, J.J., Santoyo, J., and De Haro, C. (1999) Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2alpha kinase. *Eur J Biochem* **265**: 754-762.

Boczko, E.M., Cooper, T.G., Gedeon, T., Mischaikow, K., Murdock, D.G., Pratap, S., and Wells, K.S. (2005) Structure theorems and the dynamics of nitrogen catabolite repression in yeast. *Proc Natl Acad Sci U S A* **102**: 5647-5652.

Boll, M., Daniel, H., and Gasnier, B. (2004) The SLC36 family: proton-coupled transporters for the absorption of selected amino acids from extracellular and intracellular proteolysis. *Pflugers Arch* **447**: 776-779.

Brakhage, A.A. (1998) Molecular regulation of beta-lactam biosynthesis in filamentous fungi. *Microbiol Mol Biol Rev* **62**: 547-585.

Brakhage, A.A., Sprote, P., Al-Abdallah, Q., Gehrke, A., Plattner, H., and Tuncher, A. (2004) Regulation of penicillin biosynthesis in filamentous fungi. *Adv Biochem Eng Biotechnol* **88**: 45-90.

Brakhage, A.A., Al-Abdallah, Q., Tuncher, A., and Sprote, P. (2005) Evolution of beta-lactam biosynthesis genes and recruitment of trans-acting factors. *Phytochemistry* **66**: 1200-1210.

Braus, G.H. (1991) Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*: a model system for the regulation of a eukaryotic biosynthetic pathway. *Microbiol Rev* **55**: 349-370.

Brüggeman, J., Debets, A.J., Wijngaarden, P.J., deVisser, J.A., and Hoekstra, R.F. (2003) Sex slows down the accumulation of deleterious mutations in the homothallic fungus *Aspergillus nidulans*. *Genetics* **164**: 479-485.

Bruhat, A., Jousse, C., Wang, X.Z., Ron, D., Ferrara, M., and Fafournoux, P. (1997) Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. *J Biol Chem* **272**: 17588-17593.

Busch, S., Hoffmann, B., Valerius, O., Starke, K., Düvel, K., and Braus, G.H. (2001) Regulation of the *Aspergillus nidulans hisB* gene by histidine starvation. *Curr Genet* **38**: 314-322.

Busch, S., Bode, H.B., Brakhage, A.A., and Braus, G.H. (2003) Impact of the cross-pathway control on the regulation of lysine and penicillin biosynthesis in *Aspergillus nidulans*. *Curr Genet* **42**: 209-219.

Carsiotis, M., and Jones, R.F. (1974) Cross-pathway regulation: tryptophan-mediated control of histidine and arginine biosynthetic enzymes in *Neurospora crassa*. *J Bacteriol* **119**: 889-892.

Carsiotis, M., Jones, R.F., and Wesseling, A.C. (1974) Cross-pathway regulation: histidine-mediated control of histidine, tryptophan, and arginine biosynthetic enzymes in *Neurospora crassa*. *J Bacteriol* **119**: 893-898.

Chaudhuri, J., Chowdhury, D., and Maitra, U. (1999) Distinct functions of eukaryotic translation initiation factors eIF1A and eIF3 in the formation of the 40 S ribosomal preinitiation complex. *J Biol Chem* **274**: 17975-17980.

Chen, N.H., Reith, M.E., and Quick, M.W. (2004) Synaptic uptake and beyond: the sodium- and chloride-dependent neurotransmitter transporter family SLC6. *Pflugers Arch* **447**: 519-531.

Cherkasova, V.A., and Hinnebusch, A.G. (2003) Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev* **17**: 859-872.

Clemens, M.J. (1994) Regulation of eukaryotic protein synthesis by protein kinases that phosphorylate initiation factor eIF-2. *Mol Biol Rep* **19**: 201-210.

Davis, M.A., Askin, M.C., and Hynes, M.J. (2005) Amino acid catabolism by an areA-regulated gene encoding an L-amino acid oxidase with broad substrate specificity in Aspergillus nidulans. *Appl Environ Microbiol* **71**: 3551-3555.

Deshaies, R.J. (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* **15**: 435-467.

Dever, T.E., Chen, J.J., Barber, G.N., Cigan, A.M., Feng, L., Donahue, T.F., London, I.M., Katze, M.G., and Hinnebusch, A.G. (1993) Mammalian eukaryotic initiation factor 2 alpha kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism of yeast. *Proc Natl Acad Sci U S A* **90**: 4616-4620.

Ebbole, D.J., Paluh, J.L., Plamann, M., Sachs, M.S., and Yanofsky, C. (1991) cpc-1, the general regulatory gene for genes of amino acid biosynthesis in *Neurospora crassa*, is differentially expressed during the asexual life cycle. *Mol Cell Biol* **11**: 928-934.

Eckert, S.E., Hoffmann, B., Wanke, C., and Braus, G.H. (1999) Sexual development of *Aspergillus nidulans* in tryptophan auxotrophic strains. *Arch Microbiol* **172**: 157-166.

Eckert, S.E., Kübler, E., Hoffmann, B., and Braus, G.H. (2000) The tryptophan synthase-encoding trpB gene of Aspergillus nidulans is regulated by the cross-pathway control system. *Mol Gen Genet* **263**: 867-876.

Engelberg, D., Klein, C., Martinetto, H., Struhl, K., and Karin, M. (1994) The UV response involving the Ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. *Cell* **77**: 381-390.

Fernandez, J., Lopez, A.B., Wang, C., Mishra, R., Zhou, L., Yaman, I., Snider, M.D., and Hatzoglou, M. (2003) Transcriptional control of the arginine/lysine transporter, cat-1, by physiological stress. *J Biol Chem* **278**: 50000-50009.

Forsberg, H., Gilstring, C.F., Zargari, A., Martinez, P., and Ljungdahl, P.O. (2001) The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. *Mol Microbiol* **42**: 215-228.

Furuta, A., Takashima, S., Yokoo, H., Rothstein, J.D., Wada, K., and Iwaki, T. (2005) Expression of glutamate transporter subtypes during normal human corticogenesis and type II lissencephaly. *Brain Res Dev Brain Res* **155**: 155-164.

Gagnadoux, F. (2005) [Invasive pulmonary aspergillosis complicating septic shock.]. *Rev Mal Respir*. Epub ahead of print

Galagan, J.E., Nusbaum, C., Roy, A., Endrizzi, M.G., Macdonald, P., FitzHugh, W., Calvo, S., Engels, R., Smirnov, S., Atnoor, D., Brown, A., Allen, N., Naylor, J., Stange-Thomann, N., DeArellano, K., Johnson, R., Linton, L., McEwan, P., McKernan, K., Talamas, J., Tirrell, A., Ye, W., Zimmer, A., Barber, R.D., Cann, I., Graham, D.E., Grahame, D.A., Guss, A.M., Hedderich, R., Ingram-Smith, C., Kuettner, H.C., Krzycki, J.A., Leigh, J.A., Li, W., Liu, J., Mukhopadhyay, B., Reeve, J.N., Smith, K., Springer, T.A., Umayam, L.A., White, O., White, R.H., Conway de Macario, E., Ferry, J.G., Jarrell, K.F., Jing, H., Macario, A.J., Paulsen, I., Pritchett, M., Sowers, K.R., Swanson, R.V., Zinder, S.H., Lander, E., Metcalf, W.W., and Birren, B. (2002) The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. *Genome Res* **12**: 532-542.

Galagan, J.E., Calvo, S.E., Borkovich, K.A., Selker, E.U., Read, N.D., Jaffe, D., FitzHugh, W., Ma, L.J., Smirnov, S., Purcell, S., Rehman, B., Elkins, T., Engels, R., Wang, S., Nielsen, C.B., Butler, J., Endrizzi, M., Qui, D., Ianakiev, P., Bell-Pedersen, D., Nelson, M.A., Werner-Washburne, M., Selitrennikoff, C.P., Kinsey, J.A., Braun, E.L., Zelter, A., Schulte, U., Kothe, G.O., Jedd, G., Mewes, W., Staben, C., Marcotte, E., Greenberg, D., Roy, A., Foley, K., Naylor, J., Stange-Thomann, N., Barrett, R., Gnerre, S., Kamal, M., Kamvysselis, M., Mauceli, E., Bielke, C., Rudd, S., Frishman, D., Krystofova, S., Rasmussen, C., Metzenberg, R.L., Perkins, D.D., Kroken, S., Cogoni, C., Macino, G., Catcheside, D., Li, W., Pratt, R.J., Osmani, S.A., DeSouza, C.P., Glass, L., Orbach, M.J., Berglund, J.A., Voelker, R., Yarden, O., Plamann, M., Seiler, S., Dunlap, J., Radford, A., Aramayo, R., Natvig, D.O., Alex, L.A., Mannhaupt, G., Ebbole, D.J., Freitag, M., Paulsen, I., Sachs, M.S., Lander, E.S., Nusbaum, C., and Birren, B. (2003) The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **422**: 859-868.

Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L., Wortman, J., Batzoglou, S., Lee, S., Brudno, M., Bastürkmen, M., Spevak, C.C., Clutterbuck, J., Kapitonov, V., J., J., Scazzocchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G.H., Draht, O., Busch, S., D´Enfert, C., Bouchier, C., Goldman, G.H., Griffiths-Jones, S., Vienken, K., Pain, A., Selker, E.U., Archer, D., Penalva, M.Á., Oakley, B.R., Momany, M., Sano, M., Tanaka, T., Kumagai, T., Machida, M., Nierman, W.C., Denning, D.W., Caddick, M., Hynes, M., Paoletti, M., Fischer, E.R., Miller, J.B., Dyer, P., Sachs, M.S., Osmani, S.A., and Birren, B. (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Manuscript submitted.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94**: 615-623.

Goberdhan, D.C., Meredith, D., Boyd, C.A., and Wilson, C. (2005) PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids. *Development* **132**: 2365-2375.

Gomez, E., Mohammad, S.S., and Pavitt, G.D. (2002) Characterization of the minimal catalytic domain within eIF2B: the guanine-nucleotide exchange factor for translation initiation. *Embo J* **21**: 5292-5301.

Gonzalez-Gonzalez, I.M., Cubelos, B., Gimenez, C., and Zafra, F. (2005) Immunohistochemical localization of the amino acid transporter SNAT2 in the rat brain. *Neuroscience* **130**: 61-73.

Gugnani, H.C. (2003) Ecology and taxonomy of pathogenic *aspergilli*. *Front Biosci* **8**: s346-357.

Hao, S., Sharp, J.W., Ross-Inta, C.M., McDaniel, B.J., Anthony, T.G., Wek, R.C., Cavener, D.R., McGrath, B.C., Rudell, J.B., Koehnle, T.J., and Gietzen, D.W. (2005) Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* **307**: 1776-1778.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* **6**: 1099-1108.

He, Q., Cheng, P., and Liu, Y. (2005) The COP9 signalosome regulates the *Neurospora* circadian clock by controlling the stability of the SCFFWD-1 complex. *Genes Dev*.

Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu Rev Biochem* **67**: 425-479.

Hilt, W. (2004) Targets of programmed destruction: a primer to regulatory proteolysis in yeast. *Cell Mol Life Sci* **61**: 1615-1632.

Hinnebusch, A.G. (1984) Evidence for translational regulation of the activator of general amino acid control in yeast. *Proc Natl Acad Sci U S A* **81**: 6442-6446.

Hinnebusch, A.G. (1985) A hierarchy of trans-acting factors modulates translation of an activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* **5**: 2349-2360.

Hinnebusch, A.G. (1986) The general control of amino acid biosynthetic genes in the yeast *Saccharomyces cerevisiae*. *CRC Crit Rev Biochem* **21**: 277-317.

Hinnebusch, A.G. (1997) Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. *J Biol Chem* **272**: 21661-21664.

Hinnebusch, A.G., and Natarajan, K. (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell* **1**: 22-32.

Hinnebusch, A.G., Asano, K., Olsen, D.S., Phan, L., Nielsen, K.H., and Valasek, L. (2004) Study of translational control of eukaryotic gene expression using yeast. *Ann N Y Acad Sci* **1038**: 60-74.

Hoffmann, B., Mösch, H.U., Sattlegger, E., Barthelmess, I.B., Hinnebusch, A., and Braus, G.H. (1999) The WD protein Cpc2p is required for repression of Gcn4 protein activity in yeast in the absence of amino-acid starvation. *Mol Microbiol* **31**: 807-822.

Hoffmann, B., Wanke, C., Lapaglia, S.K., and Braus, G.H. (2000) c-Jun and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans*. *Mol Microbiol* **37**: 28-41.

Hoffmann, B., Eckert, S.E., Krappmann, S., and Braus, G.H. (2001a) Sexual diploids of *Aspergillus nidulans* do not form by random fusion of nuclei in the heterokaryon. *Genetics* **157**: 141-147.

Hoffmann, B., Valerius, O., Andermann, M., and Braus, G.H. (2001b) Transcriptional autoregulation and inhibition of mRNA translation of amino acid regulator gene *cpcA* of filamentous fungus *Aspergillus nidulans*. *Mol Biol Cell* **12**: 2846-2857.

Hutson, R.G., and Kilberg, M.S. (1994) Cloning of rat asparagine synthetase and specificity of the amino acid-dependent control of its mRNA content. *Biochem J* **304 (Pt 3)**: 745-750.

Hutson, R.G., Kitoh, T., Moraga Amador, D.A., Cosic, S., Schuster, S.M., and Kilberg, M.S. (1997) Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. *Am J Physiol* **272**: C1691-1699.

Hütter, R., and DeMoss, J.A. (1967) Organization of the tryptophan pathway: a phylogenetic study of the fungi. *J Bacteriol* **94**: 1896-1907.

Hütter, R., Niederberger, P., and DeMoss, J.A. (1986) Tryptophan biosynthetic genes in eukaryotic microorganisms. *Annu Rev Microbiol* **40**: 55-77.

Hyde, R., Taylor, P.M., and Hundal, H.S. (2003) Amino acid transporters: roles in amino acid sensing and signalling in animal cells. *Biochem J* **373**: 1-18.

Irniger, S., and Braus, G.H. (2003) Controlling transcription by destruction: the regulation of yeast Gcn4p stability. *Curr Genet* **44**: 8-18.

Jackson, P.K., and Eldridge, A.G. (2002) The SCF ubiquitin ligase: an extended look. *Mol Cell* **9**: 923-925.

Jauniaux, J.C., and Grenson, M. (1990) GAP1, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. *Eur J Biochem* **190**: 39-44.

Jensen, R., and Fischer, R. (1987) The postprephenate biochemical pathways to phenylalanine and tyrosine: an overview. *Methods Enzymol* **142**: 472-478.

Jorgensen, M.U., Bruun, M.B., Didion, T., and Kielland-Brandt, M.C. (1998) Mutations in five loci affecting GAP1-independent uptake of neutral amino acids in yeast. *Yeast* **14**: 103-114.

Jurka, J. (1998) Repeats in genomic DNA: mining and meaning. *Curr Opin Struct Biol* **8**: 333-337.

Kapelari, B., Bech-Otschir, D., Hegerl, R., Schade, R., Dumdey, R., and Dubiel, W. (2000) Electron microscopy and subunit-subunit interaction studies reveal a first architecture of COP9 signalosome. *J Mol Biol* **300**: 1169-1178.

Kapp, L.D., and Lorsch, J.R. (2004) The molecular mechanics of eukaryotic translation. *Annu Rev Biochem* **73**: 657-704.

Kim, T., Hofmann, K., von Arnim, A.G., and Chamovitz, D.A. (2001) PCI complexes: pretty complex interactions in diverse signaling pathways. *Trends Plant Sci* **6**: 379-386.

Klionsky, D.J. (2005) The molecular machinery of autophagy: unanswered questions. *J Cell Sci* **118**: 7-18.

Kornitzer, D., Raboy, B., Kulka, R.G., and Fink, G.R. (1994) Regulated degradation of the transcription factor Gcn4. *Embo J* **13**: 6021-6030.

Kouzarides, T., and Ziff, E. (1989) Behind the Fos and Jun leucine zipper. *Cancer Cells* **1**: 71-76.

Krappmann, D., and Scheidereit, C. (2005) A pervasive role of ubiquitin conjugation in activation and termination of IkappaB kinase pathways. *EMBO Rep* **6**: 321-326.

Krappmann, S., Helmstaedt, K., Gerstberger, T., Eckert, S., Hoffmann, B., Hoppert, M., Schnappauf, G., and Braus, G.H. (1999) The *aroC* gene of *Aspergillus nidulans* codes for a monofunctional, allosterically regulated chorismate mutase. *J Biol Chem* **274**: 22275-22282.

Krappmann, S., Bignell, E.M., Reichard, U., Rogers, T., Haynes, K., and Braus, G.H. (2004) The *Aspergillus fumigatus* transcriptional activator CpcA contributes significantly to the virulence of this fungal pathogen. *Mol Microbiol* **52**: 785-799.

Kredics, L., Antal, Z., Szekeres, A., Hatvani, L., Manczinger, L., Vagvolgyi, C., and Nagy, E. (2005) Extracellular proteases of Trichoderma species. A review. *Acta Microbiol Immunol Hung* **52**: 169-184.

Künzler, M., Balmelli, T., Egli, C.M., Paravicini, G., and Braus, G.H. (1993) Cloning, primary structure, and regulation of the HIS7 gene encoding a bifunctional glutamine amidotransferase: cyclase from *Saccharomyces cerevisiae*. *J Bacteriol* **175**: 5548-5558.

Li, W., Wang, X., Van Der Knaap, M.S., and Proud, C.G. (2004) Mutations linked to leukoencephalopathy with vanishing white matter impair the function of the eukaryotic initiation factor 2B complex in diverse ways. *Mol Cell Biol* **24**: 3295-3306.

Luo, Z., Freitag, M., and Sachs, M.S. (1995) Translational regulation in response to changes in amino acid availability in *Neurospora crassa*. *Mol Cell Biol* **15**: 5235-5245.

Magasanik, B., and Kaiser, C.A. (2002) Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene* **290**: 1-18.

Marzluf, G.A. (1997) Genetic regulation of nitrogen metabolism in the fungi. *Microbiol Mol Biol Rev* **61**: 17-32.

