Amino Acid Biosynthesis and the COP9 Signalosome in *Aspergillus nidulans*:

Regulatory Networks in a Filamentous Fungus

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Silke Busch
aus Vechta

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Korreferent: Prof. Dr. G. Gottschalk

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Für meine Eltern

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Summary

The filamentous fungus *Aspergillus nidulans* represents an eukaryotic model system versatile in metabolism and developmental properties. This work focuses on the impact of modified enzyme activities whithin two regulatory networks on the fungal organism. The cross-pathway control ensures proper biosynthesis of amino acids upon corresponding starvation conditions in fungi. The COP9 signalosome is part of a regulatory network which is essential for the development of higher eukaryotes. Components of both systems have been isolated from *A.nidulans* and their contribution to the molecular control of physiology and morphology was examined.

Two amino acid biosynthesis genes, *hisB* and *lysA*, have been identified as target genes of the cross-pathway control. HISB, the imidazole glycerol-phosphate dehydratase (E.C. 4.2.1.19), is essential for histidine biosynthesis. Supplementation of a *hisB* deletion strain with traces of histidine enables growth of this auxotrophic strain. In contrast to the wild-type grown under the same conditions, this strain exhibits an induced cross-pathway control and arrests sexual development at the level of micro-cleistothecia. When high histidine supply represses the cross-pathway control, the sexual cycle can be complete. This indicates a link between the regulatory network of amino acid biosynthesis and sexual development in *A.nidulans*.

The *lysA* gene, encoding saccharopine dehydratase (E.C. 1.5.1.7), catalyses the ultimate step of lysine formation in the branched lysine/penicillin biosynthesis pathway. Transcription of *lysA* is increased upon amino acid starvation due to an activated cross-pathway control, whereas the *lysF* gene of the pathway's common stem as well as penicillin production were negatively affected. Thus, the regulatory network of amino acid biosynthesis is involved in a cross-talk between regulation of primary and secondary metabolism in *A.nidulans*.

The COP9 signalosome is a multiprotein complex with at least two assumed associated enzyme activities, a protein kinase and a deneddylase, which contribute to the regulation of targeted protein degradation. For the first time, two genes encoding subunits four and five of the COP9 signalosome of filamentous fungi, *csnD* and *csnE* of *A.nidulans*, were identified. Deletion of either *csn* subunit resulted in multiple mutant phenotypes. It can be concluded that the COP9 signalosome of *A.nidulans* is involved in repression of pigment production and maintenance of cell polarity in vegetative hyphae as well as in light-dependence of developmental induction and completion of the sexual cycle.

These results indicate that changes in single enzyme activities whithin genetic networks ultimately affect the metabolic and developmental potential of the entire fungal organism and suggest cross-connections between the different regulatory circuits.

Zusammenfassung

Der filamentöse Pilz *Aspergillus nidulans* ist ein eukaryontischer Modellorganismus mit vielseitigem Metabolismus und Entwicklungspotential. Diese Arbeit befasst sich mit der Auswirkung von veränderten Enzymaktivitäten innerhalb zweier regulatorischer Netzwerke auf den pilzlichen Organismus. Das System der 'Allgemeinen Kontrolle' sichert in Pilzen die Biosynthese von Aminosäuren unter entsprechenden Mangelbedingungen. Das COP9 Signalosom ist Teil eines regulatorischen Netzwerkes welches essentiell für Entwicklungsvorgänge in höheren Eukaryonten ist. Komponenten aus beiden Systemen wurden aus *A.nidulans* isoliert und ihr Einfluss auf physiologische und metabolische Prozesse untersucht.

Die beiden Aminosäure-Biosynthesegene *hisB* und *lysA* wurden als Zielgene der Allgemeinen Kontrolle identifiziert. Die Imidazol Glycerol-phophat Dehydratase HISB (E.C. 4.2.1.19) ist essentiell für die Histidin Biosynthese. Ein *hisB*-Deletionsstamm wächst wenn geringe Histidinmengen supplementiert werden, anders als beim Wildtyp zeigt er unter diesen Bedingungen jedoch eine aktivierte Allgemeine Kontrolle und arretiert seine sexuelle Entwicklung auf der Stufe der Mikro-Cleistothecien. Durch hohe Mengen an Histidin kann das Kontrollsystem abgeschaltet und die Entwicklungsblockade aufgehoben werden. Dies weist auf eine Verbindung zwischen dem regulatorischen Netzwerk der Aminosäure-Biosynthese und der sexuellen Fruchtkörper-Bildung von *A.nidulans* hin.

Das *lysA*-Gen kodiert für die Saccharopine Dehydratase (E.C. 1.5.1.7), welche den letzten Schritt der Lysin-Bildung des verzweigten Lysin/Penicillin-Biosyntheseweges katalysiert. Die Transkription von *lysA* wird bei Aminosäure-Mangel durch die Allgemeine Kontrolle erhöht, wohingegen die Transkription des *lysF*-Gens, welches im gemeinsamen Teil des Syntheseweges agiert, sowie die Penicillin Produktion negativ beeinflusst werden. Das regulatorischen Netzwerk der Aminosäure-Biosynthese ist demnach an einer Co-Regulation zwischen Primär und Sekundärmetabolismus in *A.nidulans* beteiligt.

Das COP9 Signalosom ist ein Multiprotein-Komplex mit mindestens zwei assoziierten Enzymaktivitäten, eine Proteinkinase und eine Deneddylase, welche zur Regulation gezielter Proteindegradation beitragen. Mit *csnD* und *csnE* konnten Gene der CSN-Untereinheiten vier und fünf erstmals aus einem filamentösen Pilz isoliert werden. Deletion von *csnD* oder *csnE* führte zu multiplen mutanten Phänotypen. Diese lassen darauf schliessen, dass das COP9 Signalosom in *A.nidulans* an der Repression von Pigmentbildung und dem Erhalt von Zellpolariät in vegetativen Zellen sowie an der Lichtabhängigkeit der Reproduktionszyklen und dem Abschluss der sexuellen Entwicklung beteiligt ist.

Die Ergebnisse zeigen, dass Veränderungen in einzelnen Enzymaktivitäten innerhalb komplexer genetischer Netzwerke Auswirkungen auf Metabolismus und Entwicklungvorgängen des pilzlichen Organismus haben, und lassen vermuten, dass die einzelnen Regulationskreise miteinander verbunden sind.

Chapter 1

Introduction

1.1 Scope and aim of this work

Resolution of the DNA structure about 50 years ago (Watson and Crick 1974) gave rise to a new scientific field, molecular genetics, that proceeded vastly to our current knowledge of the molecular design of life. What started with the identification of single genes has now come to a point of complete genome sequencing. It is no more primarily the discovery of distinct biochemical pathways that inspires the research community but broader studies on overall cellular organisation driven by new technologies like 'transcriptomics' and 'proteomics'. Subjects of special interest are regulatory networks that control molecular processes in the living organism in its entirety. The understanding of such sophisticated genetic regulations is indispensable for applied molecular biology, especially for industrial metabolic engineering that aims to modulate the metabolic flux to a desired biomolecule.

Several economically, medically and agriculturally important fungal species belong to the genus *Aspergillus*. For example, organic acids like citric acid and extracellular enzymes like glucoamylases are produced at industrial scale by *A. niger* and *A. awamori*, respectively. Bioactive molecules like ß-lactam antibiotics and aflatoxins are excreted by *A. fumigatus* and *A. flavus*. Additionally, *A. fumigatus* is an opportunistic pathogen causing invasive aspergillosis in immuno compromised individuals (Kontoyiannis and Bodey 2002). *A. nidulans* constitutes a representative of this fungal genus that is capable of complex biosyntheses and differentiation processes. This eukaryotic model organism is accessible to molecular methods and can be manipulated relatively easy. Thus, *Aspergillus* is particularly suited for studying regulatory networks of the molecular cross-connections between environmental stimuli, metabolism and development.

This study directs the focus on enzyme activities involved in two regulatory networks: the cross-pathway control of amino acid biosynthesis and the COP9 signalosome-dependent system cotrolling development. Components of both networks of *A. nidulans* were isolated and analysed with a special focus on their impact on metabolism and development. Two target genes of the cross-pathway control were examined, which revealed an impact of this network in sexual reproduction (Chapter 2) and penicillin biosynthesis (Chapter 3). Additionally, two subunits of the *A. nidulans* COP9 signalosome were shown to be essential for light-dependent signalling, pigment production and development (Chapter 4). Thus, the cross-pathway control and the COP9 signalosome-dependent network are involved in the regulation of environmental stimuli, physiology and morphology.

1.2 Genetic control

A major maxim of the living cell is to code information within the order of base residues of nucleic acids and selectively transfer this information as ribonucleic acids to finally produce the corresponding proteins as e.g. biocatalysts of cellular processes. This implies that different molecular levels, including DNA transcription, RNA translation and protein degradation, are point of regulatory attack (Fig. 1.1). Common regulatory themes on each level are direct modification, changes in conformation and stabilisation. For the compartmentalised eukaryotic cell and multicellular organisms, localisation and intercellular communication define an additional level of regulation. For molecular control of physiological and morphological processes, several of these regulatory principles are combined to perceive extracellular and intracellular signals and finally respond with an adequate cellular answer. Two eukaryotic regulatory networks with emphasis on metabolism and development are the cross-pathway control of amino acid biosynthesis and the COP9 signalosome-dependent system, respectively.

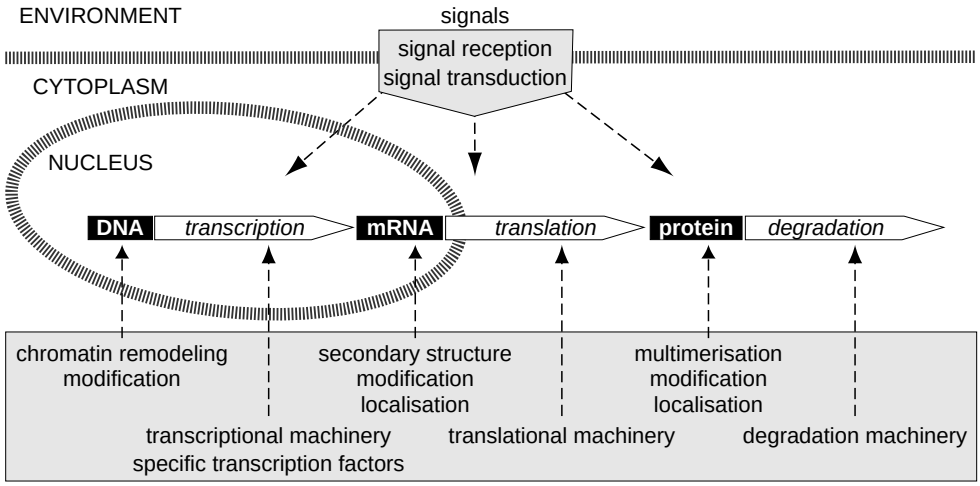

Fig. 1.1: Genetic control of protein synthesis and turnover in a eukaryotic cell. The scheme shows the central pathway of protein synthesis and degradation defining the molecular levels of regulatory attack. Grey fillings highlite examples for general mechanisms of molecular control with their target level indicated by broken-lined arrows.