McKenna, S., Moraes, T., Pastushok, L., Ptak, C., Xiao, W., Spyracopoulos, L., and Ellison, M.J. (2003) An NMR-based model of the ubiquitin-bound human ubiquitin conjugation complex Mms2.Ubc13. The structural basis for lysine 63 chain catalysis. *J Biol Chem* **278**: 13151-13158.

Meijer, A.J., and Dubbelhuis, P.F. (2004) Amino acid signalling and the integration of metabolism. *Biochem Biophys Res Commun* **313**: 397-403.

Meimoun, A., Holtzman, T., Weissman, Z., McBride, H.J., Stillman, D.J., Fink, G.R., and Kornitzer, D. (2000) Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. *Mol Biol Cell* **11**: 915-927.

Mielnicki, L.M., Hughes, R.G., Chevray, P.M., and Pruitt, S.C. (1996) Mutated Atf4 suppresses c-Ha-ras oncogene transcript levels and cellular transformation in NIH3T3 fibroblasts. *Biochem Biophys Res Commun* **228**: 586-595.

Miller, J., and Gordon, C. (2005) The regulation of proteasome degradation by multi-ubiquitin chain binding proteins. *FEBS Lett* **579**: 3224-3230.

Minehart, P.L., and Magasanik, B. (1992) Sequence of the GLN1 gene of *Saccharomyces cerevisiae*: role of the upstream region in regulation of glutamine synthetase expression. *J Bacteriol* **174**: 1828-1836.

Mo, X., and Marzluf, G.A. (2003) Cooperative action of the NIT2 and NIT4 transcription factors upon gene expression in *Neurospora crassa*. *Curr Genet* **42**: 260-267.

Mösch, H.U., Graf, R., Schmidheini, T., and Braus, G. (1990) Three GCN4 responsive elements act synergistically as upstream and as TATA-like elements in the yeast TRP4 promoter. *Embo J* **9**: 2951-2957.

Natarajan, K., Meyer, M.R., Jackson, B.M., Slade, D., Roberts, C., Hinnebusch, A.G., and Marton, M.J. (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* **21**: 4347-4368.

Nazar, R.N. (2004) Ribosomal RNA processing and ribosome biogenesis in eukaryotes. *IUBMB Life* **56**: 457-465.

Nilsson, J., Sengupta, J., Frank, J., and Nissen, P. (2004) Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. *EMBO Rep* **5**: 1137-1141.

Nishida, H., Nishiyama, M., Kobashi, N., Kosuge, T., Hoshino, T., and Yamane, H. (1999) A prokaryotic gene cluster involved in synthesis of lysine through the amino adipate pathway: a key to the evolution of amino acid biosynthesis. *Genome Res* **9**: 1175-1183.

Ogrydziak, D.M. (1993) Yeast extracellular proteases. *Crit Rev Biotechnol* **13**: 1-55.

Omura, F., Kodama, Y., and Ashikari, T. (2001) The N-terminal domain of the yeast permease Bap2p plays a role in its degradation. *Biochem Biophys Res Commun* **287**: 1045-1050.

Padbury, J.F., Diah, S.K., McGonnigal, B., Miller, C., Fugere, C., Kuzniar, M., and Thompson, N.L. (2004) Transcriptional regulation of the LAT-1/CD98 light chain. *Biochem Biophys Res Commun* **318**: 529-534.

Paluh, J.L., Orbach, M.J., Legerton, T.L., and Yanofsky, C. (1988) The cross-pathway control gene of *Neurospora crassa*, cpc-1, encodes a protein similar to GCN4 of yeast and the DNA-binding domain of the oncogene v-jun-encoded protein. *Proc Natl Acad Sci U S A* **85**: 3728-3732.

Paluh, J.L., and Yanofsky, C. (1991) Characterization of *Neurospora* CPC1, a bZIP DNA-binding protein that does not require aligned heptad leucines for dimerization. *Mol Cell Biol* **11**: 935-944.

Pavlukova, E.B., Belozersky, M.A., and Dunaevsky, Y.E. (1998) Extracellular proteolytic enzymes of filamentous fungi. *Biochemistry (Mosc)* **63**: 899-928.

Piotrowska, M., Natorff, R., and Paszewski, A. (2000) sconC, a gene involved in the regulation of sulphur metabolism in *Aspergillus nidulans*, belongs to the SKP1 gene family. *Mol Gen Genet* **264**: 276-282.

Preiss, T., and Hentze, M.W. (2003) Starting the protein synthesis machine: eukaryotic translation initiation. *Bioessays* **25**: 1201-1211.

Pries, R., Bömeke, K., Irniger, S., Grundmann, O., and Braus, G.H. (2002) Amino acid-dependent Gcn4p stability regulation occurs exclusively in the yeast nucleus. *Eukaryot Cell* **1**: 663-672.

Qiu, H., Hu, C., Yoon, S., Natarajan, K., Swanson, M.J., and Hinnebusch, A.G. (2004) An array of coactivators is required for optimal recruitment of TATA binding protein and RNA polymerase II by promoter-bound Gcn4p. *Mol Cell Biol* **24**: 4104-4117.

Qu, L.H., Henry, Y., Nicoloso, M., Michot, B., Azum, M.C., Renalier, M.H., Caizergues-Ferrer, M., and Bachellerie, J.P. (1995) U24, a novel intron-encoded small nucleolar RNA with two 12 nt long, phylogenetically conserved complementarities to 28S rRNA. *Nucleic Acids Res* **23**: 2669-2676.

Quevillon-Cheruel, S., Leulliot, N., Meyer, P., Graille, M., Bremang, M., Blondeau, K., Sorel, I., Poupon, A., Janin, J., and van Tilbeurgh, H. (2004) Crystal structure of the bifunctional chorismate synthase from *Saccharomyces cerevisiae*. *J Biol Chem* **279**: 619-625.

Rohde, J.R., Campbell, S., Zurita-Martinez, S.A., Cutler, N.S., Ashe, M., and Cardenas, M.E. (2004) TOR controls transcriptional and translational programs via Sap-Sit4 protein phosphatase signaling effectors. *Mol Cell Biol* **24**: 8332-8341.

Russnak, R., Konczal, D., and McIntire, S.L. (2001) A family of yeast proteins mediating bidirectional vacuolar amino acid transport. *J Biol Chem* **276**: 23849-23857.

Sales, A.C., and Yoshizawa, T. (2005) Updated profile of aflatoxin and Aspergillus section Flavi contamination in rice and its byproducts from the Philippines. *Food Addit Contam* **22**: 429-436.

Schmidt, A., Hall, M.N., and Koller, A. (1994) Two FK506 resistance-conferring genes in *Saccharomyces cerevisiae*, TAT1 and TAT2, encode amino acid permeases mediating tyrosine and tryptophan uptake. *Mol Cell Biol* **14**: 6597-6606.

Schneper, L., Düvel, K., and Broach, J.R. (2004) Sense and sensibility: nutritional response and signal integration in yeast. *Curr Opin Microbiol* **7**: 624-630.

Sengupta, J., Nilsson, J., Gursky, R., Spahn, C.M., Nissen, P., and Frank, J. (2004) Identification of the versatile scaffold protein RACK1 on the eukaryotic ribosome by cryo-EM. *Nat Struct Mol Biol* **11**: 957-962.

Shemer, R., Meimoun, A., Holtzman, T., and Kornitzer, D. (2002) Regulation of the transcription factor Gcn4 by Pho85 cyclin PCL5. *Mol Cell Biol* **22**: 5395-5404.

Shor, B., Calaycay, J., Rushbrook, J., and McLeod, M. (2003) Cpc2/RACK1 is a ribosome-associated protein that promotes efficient translation in *Schizosaccharomyces pombe*. *J Biol Chem* **278**: 49119-49128.

Sirtori, L.R., Dutra-Filho, C.S., Fitarelli, D., Sitta, A., Haeser, A., Barschak, A.G., Wajner, M., Coelho, D.M., Llesuy, S., Bello-Klein, A., Giugliani, R., Deon, M., and Vargas, C.R. (2005) Oxidative stress in patients with phenylketonuria. *Biochim Biophys Acta* **1740**: 68-73.

Sood, R., Porter, A.C., Olsen, D.A., Cavener, D.R., and Wek, R.C. (2000) A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2alpha. *Genetics* **154**: 787-801.

Sosa, E., Aranda, C., Riego, L., Valenzuela, L., DeLuna, A., Cantu, J.M., and Gonzalez, A. (2003) Gcn4 negatively regulates expression of genes subjected to nitrogen catabolite repression. *Biochem Biophys Res Commun* **310**: 1175-1180.

Takeuchi, J., and Tamura, T. (2004) Recombinant ATPases of the yeast 26S proteasome activate protein degradation by the 20S proteasome. *FEBS Lett* **565**: 39-42.

Thammarongtham, C., Turner, G., Moir, A.J., Tanticharoen, M., and Cheevadhanarak, S. (2001) A new class of glutaminase from *Aspergillus oryzae*. *J Mol Microbiol Biotechnol* **3**: 611-617.

Tyers, M., and Jorgensen, P. (2000) Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr Opin Genet Dev* **10**: 54-64.

Valenzuela, L., Aranda, C., and Gonzalez, A. (2001) TOR modulates GCN4-dependent expression of genes turned on by nitrogen limitation. *J Bacteriol* **183**: 2331-2334.

Valerius, O., Draht, O., Kübler, E., Adler, K., Hoffmann, B., and Braus, G.H. (2001) Regulation of *hisHF* transcription of *Aspergillus nidulans* by adenine and amino acid limitation. *Fungal Genet Biol* **32**: 21-31.

van Nocker, S., and Vierstra, R.D. (1993) Multiubiquitin chains linked through lysine 48 are abundant in vivo and are competent intermediates in the ubiquitin proteolytic pathway. *J Biol Chem* **268**: 24766-24773.

Vazquez de Aldana, C.R., Marton, M.J., and Hinnebusch, A.G. (1995) GCN20, a novel ATP binding cassette protein, and GCN1 reside in a complex that mediates activation of the eIF-2 alpha kinase GCN2 in amino acid-starved cells. *Embo J* **14**: 3184-3199.

Verrey, F., Jack, D.L., Paulsen, I.T., Saier, M.H., Jr., and Pfeiffer, R. (1999) New glycoprotein-associated amino acid transporters. *J Membr Biol* **172**: 181-192.

Volterra, A., and Meldolesi, J. (2005) Astrocytes, from brain glue to communication elements: the revolution continues. *Nat Rev Neurosci*.

Wang, H., Wang, X., and Jiang, Y. (2003) Interaction with Tap42 is required for the essential function of Sit4 and type 2A phosphatases. *Mol Biol Cell* **14**: 4342-4351.

Wanke, C., Eckert, S., Albrecht, G., van Hartingsveldt, W., Punt, P.J., van den Hondel, C.A., and Braus, G.H. (1997) The *Aspergillus niger* GCN4 homologue, CpcA, is transcriptionally regulated and encodes an unusual leucine zipper. *Mol Microbiol* **23**: 23-33.

Wipf, D., Ludewig, U., Tegeder, M., Rentsch, D., Koch, W., and Frommer, W.B. (2002) Conservation of amino acid transporters in fungi, plants and animals. *Trends Biochem Sci* **27**: 139-147.

Wittenberg, C., and Reed, S.I. (2005) Cell cycle-dependent transcription in yeast: promoters, transcription factors, and transcriptomes. *Oncogene* **24**: 2746-2755.

Yelton, M.M., Hamer, J.E., de Souza, E.R., Mullaney, E.J., and Timberlake, W.E. (1983) Developmental regulation of the *Aspergillus nidulans trpC* gene. *Proc Natl Acad Sci U S A* **80**: 7576-7580.

Zagami, C.J., O'Shea, R.D., Lau, C.L., Cheema, S.S., and Beart, P.M. (2005) Regulation of glutamate transporters in astrocytes: evidence for a relationship between transporter expression and astrocytic phenotype. *Neurotox Res* **7**: 143-149.

Zhu, S., Sobolev, A.Y., and Wek, R.C. (1996) Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate in vitro phosphorylation of eIF-2. *J Biol Chem* **271**: 24989-24994.

Chapter 4

A. nidulans CpcA localisation and nuclear localisation signal (NLS)

4.1 Abstract

The yeast transcription factor Gcn4p contains two nuclear localisation sequences NLS1 and NLS2 which are independently able to relocate the cytoplasmic protein chorismate mutase to the nucleus. Only NLS2 is highly conserved among other fungi. The counterpart of Gcn4p in *Aspergillus nidulans* is CpcA. The conserved NLS of this transcription factor is necessary to target the protein into the nucleus in *A. nidulans* and in *S. cerevisiae*. Deletion of the NLS leads to accumulation of the protein in the cytoplasm in both organisms. This suggests that only one NLS is present in CpcA. During the manual annotation of the *A. nidulans* genome two putative ORFs SrpA (AN2142.1) and KapA (AN0906.1) were found that show high similarity to the α-importin Srp1p and β-importin Kap95p which are necessary to transport Gcn4p into the nucleus.

4.2 Introduction

During protein production transcription and translation are spatially separated in eukaryotes. The transcription of the mRNA takes place in the nucleoplasm, whereas its translation takes place in the cytoplasm. The nucleoplasm and cytoplasm are spatially separated by the nuclear membrane, being continuous with the endoplasmatic reticulum and are connected by the nuclear pores. The pores are required for trafficking of involved macromolecules like mRNA, tRNA, rRNA or proteins as transcription factors of ribosome compounds and work in both directions (Kaffman and O'Shea, 1999; Lusk *et al.*, 2004). Since all proteins are synthesized in the cytoplasm nuclear proteins need to be transported into the nucleus (Görlich and Mattaj, 1996). Small proteins up to 40kDa can generally diffuse passively through the nuclear pores, whereas bigger macromolecules need to be transported by special transporter proteins which allow an active, efficient and regulated import to the nucleus. In general active nuclear import requires the formation of an heterodimeric importin α/β complex in the cytosol which recognizes the nuclear localisation sequence (NLS) of the cargo protein and translocates it through the nuclear pore into the nucleus (Görlich *et al.*, 1995). After release of the cargo protein the importins return to the cytosol. This active transport is driven by GTP hydroloysis by Ran (Görlich and Mattaj, 1996; Moore and Blobel, 1993).

Classical NLSs are either monopartite sequences that contain a single cluster of basic amino acids (Lys/Arg) or bipartite sequences that contain two clusters of basic residues separated by an unconserved linker region (Dingwall and Laskey, 1991). Only one importin α protein (Srp1p) is known in *Saccharomyces cerevisiae*, whereas thirteen proteins show similarities to importin β, eight of which being already characterized as import proteins. Gcn4p is the central transcription factor of the general control of amino acid biosynthesis in *S. cerevisiae* (Natarajan *et al.*, 2001). It controls the expression of over 500 target genes belonging to various biosynthetic pathways under amino acid limitation, purine starvation, UV radiation or glucose limitation (Engelberg *et al.*, 1994; Hinnebusch and Natarajan, 2002; Mösch *et al.*, 1990). Starvation for amino acids has two effects on Gcn4p, both the synthesis and the stability of this transcription factor are increased. Under non-limiting amino acid conditions the protein is highly instable with a half-life of approximately 5 minutes. The half-life increases to 20 minutes under limiting conditions (Kornitzer *et al.*, 1994). As a transcription factor Gcn4p has to be transported into the nucleus after synthesis in the cytoplasm (Pries *et al.*, 2002). Gcn4p has two nuclear localisation sequences NLS1 and NLS2 which are both located near the C-terminus of the protein. Each of the NLS can misdirect proteins to the

nucleus and deletion of the NLSs prevents Gcn4p from entering the nucleus (Pries *et al.*, 2002). The two karyopherins Srp1p and Kap95p are essential to transport Gcn4p into the nucleus (Pries *et al.*, 2004). Since the stability of Gcn4p seems to be only affected in the nucleus by phosphorylation and subsequent ubiquitination and degradation by the 26S proteasome the small cytoplasmic portion of Gcn4p is more stable than the nuclear fraction. Phosphorylation of Gcn4p in the nucleus requires the cyclin-dependent kinases Srb10p and Pho85p in combination with their cyclins Srb10p and Pcl5p (Chi *et al.*, 2001; Meimoun *et al.*, 2000). CpcA is the counterpart of Gcn4p in the filamentous fungus *Aspergillus nidulans* and acts as the transcription factor of the cross-pathway control of amino acid biosynthesis (Hoffmann *et al.*, 2001). So far nothing was known about the subcellular localisation of CpcA but alignments of the CpcA and the Gcn4p amino acid sequence revealed that only NLS2 is conserved among the two proteins. Furthermore it is the only NLS to be found in CpcA. Fluorescence microscopy experiments revealed that CpcA is nuclear localized if expressed in yeast and in *Aspergillus nidulans*. Deletion of the conserved NLS leads to accumulation of the protein in the cytoplasm, both in yeast and in *Aspergillus*.

4.3 Material and methods

4.3.1 Transformation and cultivation of *Aspergillus nidulans*

For CpcA localisation analysis we used the *A. nidulans* strain AGB10 (*pyrG*89, *pyroA*4) from our collection, which is a derivative of wild type strain A4 (Eckert *et al.*, 2000). Cultivation of *A. nidulans* strains was performed at 37°C on minimal medium. Transformation was carried out as previously described (Punt and van den Hondel, 1992), and transformants were selected on medium lacking uridine to select for the presence of the prototrophic marker *pyrG*. Expression of the *alcA* promoter was induced with 2% ethanol and 2% glycerol as sole carbon sources. The amount of integrations of the constructs into the genome was checked by southern experiments (data not shown). Strains with one integrated copy of the respective plasmids were further investigated.

4.3.2 Fluorescence microscopy

The microscopic experiments were carried out on a Zeiss Axiovert microscope by either differential interference contrast microscopy (DIC) or fluorescence microscopy using a GFP filter set (AHF Analysentechnik AG, Tübingen, Germany). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole) and visualized using a Hamamatsu-Orca ER digital camera and Improvision Openlab software (Improvision, Coventry, UK). For fluorescence microscopy of *A. nidulans* a thin layer of solid medium (without uridine to select for the presence of the prototrophic marker *pyrG*) was spread evenly on a glass slide, which was then tilted so that one end was in contact with a reservoir of liquid medium. The solid layer was inoculated with spores of the transformed *A. nidulans* strain and analyzed by fluorescence microscopy after formation of hyphae.