1.2.1 Expression of gene products and the cross-pathway control

1.2.1.1 The yeast central transcriptional activator Gcn4p. Instantaneous response to environmental changes requires rapid transcriptional reprogramming. The main players that co-ordinate transcriptional activation of genes attributed to a common physiological trait are specific transcription factors. These regulatory *trans*-acting proteins bind to defined nucleotide motifs in the promoter of their target genes and recruit the transcriptional machinery for initiation of transcription. These two functions generally imply at least two domains characteristic for transcription

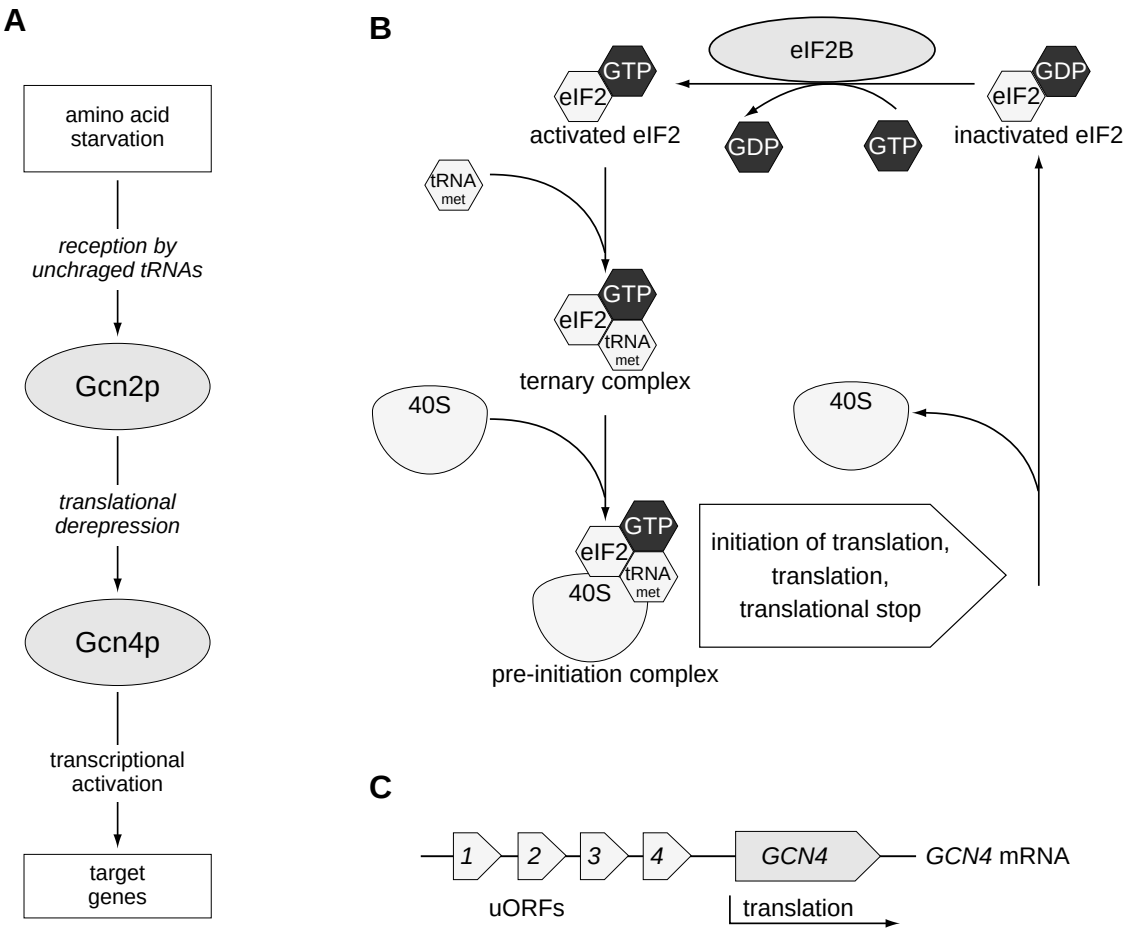

Fig. 1.2: Regulation of the synthesis of yeast Gcn4p transcriptional activity. (A) The signal "amino acid starvation" is perceived and transduced by the sensor kinase Gcn2p and finally results in translational derepression of the transcriptional activator Gcn4p which in turn activates transcription of corresponding target genes. **(B)** Of central meaning for the translational derepression mechanism is the regeneration of activated eukaryotic translation initiation factor 2 (eIF2). GTP-activated eIF2 and tRNAmet form a ternary complex that is enlarged with the small ribosomal subunit (40S) to the pre-initiation complex that is prerequisite for initiation of translation. After the translational stop, eIF2 is released in the GDP-bound, inactive form and depends on the guanosine exchange factor eIF2B to rebuild a ternary complex. Upon amino acid starvation, Gcn2p phosphorylates eIF2 which prevents GDP exchange and thus slows the process of translational initiation down. **(C)** Pivot of the translational derepression mechanism are the four small upstream open reading frames (uORFs) proximal of the *GCN4* open reading frame. The function of these uORFs in regulation of *GCN4* translation is described in the text.

factors. Recognition of the target sequence is mediated by a DNA binding domain (BD) with bZIP, bHLH or Zn-finger motif whereas recruitment of the transcriptional machinery is mainly mediated by an activation domain (AD) composed of glutamine and asparagine-, glutamine-, proline-, serine and threonine- or alanin-rich regions (Johnson and McKnight 1989; Klevit 1991). A sophisticated regulatory circuit controlled by a transcription factor is the regulation of amino acid biosynthesis in response to exogenous availability of amino acids, termed cross-pathway control (CPC) in filamentous fungi and general amino acid control (GAAC) in the yeast *Saccharomyces cerevisiae* (Piotrowska 1980; Hinnebusch 1988). The central transcription factor of this regulatory network is thoroughly studied in yeast and is described there as Gcn4p. Its carboxy terminal basic HLH domain and the

leucine zipper mediate dimerisation and DNA binding (Saudek *et al.* 1990; Pu and Struhl 1991; Ellenberger *et al.* 1992) to the general control responsive elements (GCRE) with the consensus sequence 5'-TGA C/G TCA-3' (Oliphant *et al.* 1989). The transcriptional activation domain is subdivided into an N-terminal and a central acidic part, separated by a PEST instability region (Kornitzer *et al.* 1994; Drysdale *et al.* 1995; Jackson *et al.* 1996; Drysdale *et al.* 1998). Additionally, the amino acid sequence of Gcn4p contains two functional nuclear localisation sequences (NLS) that mediate the transport of the transcriptional activator into the nucleus, independent of amino acid availability (Pries *et al.* 2002).

Extensive microarray studies revealed that 14% of the yeast's genome responds transcriptionally to amino acid starvation in a proposed Gcn4p dependent manner (Natarajan *et al.* 2001). Many of the induced genes contain a GCRE within the 5'-proximal 300 nucleotides and thus are potential direct Gcn4p target genes. They include representatives of all amino acid or amino acid precursor biosynthesis pathways and other putative targets related to synthesis or uptake of amino acids. Nevertheless, several of the identified putative targets are not related to amino acid metabolism, including several transcription factors and other cellular regulators. Some genes were even repressed under amino acid starvation conditions, but since repression affected predominantly genes of ribosomal and translational genes of which most lack GCREs, the repression effect is probably indirect and confers to decreased ribosome production and protein synthesis at amino acid starvation, or to activation of specific repressors (Hinnebusch and Natarajan 2002). It is additionally noteworthy that starvation for purine (Rolfes and Hinnebusch 1993), glucose (Yang *et al.* 2000), high salinity (Goossens *et al.* 2001) or ultraviolett light (Engelberg *et al.* 1994) also induce the Gcn4p regulatory system.

1.2.1.2 Control of Gcn4p expression. To enable rapid modulation of differentiated transcriptional levels of gene families in response to changes in the environment, several mechanisms limit abundance, distribution and activity of the corresponding transcription factors. Complex regulation of Gcn4p occurs on several molecular levels. Though amino acid starvation results in increased *GCN4* mRNA levels, regulation of *GCN4* transcription seems of only minor importance in yeast (Albrecht *et al.* 1998).

The major regulation of Gcn4p expression is a translational derepression mechanism that is activated through a signal transduction cascade upon the signal amino acid starvation (Fig. 1.2A). The molecular mechanism of this cross-pathway signal transduction cascade is explained in a widely accepted model (Hinnebusch 1997): Limitation of amino acids leads to accumulation of uncharged tRNAs in the cell that bind to the hisRS domain of the sensor kinase Gcn2p (Wek *et al.* 1995). The Gcn2p kinase domain subsequently transduces this signal to the eukaryotic translation initiation factor 2 (eIF2) by phosphorylation (Qiu *et al.* 2001), which in the end leads to shortage of ternary complex and thus slowing down of translational initiation as described in Figure 1.2B. With the resulting low rate of translational initiation, the translational barrier mediated by four small upstream open reading

frames (uORFs) in the *GCN4* mRNA leader (Fig. 1.2C) can be overcome (Hinnebusch 1997). Upon non-starvation conditions, this barrier is mainly mediated by translation of uORF4 after which about 50% of the 40S ribosomal subunits dissociate from the mRNA and never reach the GCN4 open reading frame. Upon amino acid starvation, the 40S ribosomal subunit resumes scanning the mRNA after translation of uORF1, but due to the shortage of ternary complex, the AUG start codon of the inhibitory uORF4 is passed by until a new pre-initiation complex is formed and translation re-initiates at the *GCN4* translational start.

Additionally, interference of the general control to the nitrogen sensing system has been shown to act on the translational level (Grundmann *et al.* 2001). Though nitrogen deprivation increases *GCN4* transcription, it represses its further expression. Thus, nitrogen starvation overrides the translational induction of Gcn4p but seems to keep higher *GCN4* mRNA levels in order to rapidly induce translation when nitrogen sources become available.

1.2.1.3 Post-translational control of Gcn4p. Once Gcn4p is synthesised, its stability is carefully controlled. It is a short lived protein that is rapidly degraded under non-starvation conditions (Kornitzer *et al.* 1994). Prerequisite for degradation is phosphorylation of tyrosine residues in the activation domain of Gcn4p by at least two cyclin-dependent kinases, Pho85p and Srb10p (Meimoun *et al.* 2000; Chi *et al.* 2001) (Fig. 1.3A). The control of Gcn4p degradation, as well as localisation of Pho85p, is restricted to the nucleus (Pries *et al.* 2002). Phosphorylated Gcn4p is subsequently ubiquitinylated by the SCF^{Cdc4} ubiquitin ligase which targets it to degradation by the 26S proteasome (see 1.2.2.1).