4.4 Results

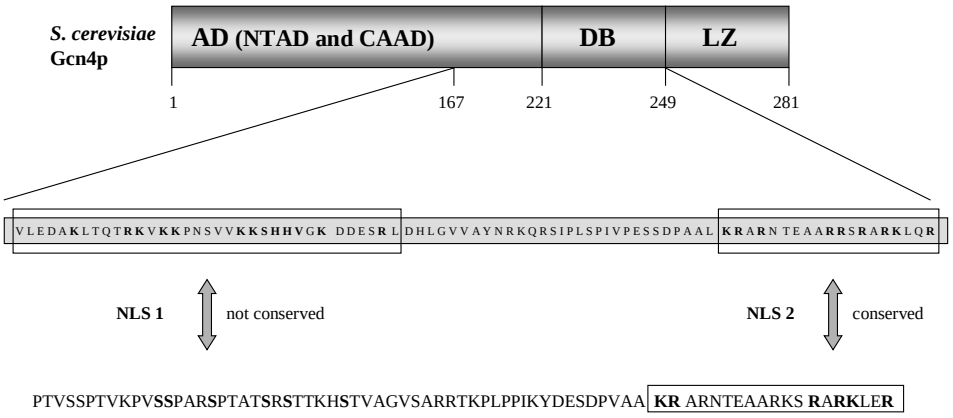
4.4.1 Comparison of Gcn4p and CpcA reveals that only NLS2 is conserved between *Saccharomyces cerevisiae* and *Aspergillus species*

The yeast transcription factor Gcn4p contains two regions which are able to target the protein after translation in the cytosol towards the nucleus. Sequence alignments revealed that the NLS2 is highly conserved among homologous proteins of other fungi, such as the filamentous fungus *Aspergillus nidulans* (Figure 18A), *A. fumigatus*, *A. niger* and *A. oryzae* CpcA and the *Neurospora crassa* CPC-1. Even in the human AP-1 transcription factor c-jun a putative NLS can be found that shows a 58% identity to Gcn4p (Figure 18B). No conserved NLS1 of Gcn4p could be found in any of the other investigated organisms. CpcA of *A. nidulans* is the transcription factor of the cross-pathway control, the counterpart of *S. cerevisiae* Gcn4p general control. Strong similarities can be found among these two proteins (40% identity) and it was shown that they can functionally substitute for each other.

4.4.2 Deletion of the conserved nuclear localisation sequence in CpcA of *Aspergillus nidulans* impairs its nuclear transport

To investigate the subcellular localisation of CpcA in the filamentous fungus *A. nidulans* a GFP-tagged version of the protein was expressed from the ethanol inducible *alcA* promoter in strain AGB10 (*pyrG*89, *pyroA*4). *A. nidulans* expressing CpcA-GFP (pME2447) was grown on a glass slide on a thin layer of solid minimal medium lacking uridine for selection on the plasmid and containing 2% ethanol and 2% glycerol as sole carbon sources to induce the *alcA* promoter. Fresh hyphae could be directly investigated by fluorescence microscopy (Figure 19A). CpcA was found to be predominantly nuclear localized, as was confirmed by DAPI staining of the hyphae, which corresponds to the localisation recently described for Gcn4p. Expression of the truncated $CpcA_{aa1\text{-}190}$-GFP lacking the putative NLS from plasmid pME2448 leads to cytoplasmic accumulation of the protein which implies that the NLS is the sole functioning NLS in CpcA and thus implies that the protein lacks the counterpart to Gcn4p NLS1. To strengthen these results both GFP-tagged versions of CpcA were expressed in *S. cerevisiae* (Figure 19B). The untruncated chimeric protein (pME2494) shows clear nuclear localisation, whereas the truncated derivative $CpcA_{aa1\text{-}190}$-GFP (pME2495), which

A



B

	NLS2	% Identity to Gcn4p
S. cerevisiae (Gcn4p)	KRARNTEAARRS RARKLQR	100
A. nidulans (CpcA)	KRARNTEAARKS RARKLER	89,4
A. fumigatus (CpcA)	KRARNTEAARKS RARKLER	89,4
A. niger (CpcA)	KRARNTEAARKS RARKLER	89,4
A. oryzae (CpcA)	KRARNTEAARKS RARKLER	89,4
N. crassa (CPC1)	KRARNTLAARKS RERKAQR	78,9
H. sapiens (c-Jun)	KRMRNRIAASKC RKRKLER	57,8

Fig. 18: NLS motif comparisons
(A) The positions of the two NLS-motifs within the entire Gcn4 protein are shown schematically. NLS1 consists of the amino acids 167 to 200 and NLS2 is represented by the amino acids 231 to 249. The different domains of Gcn4p are illustrated. AD is the activation domain, consisting of an N-terminal activation domain (NTAD) and a central acidic activation domain (CAAD). DB is the DNA binding domain and LZ the leucine zipper of Gcn4p.
An alignment with the amino acid sequence of *A. nidulans* CpcA reveals the conserved character of Gcn4p NLS2, whereas Gcn4p NLS1 is not conserved. Basic amino acid residues within the NLS motifs are written bold.
(B) Amino acid alignments of *S. cerevisiae* Gcn4p with the homologous proteins CpcA of *Aspergillus species*, CPC1 of *Neurospora crassa*, and human c-Jun revealed the highly conserved character of Gcn4p NLS 2.

lacks the nuclear localisation signal clearly accumulates in the cytoplasm as it does in *A. nidulans*. These data confirm that *A. nidulans* CpcA possesses only a single classical bipartite NLS motif, which is highly conserved among other fungi.

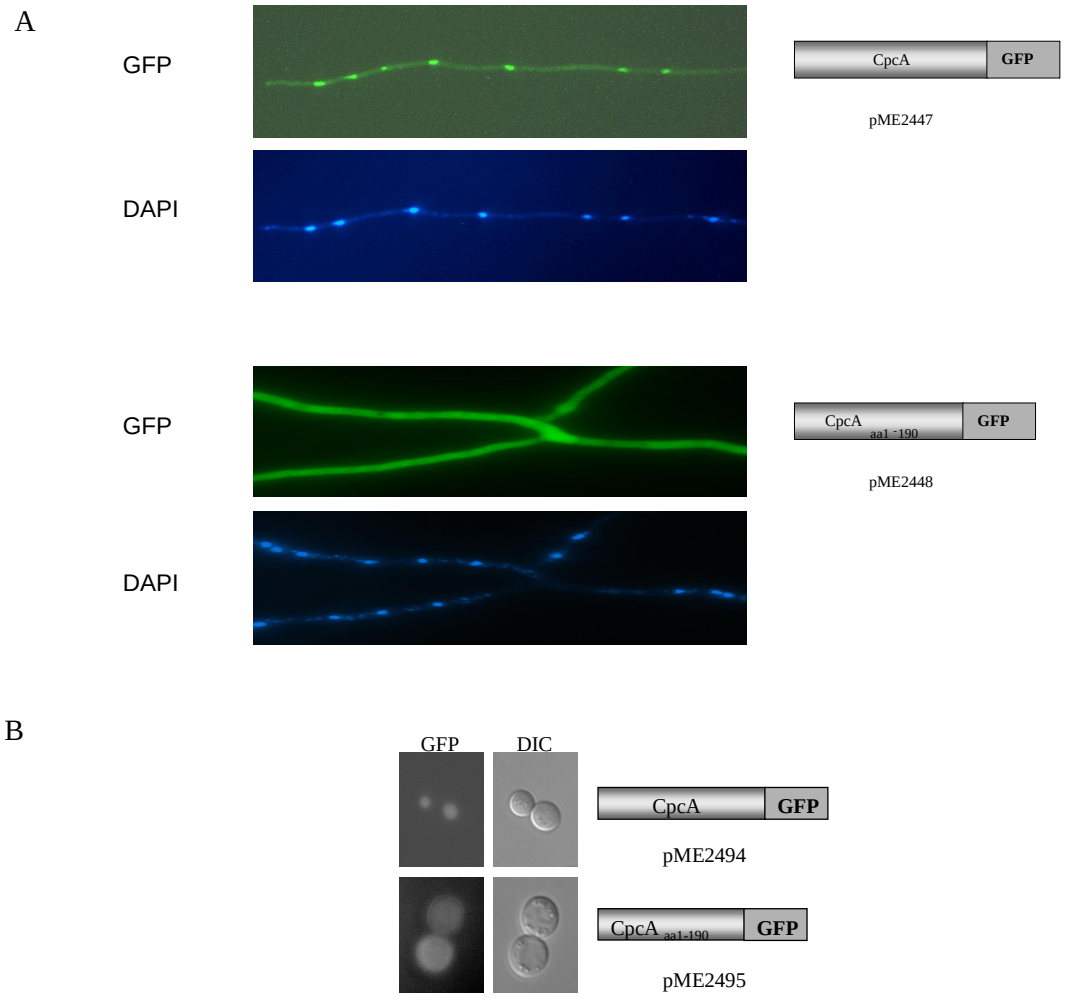


Fig. 19: Truncated CpcA$_{aa1-190}$ of *A. nidulans* accumulates in the cytoplasm
(A) Entire CpcA of *A. nidulans* was C-terminally conjugated with GFP and clearly nuclear localized, which was verified by DAPI staining of the nuclei. In contrast, truncated CpcA$_{aa1-190}$ lacking the conserved NLS motif accumulated in the cytoplasm. Both, CpcA-GFP and CpcA$_{aa1-190}$-GFP chimeric proteins were analyzed in *Aspergillus nidulans* AGB10 and driven from an inducible *alcA* promoter.
(B) Localization of entire and truncated CpcA was investigated in *S. cerevisiae*. CpcA of *A. nidulans* (pME2494) was localized in the nucleus of *S. cerevisiae*. Correspondingly to the cytoplasmic localization in *A. nidulans*, truncated CpcA$_{aa1-190}$-GFP also is impaired to enter the nucleus of *S. cerevisiae* (pME2495).

4.4.3 *In silico* investigation on putative importins of *Aspergillus nidulans*

Nuclear import is essential for Gcn4p to induce transcription of numerous genes whose products are involved in many different biosynthetic pathways in *S. cerevisiae* during response to amino acid starvation, glucose starvation and other stresses (Engelberg *et al.*, 1994; Hinnebusch and Natarajan, 2002; Mösch *et al.*, 1990; Natarajan *et al.*, 2001). CpcA as counterpart of Gcn4p in *A. nidulans* needs to be transported to the nucleus, as well, to play its role as a transcription factor during amino acid starvation response (Pries *et al.*, 2002). It was shown by Pries *et al.*, 2004 that the α-importin Srp1p and the β-importin Kap95p act as a heterodimer to channel Gcn4p into the yeast nucleus via the nuclear pores. So far no experimental procedures have been carried out to find whether similar proteins take over this task in *A. nidulans* or any other fungal species. Blast searches in the now available *Aspergillus* and *N. crassa* genomes revealed that proteins with high identities to Srp1p (over 58%) and Kap95p (over 39%) can be found to be encoded in the available *Aspergillus* and *Neurospora crassa* genomes (see Figure 20A and 20B). The assigned ORF for the Srp1p homologue SrpA was annotated as AN2142.1 and the Kap95p homologue KapA was annotated as AN0906.1 during the automated and manual annotation of the *A. nidulans* genome. Blast searches in the available *Aspergillus nidulans* genome revealed that there is only one α-importin like protein and twelve members of the importin beta superfamily present, remarkingly this is one beta importin less than is present in *S. cerevisiae*.

4.5 Discussion

4.5.1 Nuclear import of the transcription factor CpcA

It was recently shown that the entire yeast Gcn4 protein contains two nuclear localisation signals, each of which is able to direct a native cytoplasmic protein to the nucleus (Pries *et al.*, 2002). So far the emergence of multiple nuclear localisation signals is unique in yeast, whereas the human transcription factor NF-AT2 is known to possess two NLS motifs and inactivation of both motifs is required to abolish nuclear import (Beals *et al.*, 1997). The aim of this study was to investigate the capability of the *A. nidulans* CpcA NLS to guide the protein into the nucleus both in yeast, as well as in *A. nidulans*. The data presented in this work indicate that the single NLS of CpcA is necessary to guide the protein to the nucleus. This was observed expressing a GFP-tagged variant of the protein both in yeast and in *A. nidulans*. On the other hand the expressed truncated variants of CpcA accumulates in the cytoplasm in *S. cerevisiae*, as well as in its native host. This evidence lets us conclude that there is, unlike in yeast Gcn4p, only one NLS sufficient to target the CpcA protein to the nucleus and provides evidence that there is no hidden NLS1-like motive in CpcA. This is supported by the fact that we were unable to find a second NLS in the other investigated filamentous fungi. The nuclear localisation signal of the investigated CpcA proteins and yeast Gcn4p is well conserved, but nonetheless not identical. Though the experiments we carried out provides evidence, that the similarity of the bipartite NLS is high enough to properly target the transcription factor CpcA to the nucleus as well in bakers yeast, as in the filamentous fungus. Further experiments need to done to elucidate if CpcA is transported by Srp1p and Kap95p to the nucleus and if the putative proteins KapA and SrpA transport CpcA to the nucleus in *A. nidulans*.

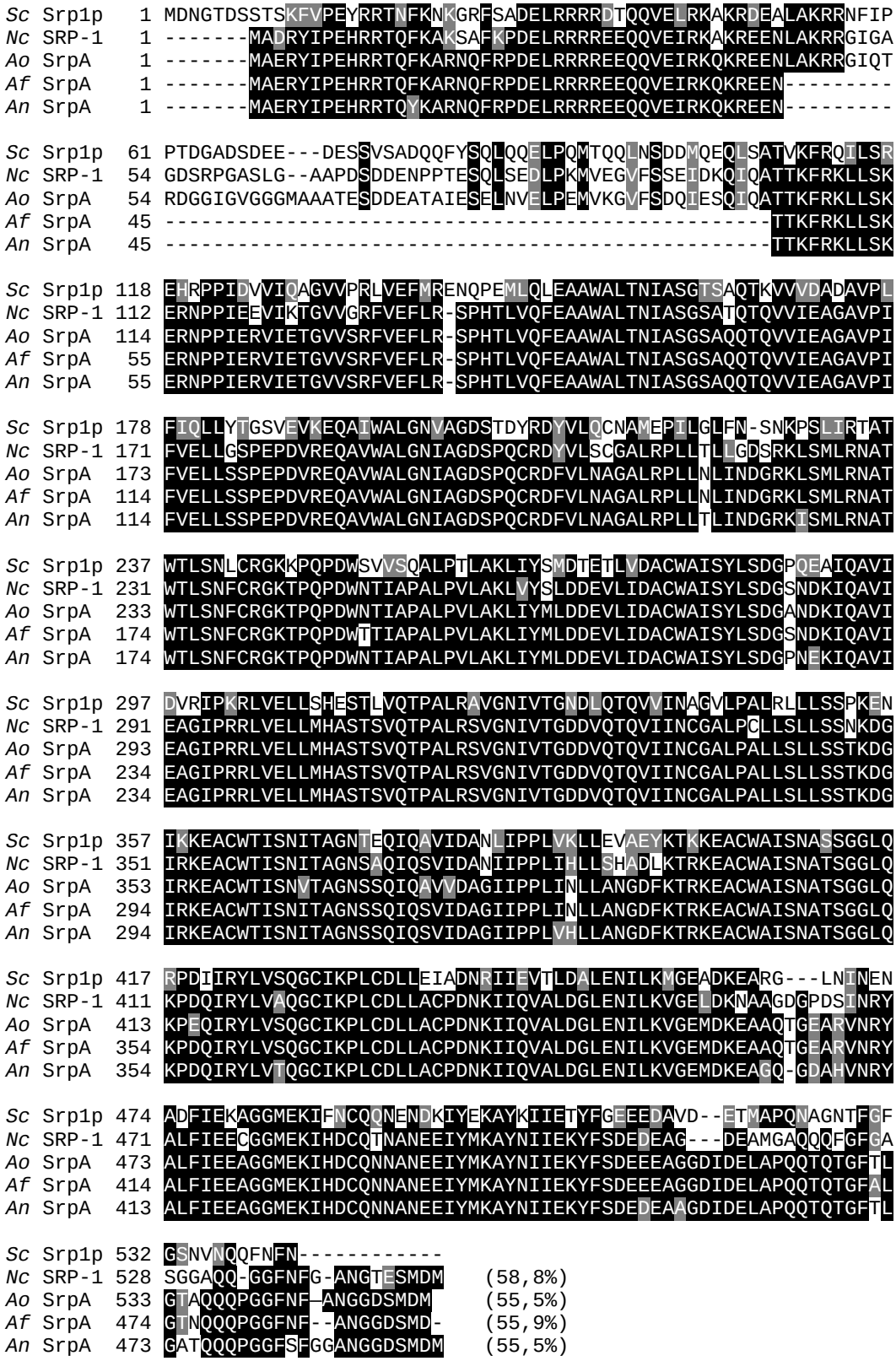
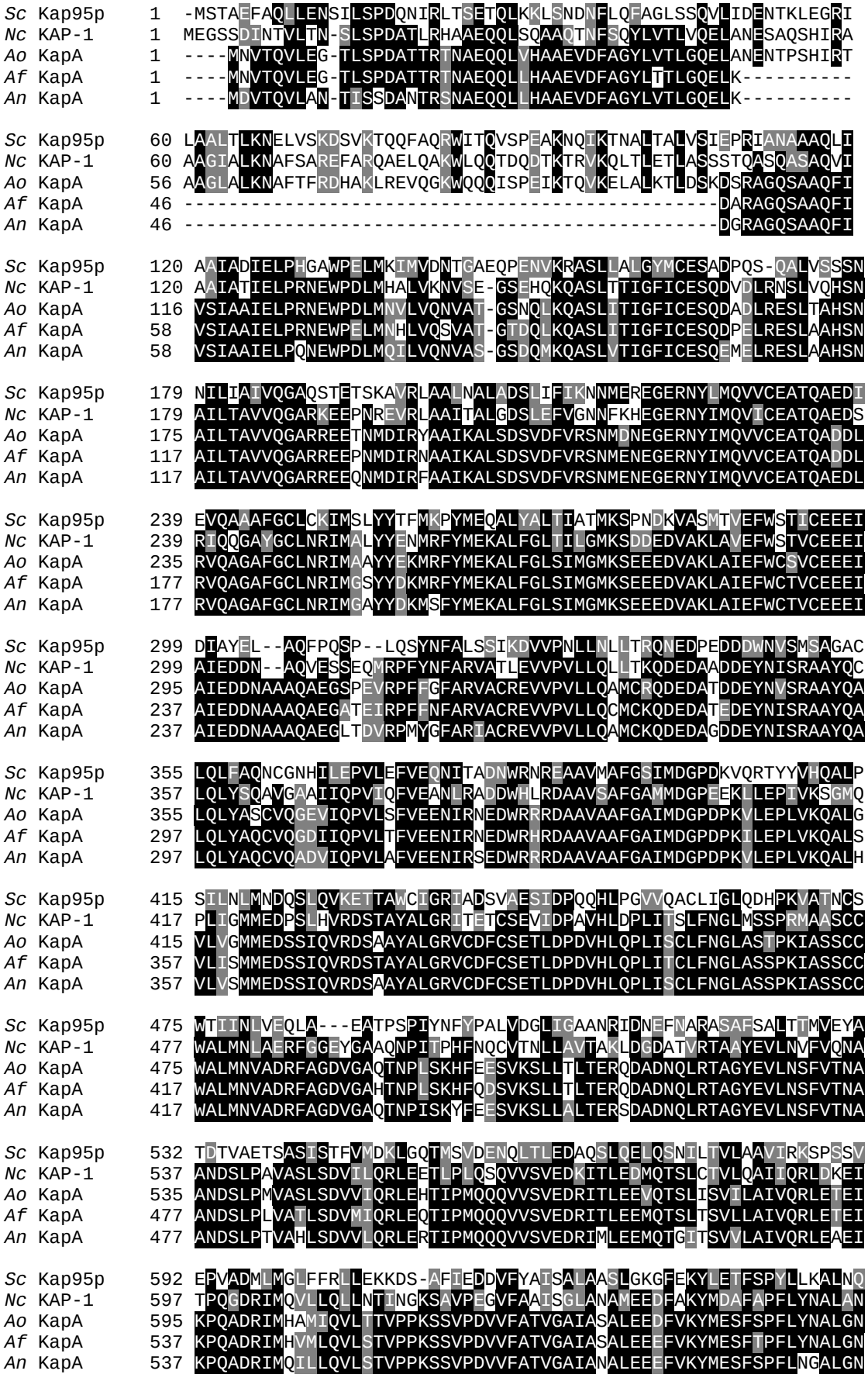