GCRE-bound Gcn4p is probably involved in the recruitment of the transcriptional machinery to the target promoter and can directly interact with at least three basal transcriptional co-activator complexes, TFIID, SAGA and MED (Drysdale *et al.* 1998; Natarajan *et al.* 1999). (i) The basal transcription factor of RNA polymerase II (TFIID) enables formation of the transcriptional pre-initiation complex. It is composed of the TATA-binding protein (TBP) that binds to promoters and several TBP associated factors (TAFs) that mediate e.g. protein kinase and acetyltransferase activity (Burley and Roeder 1996; Green 2000). TBP and the Gcn4p binding domain are connected by the co-activator multiprotein bridging factor (Mbf1p), that probably recruits the TBP to promoters where specific transcription factors are bound (Takemaru *et al.* 1998). (ii) The Spt/Ada/Gcn5 acyltransferase complex (SAGA) is composed of multiple transcriptional regulatory proteins, including several TBP-related components and the histone acetlytransferase Gcn5p. It thus links chromatin modification and the basal transcription machinery with specific transcription factors (Grant *et al.* 1997; Drysdale *et al.* 1998; Winston and Sudarsanam 1998). (iii) The RNA polymerase II holoenzyme mediator complex (MED), composed of more than 20 proteins, directly interacts with components of the RNA polymerase II and acts as an essential co-activator. It mediates response of the transcriptional machinery to specific transcriptional activators at upstream DNA binding sequences by modulating RNA polymerase II activity (Kim *et al.* 1994; Hengartner *et al.* 1995). Strikingly, the Srb10p

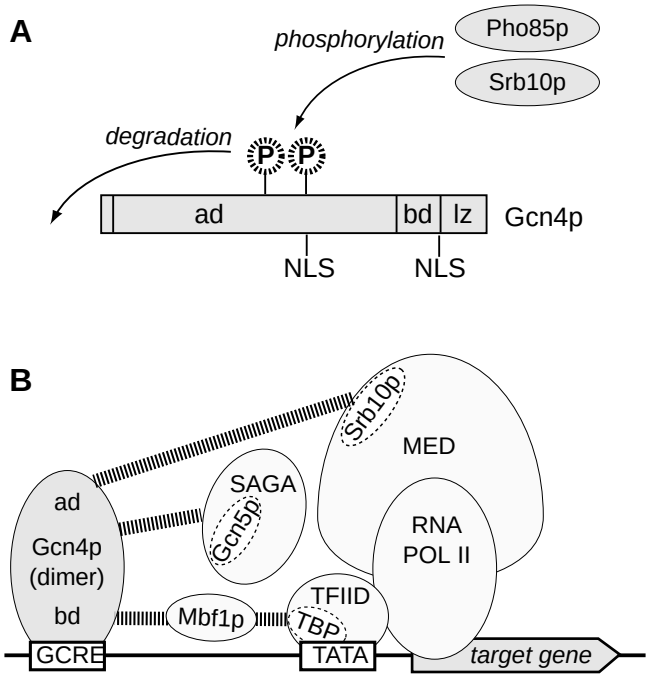

Fig. 1.3: Regulation of the turnover of yeast Gcn4p transcriptional activity. (A) Threonin residues in the Gcn4p activation domain (ad) can be phosphorylated by at least two different protein kinases, Pho85p and Srb10p. Phosphorylated Gcn4p is subject to ubiquitination by the E3 ligase SCF^{Cdc4}. Nuclear localisation motifs (NLS) are responsible for the transport of the transcription factor to its site of action: the nucleus. **(B)** Gcn4p binds as a dimer to GCRE elements in the target gene's promoter region and recruits the transcriptional machinery and accessory multiprotein complexes. Several interactions of Gcn4p with multiprotein complexes have been shown, this scheme summarises examples. RNA polymerase II (RNA POL II), the basal transcription factor IID (TFIID), the mediator complex (MED) and the Spt/Ada/Gcn5 acetyltransferase complex (SAGA). Direct protein-protein interactions are indicated by paralleled lines. In this scenario, Gcn4p is phosphorylated by Srb10 (a MED component) during its function as transcriptional activator. Abbreviations: activation domain (ad), DNA binding domain (bd), leucine zipper (lz).

protein kinase is associated with the large multiprotein mediator complex (Hengartner *et al.* 1995).

As mentioned above, Srb10p phosphorylates Gcn4p and thus targets it to the degradation machinery (Chi *et al.* 2001). This implies that promoter-bound Gcn4p specifically and probably independently recruits TFIID, SAGA and the MED-Polymerase II-holoenzyme to the promoter, enables initiation of transcription and is in turn specifically selected for proteolysis by phosphorylation. Such an interdependency of transcription factor stability and transcriptional machinery may prevent that a single promoter bound molecule activates multiple rounds of transcription initiation and thus restricts the global disruption of transcriptional control that results from squelching (Tansey 2001).

1.2.1.4 The cross-pathway control of *A. nidulans*. The cross-pathway control in filamentous fungi corresponds to the general control in yeast and is subject to intensive research. Homologues of yeast *GCN4* have been identified in the filamentous fungi *Neurospora crassa* (Paluh *et al.* 1988) and *A. niger* (Wanke *et al.* 1997) sharing in their deduced sequences high amino acid sequence identities with the mammalian oncoprotein *c-jun* (Bohmann *et al.* 1987). During the course of this work, the corresponding *A. nidulans* cross-pathway control transcriptional

activator gene *cpcA* was isolated. It is functionally exchangeable with the yeast Gcn4p, indicating that the system that mediates amino acid starvation response is highly conserved between yeast and filamentous fungi (Hoffmann *et al.* 2001). Accordingly, nucleotide motifs that resemble the yeast GCRE consensus sequence are found in the promoter region of *A. nidulans* genes that are activated by CPCA. Examples are *argB* (Piotrowska 1980) and two additional genes identified during the course of this work: *trpB* (Eckert *et al.* 2000) and *hisHF* (Valerius *et al.* 2001). Correspondingly, this consensus is termed cross-pathway response element (CPRE) in filamentous fungi. Examples like *aroC* (Krappmann *et al.* 1999) show that not all genes acting in amino acid metabolism are regulated by the cross-pathway control and, vice versa, CPCA-mediated transcriptional activation of the functionally yet uncharacterised putative DNA binding protein *jlbA* (Strittmatter *et al.* 2001) indicates that not solely genes involved in amino acid metabolism are regulated by the cross-pathway control in *A. nidulans*. The *A. nidulans* cross-pathway transcriptional activator seems to be regulated in a similar complex but not identical manner as its yeast counterpart. Only two uORFs mediate translational de-repression of the *A. nidulans cpcA* mRNA. Additionally, CPCA significantly autoregulates its own transcription by CPREs in its promoter, as it is also seen in mammalian *jun* and *fos* genes but not in the yeast *GCN4* gene (Hoffmann *et al.* 2001).

1.2.2 Degradation of gene products and the COP9 signalosome.

1.2.2.1 Ubiquitin dependent protein degradation. Controlled timely destruction of proteins is a major regulatory mechanism of complex cellular processes. The main eukaryotic protein degradation machinery, the 26S proteasome, specifically degrades ubiquitinylated proteins (Fig. 1.4A). Proteolysis is performed by the ATP-independent peptidase activity of the proteasome 20S core particle, whereas substrate specificity is mediated by the 19S regulatory particle composed of a base complex that confers ATPase and chaperon activity and the 26S lid complex (LID) with yet unknown mechanism. Substrates are targeted for degradation by conjugation of ubiquitin, a small protein of 111 amino acids with catabolic role when added as polyubiquitin chains. The corresponding ubiquitinylation cascade (Fig. 1.3B) is extensively studied and thoroughly reviewed (Hershko and Ciechanover 1998; Jackson and Eldridge 2002). An ubiquitin activating enzyme (E1) activates ubiquitin by thioester linkage prior to transfer to a lysine residue of a specific protein substrate by an ubiquitin conjugating enzyme (E2). An ubiquitin ligase (E3) catalyses substrate recognition and assists in ubiquitinylation.

Several E3 ubiquitin ligase complexes are known, most prominent is the cullin-RING-H2-family that includes SCF (Skp1/Cdc53/F-box) complexes (Deshaies 1999; Tyers and Jorgensen 2000). These E3 ubiquitin ligases are generally composed of four major subunits. The core activity is performed by a cullin (CUL1) and a RING-H2 domain protein (HRT1/ROC1/RBX1). Both are associated together with the SKP1 subunit to a special F-box protein as substrate receptor. Different E3

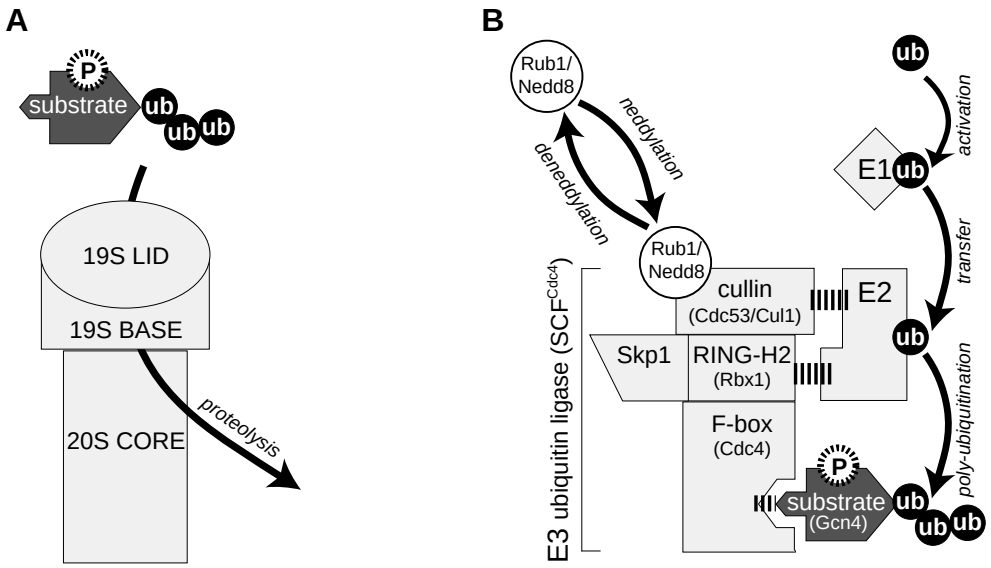

Fig. 1.4: Ubiquitin dependent protein degradation. A schematic overview of the major multiprotein complexes involved in ubiquitin-dependent proteolysis is given, modified from Tyers and Jorgensen (2000) and von Arnim (2001). **(A)** The 26S proteasome specifically degrades ubiquitinylated proteins. **(B)** Ubiquitination of the target substrates follows an enzymic cascade of an ubiquitin activating enzyme (E1), and ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3). The four critical subunits of a typical E3 ligase are shown, with the yeast protein nomenclature for the E3 SCFCdc4 that targets Gcn4p for degradation given in parentheses. Parralelled lines indicate protein-protein interaction.

ubiquitin ligase complexes are designated by their accessory F-box subunit. Since protein degradation involves regulation of essential cellular processes, the targeting of substrates to ubiquitin-dependent degradation by the 26S proteasome must be tightly connected to the actual cellular response signalling pathways. SCF ubiquitinylation activity towards potential target substrates is regulated by at least two mechanisms, phosphorylation and neddylation. (i) SCF activity towards the stability of its substrates depends on the level of substrate phosphorylation by the corresponding protein kinases. Exemplary for Gcn4p (see 1.2.1), ubiquitinylation by SCF^{CDC4} requires phosphorylation by Pho85 or Srb10 (Meimoun *et al.* 2000). (ii) The ubiquitinylation activity of the SCF itself can be modulated by reversible conjugation of the ubiquitin-related protein NEDD8/Rub1 on the cullin subunit, a process called neddylation. Cycling of neddylation and deneddylation are essential for E2-E3 complex formation (Kawakami *et al.* 2001) and cullin-dependent polyubiquitinylation of SCF target proteins (Morimoto *et al.* 2000; Podust *et al.* 2000; Read *et al.* 2000; Wu *et al.* 2000; Ohh *et al.* 2002). Proteins with specific neddylation activities were identified (del Pozo *et al.* 2002; Fan *et al.* 2002), but a distinct deneddylase was not isolated yet.

1.2.2.2 The COP9 signalosome. A regulatory complex tightly connected to the control of ubiquitin dependent protein degradation is the constitutive photomorphogenesis 9 complex (COP9) termed COP9 signalosome (CSN). Together with the 19S proteasome lid (LID) and the eukaryotic translation initiation factor 3 (eIF3), the CSN belongs to the PCI family of multiprotein complexes that is highly conserved among higher eukaryotes. Their subunits are characterised by either a PCI (proteasome, COP9, eIF3) or a MPN (Mpr1p, Pad1 N-terminal) domain

of about 200 and 140 amino acids, respectively. These domains might mediate stabilisation of protein-protein interactions, though their precise function remains obscure (Glickman *et al.* 1998; Hofmann and Bucher 1998; Kim *et al.* 2001). The COP9 signalosome (Fig. 1.5A) is composed of the eight subunits CSN1-CSN8 (Deng *et al.* 2000). These exhibit several subunit-subunit and non-subunit interactions (Tab. 1.1) and can partially be phosphorylated (Kapelari *et al.* 2000; Bech-Otschir *et al.* 2002). The proteasomal lid shows a similar subunit composition with Rpn7, 6, 3, 5, 11, 8, 9 and 12 corresponding to CSN1-8 (Kim *et al.* 2001). But electron microscopy of subunit arrangements revealed that the overall architecture of CSN and LID is not identical, though at least both share an asymmetric shape enclosing a central cave (Kapelari *et al.* 2000; Fu *et al.* 2001). Both complexes share a similar size of about 500 kDa and are localised mainly in or close to the nucleus. A common evolutionary origin of the PCI complexes is thus discussed, with eIF3 being more distantly related (Hofmann and Bucher 1998; Kapelari *et al.* 2000; Fu *et al.* 2001; Kim *et al.* 2001; Tsuge *et al.* 2001).

Tab. 1.1: Seletion of interactions of mammalian COP9 signalosome subunits

protein		interaction / CSN-mediated activity[1]	
degradation machinery:			
CUL1	SCF cullin subunit	CSN2 / D	Lyapina *et al*. 2001
Rpt6	26S proteasome LID subunit	CSN3	Kwok *et al*. 1999
regulatory proteins:			
Bcl3	IκB multigene family member	CSN5 / +	Dechend *et al*. 1999
c-Jun	transcription factor / AP-1	CSN5 / + P	Naumann *et al*. 1999
ICSBP	interferon consensus sequence binding protein	CSN2 / + P	Cohen *et al*. 2000
IKKγ	IκB-kinase complex component	CSN3 / -	Hong *et al*. 2001
LFA-1	integrin	CSN5	Bianchi *et al*. 2000
MIF	cytokine macrophage migration inhibitory factor	CSN5	Kleemann *et al*. 2000
$p27^{Kip1}$	cyclin-dependent kinase inhibitor	CSN5 / -	Tomada *et al*. 1999
p53	tumor suppressor	CSN5 / - P	Bech-Otschir *et al*. 2001
PR	progesteron receptor	CSN5 / +	Chaucherau *et al*. 2000
rLHR	lutropin/choriogonadotropin receptor precursor	CSN5 / -	Li *et al*. 2000
SCR-1	steroid receptor co-activator	CSN5 / +	Chaucherau *et al*. 2000
THR	thyroid hormone receptor	CSN2	Lee *et al*. 1995
Vpr	HIV-1 accessory protein	CSN6	Mahalingam *et al*. 1998

[1] CSN-mediated stability (+) or proteolysis (-) effects and proven phosphorylation (P) or deneddylation (D) activities are indicated.

The available data on CSN function suggest that it contributes to the regulation of ubiquitin dependent degradation by modification of E3 ubiquitin ligase activity by at least two different functions (Seeger *et al.* 2001; Bech-Otschir *et al.* 2002). The COP9 signalosome promotes deneddylation of cullins by a proposed associated isopeptidase that mediates deneddylase activity, as shown for mammalian cells, *A. thaliana* and *S. pombe* (Zhou *et al.* 2001; Wang *et al.* 2002; Yang *et al.* 2002). Additionally, an associated Ser/Thr kinase activity of the CSN phosphorylates proteins like p53 (Bech-Otschir *et al.* 2001), c-Jun (Musti *et al.* 1997; Naumann *et al.* 1999) and ICSBP (Cohen *et al.* 2000) and thus alters their susceptibility to ubiquitinylation. The precise mechanisms of the deneddylation and phosphorylation activities associated with CSN and the connections between both are not yet understood. To date, two major working hypotheses for the CSN

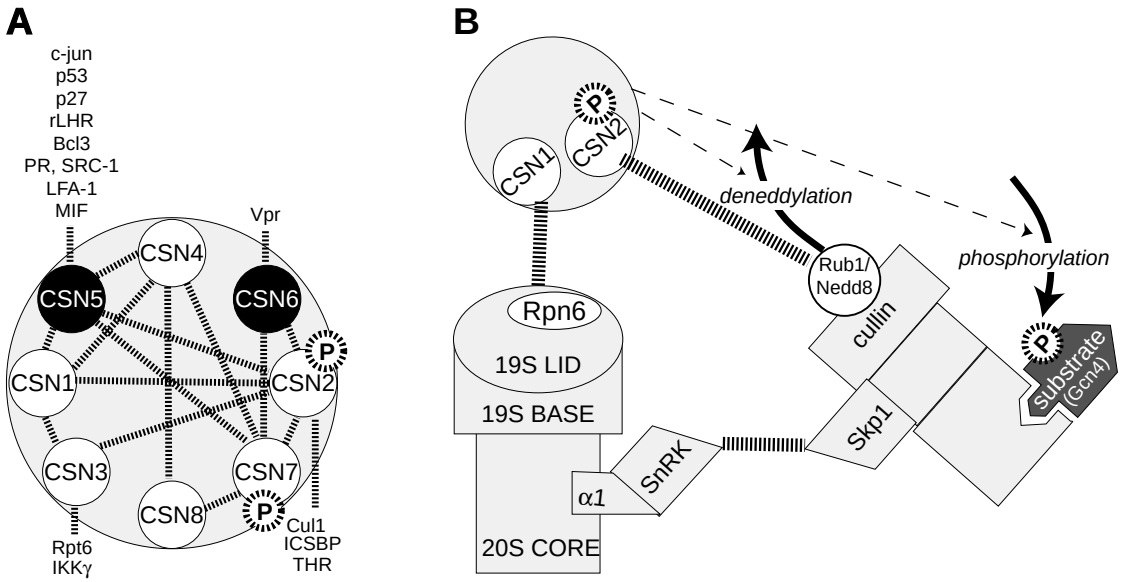

Fig. 1.5: The COP9 signalosome (CSN) and its proposed functions. Protein-protein interaction are indicated by parralelled bars. **(A)** The eight subunits of the CSN are interconnected by several protein-protein interactions, and single subunits additionally interact with non-subunit proteins (see also Tab. 2) as summarised in Kappellari *et al*., 2000 and Bech-Otschir *et al*., 2002. **(B)** Several ineractions between proteasome, SCF and CSN suggest physical association of the three multiprotein complexes (Kwok *et al*.1999, Schwechheimer *et al*. 2001; Farras *et al*. 2001). Additionally, the CSN-associated functions are indicated by broken arrow lines.

associated activities have been suggested. One model relies on the interaction between CSN and the 26S proteasome, since subunits of both complexes co-purify (Seeger *et al.* 1998) and specific protein-protein interactions were identified between CSN and subunits of the proteasome regulatory complex (Kwok *et al.* 1999). The CSN might thus act as an alternative or additional proteasome lid or modulate proteasome activity by altering the activity of the lid complex (Wei and Deng 1999; Schwechheimer and Deng 2000). A second model suggests the CSN as a platform with scaffolding function for associated enzymes like kinase(s), deneddylase(s), and E3 ubiquitin ligase(s) (Bech-Otschir *et al.* 2002). More precisely, the CSN was suggested to directly interact with SCF type 3 ubiquitin ligases or associate with the complete E2-E3 complex to modulate E3 activity (Schwechheimer and Deng 2001; Bech-Otschir *et al.* 2002; Suzuki *et al.* 2002). Notably, also the 26S proteasome and the E2-E3 complex interact, which implies that the processes of ubiquitinylation and proteolysis are probably not separated. The Snf1-like protein kinase SnRK interacts with the α4 subunit of the proteasome core complex and with the Skp1 subunit of the SCF and might thus constitute a proteasomal docking station for the SCF. This led to a model which proposes that the SCF changes between proteasome associated and a free status (Tongaonkar *et al.* 2000; Verma *et al.* 2000; Xie and Varshavsky 2000; Farras *et al.* 2001; von Arnim 2001). Taken together (Fig. 1.5B) it seems conceivable that further studies will reveal complex and non-statically physical associations between the three multiprotein complexes 26S proteasome, E3 ubiquitin ligases and the COP9 signalosome.

1.2.2.3 Physiological role of the COP9 signalosome. The targets of the COP9 signalosome include a variety of proteins, particularly transcription factors, cell cycle regulators and hormone receptors (Tab. 1.1). Physiologically, the CSN is thus involved in the regulation of several processes predominately affecting cellular homeostasis, growth, differentiation and development. Correspondingly, defects in CSN function display a pleiotrophic mutant phenotype higher eukaryotes. In plants and insects, a functional COP9 signalosome is essential for survival: though CSN mutants successfully pass embryogenesis, they die during later developmental stages. This post-embryonic lethality becomes evident in CSN mutant seedlings of the plant *A. thaliana* that cease development after a few days (Wei *et al.* 1994). Comparably, larvae of the invertebrate *D. melangolaster* stop differentiation after pupation (Freilich *et al.* 1999). Thus, mutant analyses are restricted to the first days of growth and development or to reduced-function mutants.

The model organism for studies concerning the COP9 signalosome is *A. thaliana*, where mutations in CSN subunits result in the loss of complex formation and pleiotrophic mutant phenotypes (Wei and Deng 1999). With light and auxin responses, two major target pathways of CSN regulation have been described thoroughly. (i) Light response includes the integration of a physical parameter into regulation of development. Photomorphogenesis of plant seedlings proceeds in the light but is repressed in the dark. In *A. thaliana*, the corresponding light signal transduction depends on ubiquitin-dependent degradation of HY5, the transcriptional activator of light regulated genes (Osterlund *et al.* 2000). At least three constitutive photomorphogenesis (COP) complexes are involved in this process: the CSN, COP1 and COP10 (Ang *et al.* 1998; Schwechheimer and Deng 2000). It is assumed that they define a protein ubiquitinylation pathway that directs the degradation of HY5 in the dark (Suzuki *et al.* 2002). COP1, that accumulates in the nucleus in the dark, probably functions as E3 ubiquitin ligase (Osterlund *et al.* 1999; Osterlund *et al.* 2000), COP10 might mediate the corresponding E2 activity. The COP9 signalosome probably contributes to the ubiquitinylation process by its associated activities as E3-modulator (Suzuki *et al.* 2002). (ii) Auxin response describes the integration of a chemical parameter into regulation of differentiation. Plant response to the phytohormone auxin is responsible for a number of developmental processes like proper cell elongation and suppression of secondary inflorescences. In *A. thaliana* it is mediated by expression of the AUX/IAA genes encoding short-lived transcriptional repressor proteins that are supposed substrates of the E3 ubiquitin ligase SCF^{TIR1} (Abel *et al.* 1994; Gray *et al.* 2001). The CSN deneddylates the cullin of SCF^{TIR1} and thus probably diminishes auxin signalling by degradation of the AUX/IAA repressors (Schwechheimer *et al.* 2001). Moreover, the studies on auxin responses support the model of tight association between proteasome and E3 ligase (Fig. 1.5B). The regulatory WD40 protein PRL1 interacts with SnRK and might thus control docking of the SCF to the proteasome by attenuation of SnRP binding. It seems striking that, in respects to auxin response, *PRL1* mutations cause the opposite effect of *CSN* mutations in *A. thaliana*, suggesting that proteosomal binding and deneddylation of the SCF are essential for proper auxin response (Nemeth *et al.* 1998; Gray and Estelle 2000;

Farras *et al.* 2001).