Fig. 20: Global multialignment of α and β type karyophyrins (A)
The multialignment of fungal α-importin Srp1p of *S. cerevisiae* (*Sc*), SrpA of *A. nidulans* (*An*), SrpA of *A. fumigatus* (*Af*), SrpA of *A. oryzae* (*Ao*) and srp-1 of *N. crassa* (*Nc*) shows a high conservation level. The percentage of identity of each single protein sequence to *S. cerevisiae* Srp1p is given in parentheses.


Sc Kap95p 1 -MSTAEFAQLLENSILSPDQNIRLTSETQLKKLSNDNFLQFAGLSSQVLIDENTKLEGRI
Nc KAP-1 1 MEGSSDINTVLTN-SLSPDATLRHAAEQQLSQAAQTNFSQYLVTLVQELANESAQSHIRA
Ao KapA 1 ----MNVTQVLEG-TLSPDATTRTNAEQQLVHAAEVDFAGYLVTLGQELANENTPSHIRT
Af KapA 1 ----MNVTQVLEG-TLSPDATTRTNAEQQLLHAAEVDFAGYLTTLGQELK----------
An KapA 1 ----MDVTQVLAN-TISSDANTRSNAEQQLLHAAEVDFAGYLVTLGQELK----------
Sc Kap95p 60 LAALTLKNELVSKDSVKTQQFAQRWITQVSPEAKNQIKTNALTALVSIEPRIANAAAQLI
Nc KAP-1 60 AAGIALKNAFSAREFARQAELQAKWLQQTDQDTKTRVKQLTLETLASSSTQASQASAQVI
Ao KapA 56 AAGLALKNAFTFRDHAKLREVQGKWQQQISPEIKTQVKELALKTLDSKDSRAGQSAAQFI
Af KapA 46 ----------DARAGQSAAQFI
An KapA 46 ----------DGRAGQSAAQFI
Sc Kap95p 120 AAIADIELPHGAWPELMKIMVDNTGAEQPENVKRASLLALGYMCESADPQS-QALVSSSN
Nc KAP-1 120 AAIATIELPRNEWPDLMHALVKNVSE-GSEHQKQASLTTIGFICESQDVDLRNSLVQHSN
Ao KapA 116 VSIAAIELPRNEWPDLMNVLVQNVAT-GSNQLKQASLITIGFICESQDADLRESLTAHSN
Af KapA 58 VSIAAIELPRNEWPELMNHLVQSVAT-GTDQLKQASLITIGFICESQDPELRESLAAHSN
An KapA 58 VSIAAIELPQNEWPDLMQILVQNVAS-GSDQMKQASLVTIGFICESQEMELRESLAAHSN
Sc Kap95p 179 NILIAIVQGAQSTETSKAVRLAALNALADSLIFIKNNMEREGERNYLMQVVCEATQAEDI
Nc KAP-1 179 AILTAVVQGARKEEPNREVRLAAITALGDSLEFVGNNFKHEGERNYIMQVICEATQAEDS
Ao KapA 175 AILTAVVQGARREETNMDIRYAAIKALSDSVDFVRSNMDNEGERNYIMQVVCEATQADDL
Af KapA 117 AILTAVVQGARREEPNMDIRNAAIKALSDSVDFVRSNMENEGERNYIMQVVCEATQADDL
An KapA 117 AILTAVVQGARREEQNMDIRFAAIKALSDSVDFVRSNMENEGERNYIMQVVCEATQAEDL
Sc Kap95p 239 EVQAAAFGCLCKIMSLYYTFMKPYMEQALYALTIATMKSPNDKVASMTVEFWSTICEEEI
Nc KAP-1 239 RIQQGAYGCLNRIMALYYENMRFYMEKALFGLTILGMKSDDEDVAKLAVEFWSTVCEEEI
Ao KapA 235 RVQAGAFGCLNRIMAAYYEKMRFYMEKALFGLSIMGMKSEEEDVAKLAIEFWCSVCEEEI
Af KapA 177 RVQAGAFGCLNRIMGSYYDKMRFYMEKALFGLSIMGMKSEEEDVAKLAIEFWCTVCEEEI
An KapA 177 RVQAGAFGCLNRIMGAYYDKMSFYMEKALFGLSIMGMKSEEEDVAKLAIEFWCTVCEEEI
Sc Kap95p 299 DIAYEL--AQFPQSP--LQSYNFALSSIKDVVPNLLNLLTRQNEDPEDDDWNVSMSAGAC
Nc KAP-1 299 AIEDDN--AQVESSEQMRPFYNFARVATLEVVPVLLQLLTKQDEDAADDEYNISRAAYQC
Ao KapA 295 AIEDDNAAAQAEGSPEVRPFFGFARVACREVVPVLLQAMCRQDEDATDDEYNVSRAAYQA
Af KapA 237 AIEDDNAAAQAEGATEIRPFFNFARVACREVVPVLLQCMCKQDEDATEDEYNISRAAYQA
An KapA 237 AIEDDNAAAQAEGLTDVRPMYGFARIACREVVPVLLQAMCKQDEDAGDDEYNISRAAYQA
Sc Kap95p 355 LQLFAQNCGNHILEPVLEFVEQNITADNWRNREAAVMAFGSIMDGPDKVQRTYYVHQALP
Nc KAP-1 357 LQLYSQAVGAAIIQPVIQFVEANLRADDWHLRDAAVSAFGAMMDGPEEKLLEPIVKSGMQ
Ao KapA 355 LQLYASCVQGEVIQPVLSFVEENIRNEDWRRRDAAVAAFGAIMDGPDPKVLEPLVKQALG
Af KapA 297 LQLYAQCVQGDIIQPVLTFVEENIRNEDWRHRDAAVAAFGAIMDGPDPKILEPLVKQALS
An KapA 297 LQLYAQCVQADVIQPVLAFVEENIRSEDWRRRDAAVAAFGAIMDGPDPKVLEPLVKQALH
Sc Kap95p 415 SILNLMNDQSLQVKETTAWCIGRIADSVAESIDPQQHLPGVVQACLIGLQDHPKVATNCS
Nc KAP-1 417 PLIGMMEDPSLHVRDSTAYALGRITETCSEVIDPAVHLDPLITSLFNGLMSSPRMAASCC
Ao KapA 415 VLVGMMEDSSIQVRDSAAYALGRVCDFCSETLDPDVHLQPLISCLFNGLASTPKIASSCC
Af KapA 357 VLISMMEDSSIQVRDSTAYALGRVCDFCSETLDPDVHLQPLITCLFNGLASSPKIASSCC
An KapA 357 VLVSMMEDSSIQVRDSAAYALGRVCDFCSETLDPDVHLQPLISCLFNGLASSPKIASSCC
Sc Kap95p 475 WTIINLVEQLA---EATPSPIYNFYPALVDGLIGAANRIDNEFNARASAFSALTTMVEYA
Nc KAP-1 477 WALMNLAERFGGEYGAAQNPITPHFNQCVTNLLAVTAKLDGDATVRTAAYEVLNVFVQNA
Ao KapA 475 WALMNVADRFAGDVGAQTNPLSKHFEESVKSLLTLTERQDADNQLRTAGYEVLNSFVTNA
Af KapA 417 WALMNVADRFAGDVGAHTNPLSKHFQDSVKSLLTLTERQDADNQLRTAGYEVLNSFVTNA
An KapA 417 WALMNVADRFAGDVGAQTNPISKYFEESVKSLLALTERSDADNQLRTAGYEVLNSFVTNA
Sc Kap95p 532 TDTVAETSASISTFVMDKLGQTMSVDENQLTLEDAQSLQELQSNILTVLAAVIRKSPSSV
Nc KAP-1 537 ANDSLPAVASLSDVILQRLEETLPLQSQVVSVEDKITLEDMQTSLCTVLQAIIQRLDKEI
Ao KapA 535 ANDSLPMVASLSDVVIQRLEHTIPMQQQVVSVEDRITLEEVQTSLISVILAIVQRLETEI
Af KapA 477 ANDSLPLVATLSDVMIQRLEQTIPMQQQVVSVEDRITLEEMQTSLTSVLLAIVQRLETEI
An KapA 477 ANDSLPTVAHLSDVVLQRLERTIPMQQQVVSVEDRIMLEEMQTGITSVVLAIVQRLEAEI
Sc Kap95p 592 EPVADMLMGLFFRLLEKKDS-AFIEDDVFYAISALAASLGKGFEKYLETFSPYLLKALNQ
Nc KAP-1 597 TPQGDRIMQVLLQLLNTINGKSAVPEGVFAAISGLANAMEEDFAKYMDAFAPFLYNALAN
Ao KapA 595 KPQADRIMHAMIQVLTTVPPKSSVPDVVFATVGAIASALEEDFVKYMESFSPFLYNALGN
Af KapA 537 KPQADRIMHVMLQVLSTVPPKSSVPDVVFATVGAIASALEEEFVKYMESFTPFLYNALGN
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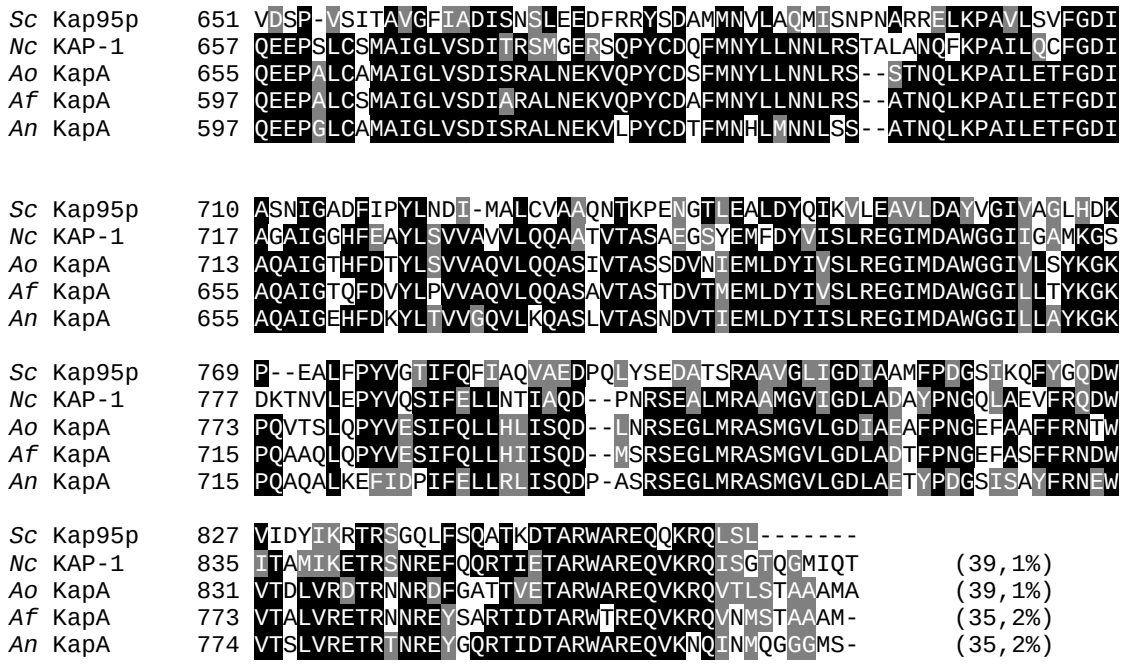