The CSN subunits were identified in several vertebrate tissue cells including mouse and human (Wei and Deng 1998; Bounpheng *et al.* 2000). They are biochemically related to the ubiquitinylation system in regulating stability of transcription factors and cell cycle regulators. They interact with cullin subunits (Lyapina *et al.* 2001; Yang *et al.* 2002) and are probably also involved in developmental processes. One mammalian CSN target is of major interest, because of an implication in tumor growth. This is the transcription factor c-Jun, the mammalian homologue of yeast Gcn4p, that increases specificity of target gene activation by AP-1 proteins through c-Jun / AP-1 site complex stabilisation (Claret *et al.* 1996). Because of its short half life, c-Jun protein levels are constitutively low. c-Jun half-life is increased when it is phosphorylated by mitogen- and stress activated Jun-terminal kinases (JNK), since phosphorylation prevents ubiquitin-dependent degradation of c-Jun (Karin *et al.* 1997). The CSN stabilises c-Jun towards the ubiquitin system by phosphorylation independent of the known JNK pathway (Naumann *et al.* 1999; Chamovitz and Segal 2001). This CSN-directed c-Jun stabilisation results in increased AP-1 activity which in turn affects expression of regulatory proteins like the vascular endothelial growth factor (VEGF) that controls tumor angiogenesis (Pollmann *et al.* 2001). Additionally, the c-Jun/p53 balance - that is disturbed in many tumor cells - is at least partially controlled by the CSN mediated ubiquitin system. Both proteins directly interact with CSN5 for CSN-mediated phosphorylation, with c-Jun being stabilised (Musti *et al.* 1997) and p53 destabilised upon phosphorylation (Bech-Otschir *et al.* 2001).

1.2.2.4 The COP9 signalosome in lower eukaryotes. The COP9 signalosome, well conserved in higher eukaryotes, was not identified in any of the prokaryotic genome sequences known to date. Knowledge about the COP9 signalosome in lower eukaryotes is scarce. The complete genome sequence of the baker's yeast *S. cerevisiae* revealed with the open reading frame of *RRI1* only one putative coding region with similarity to the COP9 signalosome subunit 5. Subunits CSN1-CSN6 were identified in *S. pombe* (Mundt *et al.* 1999; Peng *et al.* 2001b; Peng *et al.* 2001a; Zhou *et al.* 2001; Mundt *et al.* 2002), all of which are essential for proper cullin deneddylaton suggesting the existence of the complete complex in fission yeast. *S. pombe csn* mutant strains show less severe defects than their metazoan counterparts. Moreover, deletion of various subunits revealed different mutant phenotypes. Loss of CSN1 resulted in a cell cycle defect, slow growth with reduced cell elongation and increased UV sensitivity, whereas deletion of other CSN subunits did not display these mutant phenotypes. Distinct functions for different CSN subunits of fission yeast were proposed. A COP9 signalosome is not yet identified in filamentous fungi. In *A. nidulans*, few components of an ubiquitin-related protein degradation machinery are yet described: two ubiquitin genes (Noventa-Jordao *et al.* 2000) and two open reading frames encoding a putative cullin and a putative CSN4-like component (Eckert 2000), respectively. Additionally, the pre-competence gene *acoB* shows similarity to CSN subunit 7, though overall amino acid identities are rather weak (Lewis and Champe 1995).

1.3 Regulation of metabolism in *A. nidulans*

Metabolism defines the sum of biosynthetic processes occurring within a living organism (Fig. 1.6). The division between primary and secondary metabolism is rather artificial and boundaries are blurred. Generally, primary metabolism is defined to be essential since it uses energy from nutritional sources to ensures production of cellular components. Contrary, secondary metabolites comprise a variety of complex, according to the definition non-essential organic compounds that are normally produced when growth ceases (Firn and Jones 2000). Cascades of enzymatic reactions define metabolic pathways that lead to the biosynthesis of a specific metabolite. Such pathways are often not linear since substrates, intermediates and products are often shared for production of different metabolites. Thus, metabolism can be regarded as an interconnected network of enzymatic activities that are modulated according to the immediate situation of the cell. This implies highly sophisticated regulatory systems that control production of various metabolites in response to the environmental conditions. Since the *A. nidulans* genome sequence is not yet fully open to the research community, the complete metabolic properties of this fungus remain to be discovered. Several regulatory proteins that impact metabolic pathways have already been described, though overall knowledge about their role in the whole cellular composition is rather fragmentary.

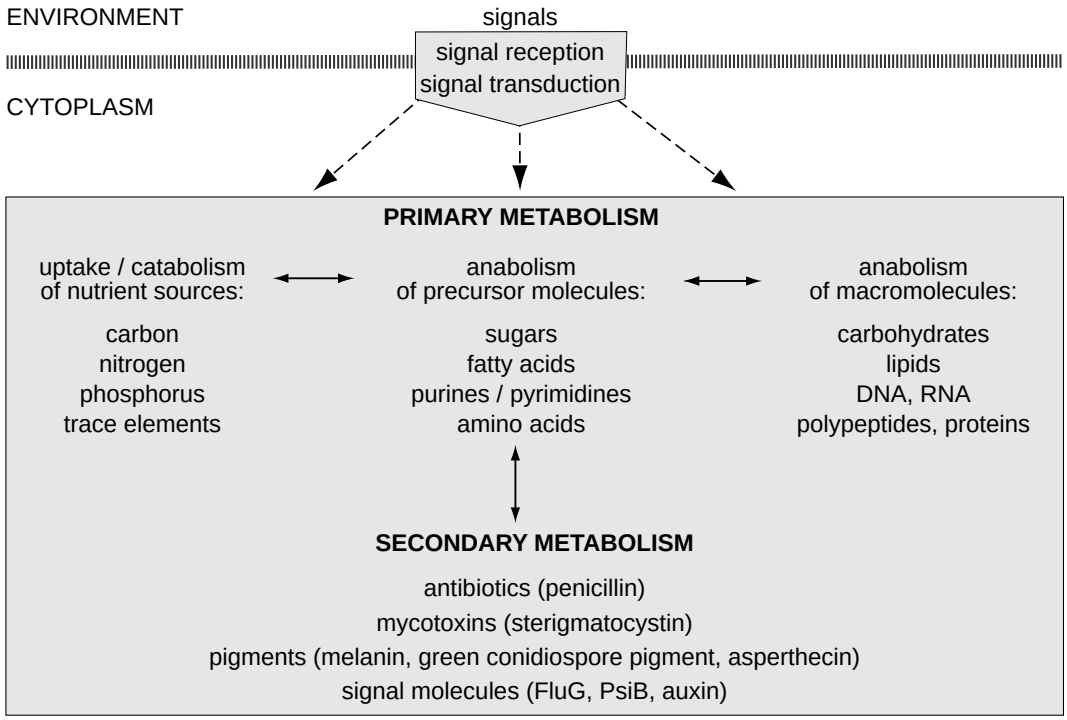

Fig. 1.6: Fungal metabolism. Enzyme activities of primary metabolism are modulated according to the availability of nutrient sources. Primary metabolites serve as precursor molecules for secondary metabolites. Dashed arrow lines indicate the dependency of metabolism on environmental signals.

1.3.1 Primary metabolism

1.3.1.1 Nutrition. *A. nidulans* can use various nutrient sources and tolerates a broad pH spectrum, especially in the acidic region where numerous prokaryotic microorganisms have difficulties to survive (Pontecorvo *et al.* 1953). Expression of metabolic enzymes is restricted to their need in order to minimise energetic expenses, and several specific transcription factors are known to regulate metabolic traits in *A. nidulans* (Tab. 1.2). The major transcription factor of pH signalling in *A. nidulans* is PACC, ensuring that genes are expressed at a pH where the corresponding enzymes can function (Espeso and Penalva 1996). Utilisation of easily accessible nutrients at low energetic expense is normally preferred. Thus, uptake systems as well as the corresponding metabolic pathways are co-ordinately controlled by transcription factors of global metabolite repression

Tab. 1.2: Selection of important *A. nidulans* regulatory factors

regulator[1]	regulatory circuit	references
transcription factors:		
ABAA	asexual development	Andrianopoulos and Timberlake 1994
ALCR	ethanol utilisation	Felenbok *et al.* 1988
AMDR	acetamide utilization	Andrianopoulos and Hynes 1990
AnCF	unknown	Steidl *et al.* 1999
AFLR	sterigmatocystein synthesis	Yu *et al.* 1996a
AREA	ammonium repression	Punt *et al.* 1995
AREB	ammonium repression	Conlon *et al.* 2001
BRLA	asexual development	Prade and Timberlake 1993
CPCA	amino acid control	Hoffmann *et al.* 2001
CREA	glucose repression	Panozzo *et al.* 1998
DOPA	growth and development	Pascon and Miller 2000
MEDA	developmental modifier	Busby *et al.* 1996
NIR	inorganic nitrogen assimilation	Burger *et al.* 1991
NSDD	sexual development	Han *et al.* 2001
PACC	pH regulation	Espeso *et al.* 1997
SREA	iron uptake	Haas *et al.* 1999
STEA	sexual development	Vallim *et al.* 2000
STUA	developmental modifier	Dutton *et al.* 1997
UAY	purine utilization	Suarez *et al.* 1995
(VEA)	developmental light response	Mooney and Yager 1990
(WETA)	conidiosporogenesis	Marshall and Timberlake 1991
signal transduction:		
FADA	sterigmatocystin, penicillin (Gα- protein)	Tag *et al.* 2000
FLBA	growth, dev. (regulator of G-protein)	Yu *et al.* 1996b
MPKA	polarised growth (mitogen-activated kinase)	Bussink and Osmani 1999
NIMA	cell cycle (kinase)	Lu *et al.* 1993
NIMO	cell cycle (kinase)	James *et al.* 1999
PCLA	cell cycle (cyclin)	Schier *et al.* 2001
PHOA	phosphorus, dev. (cyclin-dep. kinase)	Bussink and Osmani 1998
PPHA	hyphal growth (2A protein phosphatase)	Kosmidou *et al.* 2001
RASA	carbon, dev. (small G protein)	Som and Kolaparthi 1994
SFAD	sterigmatocystin, penicillin (Gβ- protein)	Rosen *et al.* 1999

[1] Parentheses indicate that the function as transcription factor is conceivable but questioned. Abbreviation: development (dev.).

systems. The carbon catabolite repression system relies on the central transcriptional repressor CREA (Strauss *et al.* 1999) that blocks expression of enzymes for alternative carbon source consumption as long as glucose is available. An analogous mechanism mediated by the transcriptional repressor AREA (Platt *et al.* 1996) ensures that ammonium and glutamine are favoured as nitrogen sources. Additionally, the transcription factor SREA (Haas *et al.* 1999) specifically regulates transcription of genes for siderophore biosynthesis and iron uptake.

1.3.1.2 Amino acid metabolism. An important group of precursors for all proteins that are produced during primary metabolism are amino acids. Fungi are generally capable of the *de novo* synthesis of all 20 proteinogenic amino acids. Amino acids are not only building blocks for proteins but also serve as precursor molecules of other primary and secondary metabolites. Their production thus interconnects several pathways. An example is the tight link to carbohydrate metabolism: amino acids are anabolised from intermediates of glycolysis, pentosephosphate cycle or citric acid cycle and, vice versa, can often be catabolised into such intermediates. According to the carbon compound they originate from, amino acids are subdivided into 6 biosynthesis families which implies that the corresponding biosynthesis pathways are branched. One example is production of the aromatic amino acids (Fig. 1.7A) that share chorismate as last common key intermediate (Bentley 1990). Additionally, pathway intermediates are shared between biosynthesis of amino acids and other primary metabolites. Exemplary, biosynthesis of histidine (Fig. 1.7B) is not directly connected to the production of other amino acids but to *de novo* synthesis of purine via the by-product 5'-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR) (Alifano *et al.* 1996). Additionally, amino acids are precursors for a variety of secondary metabolites. A direct connection of primary and secondary metabolism is represented by the non-proteinogenic amino acid α-aminoadipate (Fig. 1.7C) that constitutes a branch point of fungal lysine and penicillin biosynthesis (Brakhage 1998).

Only few components of amino acid biosynthesis pathways have been isolated from *A. nidulans* to date, and their regulation is subject to current research. The co-ordinated transcriptional activation of the *argB* (Goc and Weglenski 1988), *trpB* (Eckert *et al.* 2000) and *hisHF* (Valerius *et al.* 2001) amino acid biosynthesis genes in response to external amino acid availability is mediated by the transcriptional activator CPCA of the cross-pathway control (Hoffmann *et al.* 2001), which corresponds to yeast Gcn4p. Knowledge about the mechanism of amino acid specific regulation and their biochemical connections to other pathways of primary and secondary metabolism in *A. nidulans* is scarce. Selected examples are given in Fig. 1.7: (i) On the transcriptional level, the *hisHF* gene encoding the branch point enzyme of histidine biosynthesis and AICAR cycle (heterodimeric imidazole-glycerole-phosphate synthase; E.C. 2.4.2.14 and E.C. 4.3.2.4) is not repressed by histidine but by adenine (Fig. 1.7B) (Valerius *et al.* 2001). (ii) On protein level, regulation by the pathways end-product has been shown for the *aroC*-encoded chorismate mutase (E.C. 5.4.99.5) that is allosterically regulated with

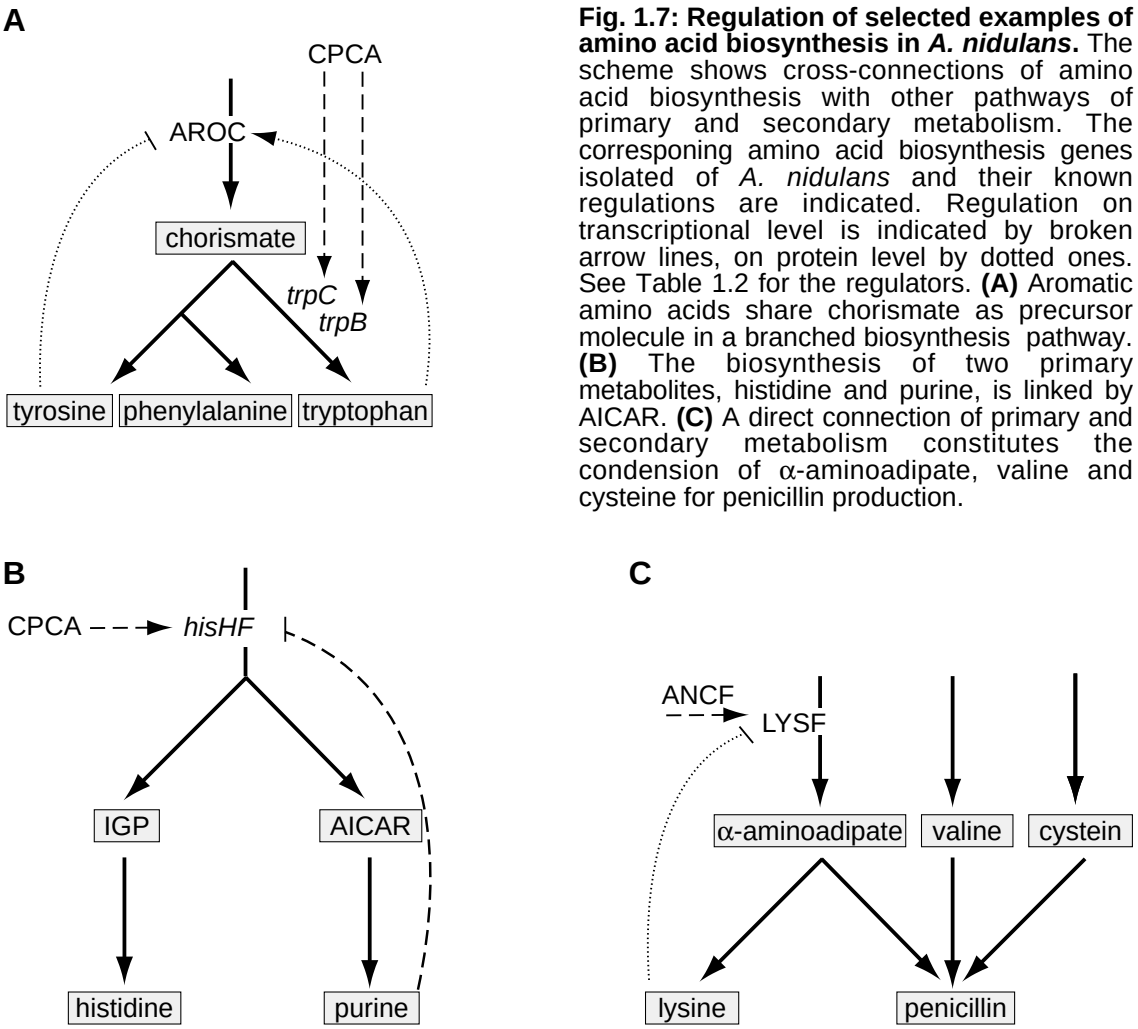

Fig. 1.7: Regulation of selected examples of amino acid biosynthesis in *A. nidulans*. The scheme shows cross-connections of amino acid biosynthesis with other pathways of primary and secondary metabolism. The corresponing amino acid biosynthesis genes isolated of *A. nidulans* and their known regulations are indicated. Regulation on transcriptional level is indicated by broken arrow lines, on protein level by dotted ones. See Table 1.2 for the regulators. **(A)** Aromatic amino acids share chorismate as precursor molecule in a branched biosynthesis pathway. **(B)** The biosynthesis of two primary metabolites, histidine and purine, is linked by AICAR. **(C)** A direct connection of primary and secondary metabolism constitutes the condension of α-aminoadipate, valine and cysteine for penicillin production.

tryptophan acting as cross-pathway activator and tyrosine as feedback inhibitor (Fig. 1.7A) (Krappmann *et al.* 1999). (iii) Expression of LYSF, the homoaconitase acting in the common stem of lysine and penicillin biosynthesis, is regulated on at least two levels. Transcription of the *lysF* gene is repressed by the HAP-like transcription factor AnCF and activated by an yet unidentified GATA-like transcription factor (Weidner *et al.* 2001). On protein level, LYSF is subject to end product repression by lysine (Weidner *et al.* 1997).

1.3.2 Secondary metabolism

1.3.2.1 Antibiotics and toxins. Secondary metabolites, whose biosynthesis relies on precursor molecules of primary metabolism, include the economically important microbial products like antibiotics and pigments. It is still obscure why secondary metabolites are produced at all, with hypotheses ranging from waste products to signal molecules (Firn and Jones 2000). Among the most prominent secondary metabolites are β-lactam antibiotics. Since penicillin is a block buster of pharmacological industry, much effort was directed into the study of mechanisms that regulate penicillin biosynthesis (Brakhage 1998). All three penicillin biosynthesis genes of *A. nidulans*, *acvA*, *ipnA* and *aatA*, have been cloned

(MacCabe *et al.* 1990). The genes *acvA* and *ipnA* are divergently transcribed from a shared promoter region, whereas *aatA* transcription is driven by a separate promoter. To date, no penicillin-specific regulators have been thoroughly characterised, but several regulators affect the synthesis of the antibiotic. This underscores the implication of regulatory networks that connect control of primary and secondary metabolism. The transcriptional activator AnCF (also known as PENR1) increases *ipnA* and *aatA* expression (Litzka *et al.* 1999). Penicillin production is subject to pH regulation by PACC being highest at alkaline pH, whereas glucose repression of penicillin biosynthesis is mediated in a CREA-independent manner (Shah *et al.* 1991; Martin *et al.* 1999).

Another prominent secondary metabolite of *A. nidulans* is the polyketide-derived mycotoxin sterigmatocystin, a precursor of aflatoxin. Its biosynthesis requires a cluster of at least 20 genes (Keller and Adams 1995). Regulation of penicillin biosynthesis seems to be connected to production of sterigmatocystin. The α subunit of a heterotrimeric G protein (FADA) acts negatively on sterigmatocystin and positive on penicillin syntheses (Tag *et al.* 2000). Moreover, sterigmatocystin synthesis is additionally linked to development (see 5.2.2.1).

1.3.2.2 Pigments and signal molecules. Beneath antibiotic and aflatoxin production, the fields of pigmentation and production of signal molecules are of major concern. *A. nidulans* produces pigments in various cell types, and again, amino acids are crucial precursor molecules. The brownish pigment melanin is formed from tyrosine by a phenoloxidase, a tyrosinase. It is partly secreted and partly accumulates as microfibrilles on the exterior of elder fungal hyphae (Martinelli *et al.* 1969; Pirt and Rowley 1969). Other pigments are restricted to the reproductive cycles (see 1.4.3 and 1.4.4), where they probably function to protect nucleic acids from damage by ultraviolet light. The conidiospore colour probably originates from tryptophan: 6-hydroxy-N-acetyl-tryptophan is converted to a yellow pigment precursor and subsequently to the green pigment under contribution of a phenol oxidase, a polyketide synthase and a *p*-diphenol-oxidase, encoded by the genes *ivoB* (Clutterbuck 1990), *wA* (Mayorga and Timberlake 1990) and *yA* (Aramayo and Timberlake 1993), respectively. Expression of these gene products of conidiosporogenesis is mainly regulated by regulators specific for asexual development (see 1.4.3). The synthesis pathway of the red ascospore colour asperthecin is yet unknown. This anthraquinone is difficult to isolate because of its resistance to solvent extraction (Howard and Raistrick 1955). A phenol oxidase (laccase II) is specifically expressed during early sexual development in specialised structures (primordia and Hülle cells), but its contribution to pigment synthesis remains to be proven (Scherer and Fischer 1998).

Knowledge about biosynthesis of hormones, pheromones and other signal molecules in *A. nidulans* is even scarce. Two proposed signal molecules are probably linked to amino acid metabolism and one to fatty acid metabolism - and all three of them mediate developmental responses (see Chapter 4). During the course of this work, the tryptophan-related secondary metabolite auxin was identified as a probable contributor for differentiation processes (Eckert *et al.* 1999).

Also, the diffusible extracellular ammonium-containing FluG factor is supposedly derived from amino acids, since its production depends on *fluG* that encodes a proposed glutamine synthetase activity (Lee and Adams 1994a; Yager *et al.* 1998). An endogenous mixture of hydroxylinoleic acid moieties, is known as Psi factor, precocious sexual inducer, a structural relative to vertebrate eicosanoid hormones. They comprise three interconvertible compounds, with the 5,8-dihydroxylinoleic acid PsiC as the most active compound that can be converted into its cyclic lactone PsiA. The 8-hydroxylinoleic acid PsiB is presumably an intermediate (Champe and el-Zayat 1989; Calvo *et al.* 1999; Calvo *et al.* 2001; Braus *et al.* 2002)

1.4 Regulation of growth and development in *A. nidulans*

A. nidulans is an ubiquitous saprophytic soil organism. A single colony is generally capable of hyphal growth as well as reproduction and can be regarded as a developmental unit. Growth and reproduction require sophisticated regulatory mechanisms, particularly in multicellular organisms that differentiate complex three dimensional structures (Fig. 1.8). Metabolite synthesis, nuclear distribution and cellular transport systems have to be remodelled, and processes as filamentous versus bud-like growth, cell cycle synchronisation or dis-synchronisation, mitosis versus meiosis and cellular differentiation must be initiated at the correct time and place. Genetic control of growth and development of *A. nidulans* is subject to intensive research. Several regulatory proteins (Tab. 1.2) and dependencies have been proposed, but their precise contribution to developmental decisions in *A. nidulans* remains to be elucidated.