Fig. 20: Global multialignment of α and β type karyophyrins (B)
The multialignment of fungal β-importin Kap95p of *S. cerevisiae*, KapA of *A. nidulans*, KapA of *A. fumigatus*, KapA of *A. oryzae* and kap-1 of *N. crassa* shows a high conservation level. The percentage of identity of each single protein sequence to *S. cerevisiae* Kap95p is given in parentheses.

4.5.2 Importins of *Aspergillus nidulans*

Directed nuclear import of proteins requires energy and nuclear localisation signals and importins. In Pries *et al*., 2004 we have shown that the α-importin Srp1p and the β-importin Kap95p are necessary to import Gcn4p into the nucleus of *S. cerevisiae*, here we are able to enlarge the scale of results with the help of *in silico* analyses of the *A. nidulans* genome. Srp1p is so far the only known member of the α-importin family in *S. cerevisiae* and was found to recognize the bipartite NLS2 of Gcn4p and to trigger its nuclear import. Kap95p was shown to form heterodimers with Srp1p and in the publication evidence is provided that the β-karyopherin in combination with the α-importin Srp1p is necessary to target Gcn4p to the nucleus (Pries *et al.*, 2004). So far no experimental investigations were carried out, whether orthologs of these proteins play a similar role in *A. nidulans*. The availability to the *A. nidulans* genome and the deduced amino acid sequences made it possible to find first hints on these questions. Putative proteins with high similarity to both Srp1p and Kap95 can be found in *A. nidulans*, indicating that homologous proteins might play a similar role in nuclear uptake of the transcription factor CpcA.

4.6 References

Beals, C.R., Clipstone, N.A., Ho, S.N., and Crabtree, G.R. (1997) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. *Genes Dev* **11**: 824-834.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev* **15**: 1078-1092.

Dingwall, C., and Laskey, R.A. (1991) Nuclear targeting sequences - a consensus? *Trends Biochem Sci* **16**: 478-481.

Eckert, S.E., Kübler, E., Hoffmann, B., and Braus, G.H. (2000) The tryptophan synthase-encoding *trpB* gene of *Aspergillus nidulans* is regulated by the cross-pathway control system. *Mol Gen Genet* **263**: 867-876.

Engelberg, D., Klein, C., Martinetto, H., Struhl, K., and Karin, M. (1994) The UV response involving the Ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. *Cell* **77**: 381-390.

Görlich, D., Vogel, F., Mills, A.D., Hartmann, E., and Laskey, R.A. (1995) Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377**: 246-248.

Görlich, D., and Mattaj, I.W. (1996) Nucleocytoplasmic transport. *Science* **271**: 1513-1518.

Hinnebusch, A.G., and Natarajan, K. (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell* **1**: 22-32.

Hoffmann, B., Valerius, O., Andermann, M., and Braus, G.H. (2001) Transcriptional autoregulation and inhibition of mRNA translation of amino acid regulator gene *cpcA* of filamentous fungus *Aspergillus nidulans*. *Mol Biol Cell* **12**: 2846-2857.

Kaffman, A., and O'Shea, E.K. (1999) Regulation of nuclear localization: a key to a door. *Annu Rev Cell Dev Biol* **15**: 291-339.

Kornitzer, D., Raboy, B., Kulka, R.G., and Fink, G.R. (1994) Regulated degradation of the transcription factor Gcn4. *Embo J* **13**: 6021-6030.

Lusk, C.P., Makhnevych, T., and Wozniak, R.W. (2004) New ways to skin a kap: mechanisms for controlling nuclear transport. *Biochem Cell Biol* **82**: 618-625.

Meimoun, A., Holtzman, T., Weissman, Z., McBride, H.J., Stillman, D.J., Fink, G.R., and Kornitzer, D. (2000) Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. *Mol Biol Cell* **11**: 915-927.

Moore, M.S., and Blobel, G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* **365**: 661-663.

Mösch, H.U., Graf, R., Schmidheini, T., and Braus, G. (1990) Three GCN4 responsive elements act synergistically as upstream and as TATA-like elements in the yeast TRP4 promoter. *Embo J* **9**: 2951-2957.

Natarajan, K., Meyer, M.R., Jackson, B.M., Slade, D., Roberts, C., Hinnebusch, A.G., and Marton, M.J. (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* **21**: 4347-4368.

Pries, R., Bömeke, K., Irniger, S., Grundmann, O., and Braus, G.H. (2002) Amino acid-dependent Gcn4p stability regulation occurs exclusively in the yeast nucleus. *Eukaryot Cell* **1**: 663-672.

Pries, R., Bömeke, K., Draht, O., Künzler, M., and Braus, G.H. (2004) Nuclear import of yeast Gcn4p requires karyopherins Srp1p and Kap95p. *Mol Genet Genomics* **271**: 257-266.

Punt, P.J., and van den Hondel, C.A. (1992) Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods Enzymol* **216**: 447-457.

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