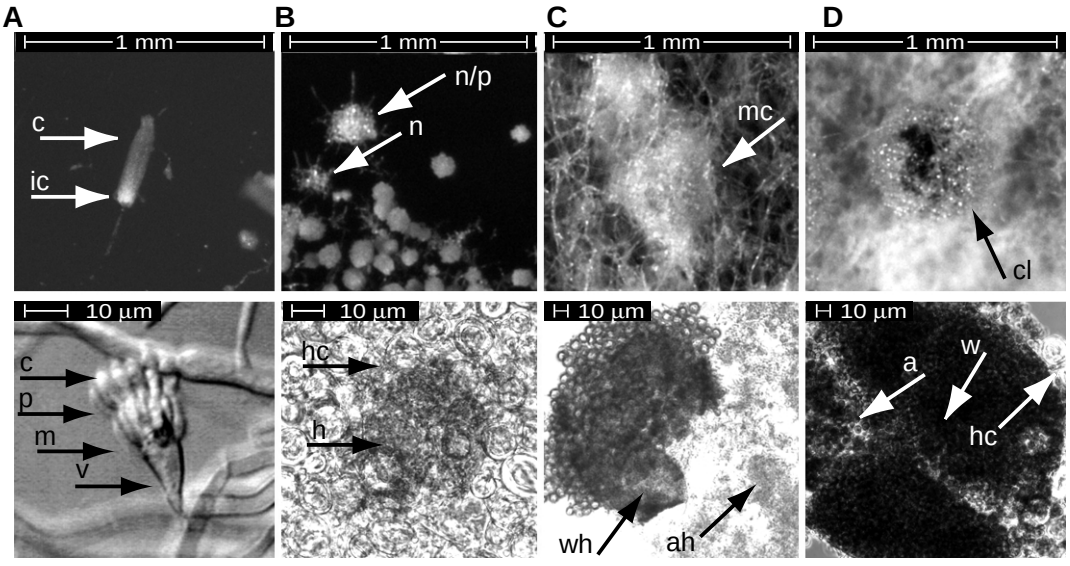

Fig. 1.8: Morphology of developmental structures of *A. nidulans*. Pictures were taken under the binocular (upper row) and microscope (lower row). **(A)** Asexual development. Conidiophore architechture includes a vesicle (v), the metulae (m) and phialides (p) and mature (c) and immature (ic) conidiospores. **(B)** The first visible structure of sexual development are nest (n) that contain the characteristic Huelle cells (hc). The primordium (p) looks makroscopically like a ball of hyphae (h). **(C)** In the mikro-cleistothecium, the dissection into inner ascogenous hphae (ah) and outer wall hyphae (wh) becomes obvious. **(D)** The mature cleistothecium (cl) is composed of a hard wall (w) and mature ascospores (a).

1.4.1 Germination and vegetative growth

1.4.1.1 Morphology of growth. During vegetative growth, *A. nidulans* spreads over surfaces with filamentous, branched hyphae to occupy new nutritional sources. At germination, the G1-arrested asexual spores re-enter the cell cycle, swell by isotropic extension and switch to polarised hyphal growth to form the germ tube. In the growing hyphae, the apical cell accomplishes mitotic cycles resulting in an accumulation of nuclei. While the hyphal tip grows by apical extension, the cytoskeleton transports vesicles containing wall material and exoenzymes which fuse with the cell membrane to form the rigid chitineous cell wall and excrete exoenzymes. During growth, perforated septae composed of actin microfilaments isolate 3-4 nuclei in the subapical cells. These cells have a length of about 40 μm and are arrested for growth and mitosis. They establish new polarity axes to form lateral branches with new apical cells under break-down of the cell wall at the future branch spot (McGoldrick *et al.* 1995; Momany *et al.* 1999).

1.4.1.2 Regulation of cellular organisation. On the molecular level, little is known about the onset of germination that requires co-ordination of growth, nuclear division and septation. Defects in vegetative hyphal growth mainly reflect general problems in cellular organisation processes like cell cycle control, cytoskeleton and polarity and thus often additionally effect the propagation cycles. Several components of these events have been identified by mutant analyses, while a complete picture of the regulatory connections remains to be established.

In eukaryotes, an important switch for perception of extracellular signals and subsequent transduction of this signal are heterotrimeric G proteins composed of subunits α, β and γ, for example Ras proteins. Activated Ras proteins stimulate the adenylyl cyclase that converts ATP into the regulatory cAMP. This 'second messenger' in turn activates the protein kinase A which modifies the activity of specific transcription factors by phosphorylation. For example, yeast Ras- and cAMP signalling is involved in the starvation-dependent control of budding versus pseudohyphal growth (Mösch 2000). An overall regulatory role in the integration of carbon source availability and the onset of germination in *A. nidulans* is proposed for RASA: initial high levels of RASA are essential for germination but subsequent gradual decrease of RASA activity is prerequisite for further development (Som and Kolaparthi 1994; Osherov and May 2000). This Ras signalling during germination is independent of cAMP signalling which also affects vegetative growth (Fillinger *et al.* 2002).

The cytoskeleton is characteristic for eukaryotic cells. Its dynamic structure of microfilaments and microtubules is important for the cellular scaffold and enables contractile functions. Moreover, transport of vesicles and cell organelles is dependent on this intercellular scaffold. Major components of this system are the structural tubulins and actins as well as motor proteins like dyneins and kinesins. Several factors that contribute to localisation events via the cytoskeleton have been identified in *A. nidulans*: nuclear distribution is affected in mutants of the *nud* (Ahn and Morris 2001) or *tub* genes (Kirk and Morris 1991; Kirk and Morris 1993)

resulting in vegetative growth defects and *apsA*,*B* mutants produce anucleate primary sterigmata (Fischer and Timberlake 1995; Sülmann *et al.* 1997). Strikingly, *aps* mutations are suppressed by the *samB* gene product that is involved in polarised growth (Krüger and Fischer 1998).

Polarity is an important feature of organisms that exhibit morphogenetic traits. A key feature of germination is the switch from isotropic swelling to polarised growth. Polarised growth requires the gene products of the *pod*, *sep* and *hyp* genes (Kaminskyj and Hamer 1998; Harris *et al.* 1999; Sharpless and Harris 2002), a mitogen-activated protein kinase MPKA (Bussink and Osmani 1999) and the protein phosphatase PPHA (Kosmidou *et al.* 2001).

Another characteristic of eukaryotic cells is the controlled proliferation program based on the cell cycle. Key components in the regulation of the cell cycle are cyclin-dependent protein kinases, highly conserved among eukaryotes, that control the transition between different cell cycle phases. Correspondingly, expression and degradation of the specific regulatory cyclin subunits are highly regulated. The growth or developmental mutant phenotype of several *A. nidulans* are founded on impaired cell cycle control and mitosis. Corresponding regulatory proteins have been identified, for example the "never in mitosis" kinases NIMA and NIMO (Lu *et al.* 1993; James *et al.* 1999) and the cyclin homologue PCLA (Schier *et al.* 2001; Schier and Fischer 2002). A striking effect resulting in binuclear conidiospores is seen in strains carrying the mutant *bncA1* allele (Castiglioni Pascon *et al.* 2001).

1.4.2 Developmental competence

1.4.2.1 Environmental factors. About 16 to 20 hours after germination, elder subapical vegetative hyphae establish competence for further differentiation which implies an intrinsic time factor that controls developmental initiation (Axelrod *et al.* 1973). The knowledge about acquisition of developmental competence and subsequent induction of the reproductive cycles is scarce, but various physical, biochemical and genetic factors are involved (Fig. 1.9A). Only few pre-competence genes (*aco)* contributing to the establishment of competence have been identified in a genetic screen (Butnick *et al.* 1984a; Butnick *et al.* 1984b; Lewis and Champe 1995).

The two propagation cycles of *A. nidulans* probably have the potential to proceed independently, though only extreme conditions lead to formation of exclusively conidiophores or cleistothecia. As soon as competence is established, reproduction is induced strongly dependent on the environmental conditions. Nutrient availability plays an important role in developmental decisions. Either carbon or nitrogen starvation initiate asexual sporulation (Han *et al.* 1994; Skromne *et al.* 1995). It is suggested that carbon source availability activates a Ras-dependent sensory mechanism with controlled *rasA* expression levels as mediator of ordered growth and development (Som and Kolaparthi 1994; Osherov and May 2000). RASA genetically interacts with the transcription factor DOPA, that in turn

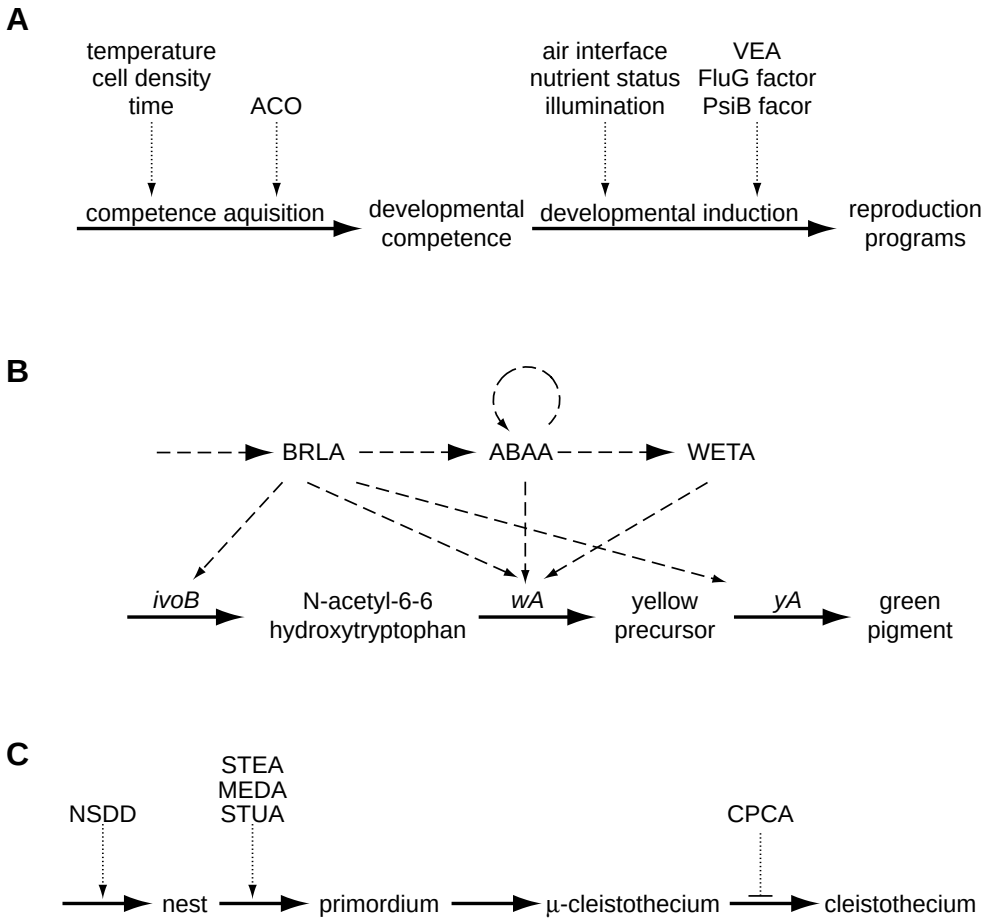

Fig. 1.9: Regulation of development in *A. nidulans*. The schemes depict selected dependencies of regulatory proteins. Arrows show biochemical or morphological processes whereas broken arrow lines indicate a positive transcriptional regulation and dotted arrow lines a yet uncharacterised regulatory impact. **(A)** Competence aquisition and initiation of development. **(B)** Asexual development. The central cascade of transcription factors (BRLA, ABAA, WETA) controls several processes of conidiosporogenesis including the biosynthesis of the green conidiospore pigment by transcriptional activation of the corresponding genes. **(C)** Sexual development. The main morphological stages are shown with regulatory proteins whose level of action was proposed from mutant analyses.

mediates proper expression of several developmental transcriptions factors (*brlA*, *abaA* and *steA;* see Tab. 1.2) (Pascon and Miller 2000). Another important nutritional signal is the availability of phosphate. The putative cyclin dependent protein kinase PHOA, corresponding to the yeast Pho85 kinase, is involved in the developmental responses to phosphorus concentration, inoculation density and pH and probably integrates these environmental signals with developmental decisions in *A. nidulans* (Bussink and Osmani 1998).

Additionally, several physical factors are involved in the onset of differentiation processes. Prerequisite for development is a medium / air interface, though special growth conditions low in nitrogen exceptionally allow differentiation in submerged culture. Aeration directs the developmental program towards asexual reproduction, whereas high CO_2 tension favours the sexual cycle (Axelrod *et al.* 1973). An important physical environmental factor that controls induction of development is illumination. Generally, *A. nidulans* produces predominantly conidiophores in the light and cleistothecia in the dark, though this light effect is

dependent on medium composition and inoculation density (Raper and Fennell 1965; Zonneveld 1977; Mooney and Yager 1990). Moreover, the light-regulation of developmental decisions is dependent on the *veA* gene product. Most of the frequently used laboratory strains carry the mutant *veA1* allele, causing light-independent induction of asexual sporulation with concomitant increased levels of the asexual-specific transcription factor BRLA - and only rare induction of the sexual cycle. It is assumed that the truncated *veA1* gene product mediates low functionality of the corresponding protein. VEA is thus hypothesised to repress initiation of asexual development and promotes the onset of sexual development (Mooney and Yager 1990).

1.4.2.2 Signal molecules. Two signal molecules involved in the initiation of developmental processes are the FluG and PsiC factors (see 1.3.2.2), and both seem to be linked to light response and the VEA protein. The FluG factor probably initiates asexual development at a certain threshold (Lee and Adams 1994a; Lee and Adams 1994b; Lee and Adams 1996). VEA was proposed to interact with a protein involved in the production of the FluG factor: FLUG (Yager *et al.* 1998). The *fluG* gene product in turn is dependent on the *flbA* gene product, a proposed regulator of G protein signalling. FLBA probably controls the activity of a heterotrimeric G protein (*fadA*, *sfaD*) whose inactivation is essential for both asexual and sexual sporulation (Yu *et al.* 1996b; Rosen *et al.* 1999). Interestingly, *A. nidulans flbA* overproduction strains, as well as *fluG* and *flbD* overproducers, show increased expression of *brlA* and develop wild-type like conidiophores in submerged culture (Wieser and Adams 1995), emphasising their role in initiation of the asexual regulatory cascade. Mutants defective in these or the additional *flbB*, *flbC*, *flbD* and *flbE* loci show a characteristic phenotype of profuse aerial hyphae (Wieser and Adams 1995).

The PsiC factor acts as inducer of sexual development (Champe *et al.* 1987). Strikingly, the sexual inducing activity of the PsiC factor is abolished in *veA1* mutant strains. A specific hormone receptor coupled to a signal transduction pathway is not yet identified for PsiC, but might be conceivable. The second hypothesis on PsiC function is its proposed activity in modification of membrane properties that might contribute to the fusion of specialised hyphae during sexual development. (Champe and el-Zayat 1989).

1.4.3 Asexual reproduction

1.4.3.1 Conidiophore morphology. Asexual reproduction of *A. nidulans* results in the differentiation of mitotically derived conidiospores about 48 hours after germination. Production of the asexual developmental unit, the conidiophore (Fig. 1.8A), can be microscopically observed as a linear cascade of morphological events as thoroughly reviewed (Adams *et al.* 1998). At first, the vegetative mycelium differentiates a thick-walled foot cell from which an aerial filament grows by vertical apical extension. This conidiophore stalk of about 100 µm height and 4 µm width swells at its tip to form a vesicle. Synchronised mitosis of nuclei and budding in a

pseudohyphal-like appearance result in two rows of uninucleate sterigmata, the metulae and phialides. Each phialide mother successively buds at its tip with concurrent mitotic divisions, resulting in isogenic haploid nuclei encapsulated in a cell wall. The nucleus most distal to the phialide mother cell arrests at G1-phase and, during the following conidiosporogenesis, the cell wall gets impregnated and coloured characteristically green. Each phialide mother cell produces chains of up to 100 conidiospores that look like an aspergill used for catholic consecration - inspiring the naming of the genus *Aspergillus*.

1.4.3.2 The asexual regulatory cascade. Specific regulation of asexual development is driven by a central regulatory cascade constituted of two major transcription factors: BRLA and ABAA (Fig. 1.9B). The *brlA* gene product is responsible for a developmental switch from apical growth to swelling of the vesicle and budding of the sterigmata (Miller *et al.* 1992). The *brlA* locus includes two overlapping transcription units, *brlA*α and *brlA*β, that both accumulate early during development and probably have redundant functions. Main effector is the *brlA*β gene product that is expressed about 20 hours after germination and activates transcription of developmental genes like *abaA*, *wetA* and *rodA* (Chang and Timberlake 1993; Han *et al.* 1993; Prade and Timberlake 1993). ABAA is required for the switch from sterigmata budding to formation of conidiospores. The *abaA* gene product is expressed about 24 hours after germination and activates transcription of several conidiation-specific genes including *wetA*, *rotA*, *yA*, *wA*.

The two-component protein kinase TCSA is essential for conidiation (Virginia *et al.* 2000), though it was not yet integrated into the regulatory cascade that drives the asexual circuit. Additionally, several regulators of conidiosporogenesis have been identified. The putative transcription factor WETA is essential for maturation of conidia including the inner wall layer and expression of the *wA* gene (Marshall and Timberlake 1991), the *rodA* and *dewA* gene products contribute to formation of the outer hydrophobic protein layer (Stringer *et al.* 1991; Strauss *et al.* 1999) and the genes *ivoA*, *ivoB, wA* and *yA* are involved in the synthesis of the green polyketide-derived conidiospore pigment (see 1.3.2.2). Conidiosporogenesis is mainly driven by the regulatory backbone of conidiophore regulation (Fig. 1.9B) as seen for the transcriptional activation of the genes dedicated to pigment synthesis by BRLA and ABAA (Mayorga and Timberlake 1990; Aramayo and Timberlake 1993; Andrianopoulos and Timberlake 1994; Timberlake and Clutterbuck 1994).

1.4.4 Sexual reproduction and developmental co-ordination

1.4.4.1 Morphology of the cleistothecium. One of few representatives of the genus *Aspergillus* that are capable of meiotic reproduction is *A. nidulans*, in its sexual form also known as *Emericella nidulans*. Sexual propagation results in the differentiation of meiotically derived ascospores about 100 hours after germination. Development of the sexual reproduction unit, the cleistothecium, comprises several morphological process (Champe *et al.* 1994; Braus *et al.* 2002). About 50 hours

after germination, the onset of sexual development is probably mediated by fusion of fertile hyphae. These are wrapped up with unordered mycelium of specialised vegetative hyphae that bud from the tip to produce Hülle cells. These globose cells contain tissue-specific proteins like a phenol oxidase (laccase II) and probably function as nurse cells. (Hermann *et al.* 1983; Scherer and Fischer 1998). The conglomerate of specialised hyphae and Hülle cells, the first visible structure of sexual development, is generally termed nest (Fig. 1.8B) - and was inspiring for the naming of the species as nest-former (*nidulans*). Within this nest, a cleistothecial primordium develops (Fig. 1.8B). After further differentiation to a structure termed micro-cleistothecium (Fig. 1.8C), a morphological bipartition into an outer sterile hyphal layer and an inner fertile cellular mass becomes visible under the microscope. The surrounding nest mycelium differentiates to form an outer network of flat and highly branched hyphae. The network is glued by the yet uncharacterised substance cleistin and finally matures to a compact surface, the cleistothecial wall (Champe *et al.* 1994). Within this developing shell, the dikaryotic hyphae undergo co-ordinated cellular and nuclear divisions. About 80 hours after germination, two nuclei are trapped in the topmost cell of the co-called crozier and fuse to form a zygote (Pontecorvo *et al.* 1953). From this diploid ascus mother cell, meiosis and two rounds of mitosis finally result in asci that contain eight nuclei. Each nucleus is encapsulated by a membrane and undergoes an additional mitotic division, resulting in binucleate ascospores. During ascosporogenesis, the membrane is differentiated into the ascospore wall by accumulation of a characteristic red pigment called asperthecin (see 1.3.2.2) and surface ornamentation. Mature cleistothecia (Fig. 1.8D) can reach a size of 200 μm and enclose up to 80,000 ascospores (Champe *et al.* 1994).

1.4.4.2 Sexual tissue type-specific regulation. The precise timing of the events leading to a cleistothecium is not known to date. Also, knowledge about the regulatory circuits that drive sexual development in *A. nidulans* is rather fragmentary and is deduced mainly from the physiological defects of mutant strains (Fig. 1.9C). Two transcription factors, NSDD and STEA, are known to exclusively impact cleistothecia formation. *A. nidulans nsdD* null mutants lack all sexual tissue types (Han *et al.* 2001) whereas *steA* deletion mutants are acleistothecial but still produce Hülle cells (Vallim *et al.* 2000). Other mutants with defects in the sexual cycle are less characterised. Several sexual sporulation mutants form externally normal cleistothecia but do not differentiate mature ascospores due to defects in crozier formation, karyogamy, meiosis or post-meiotic mitosis (Swart *et al.* 2001). Additionally, several mutants specifically abort the developmental process. For example, reduced carbon sources result in limited and blocked sexual development, probably because of absence of α-1,3 glucan components for cleistothecial wall synthesis (Zonneveld 1975). Accordingly, strains lacking the Hülle cell-specific α-1,3 glucanase *mutA* are acleistothecial (Wei *et al.* 2001). An effect of the cross-pathway activator CPC (Hoffmann *et al.* 2001) and auxin (Eckert *et al.* 1999) on sexual development was shown during the course of this work and will be discussed together with the results of this study in Chapter 5.

1.4.4.3 Developmental modifiers. Once developmental cycles are induced, several regulatory mechanisms seem to co-ordinate their molecular control. Two transcription factors have been termed as developmental modifiers of both pathways, STUA and MEDA. Transcript levels of *stuA* rise at competence, and increasing developmental *stuA* thresholds probably confer to vegetative growth, asexual propagation and sexual development (Wu and Miller 1997). *A. nidulans stuA* deletion strains develop disorganised asexual structures with conidiospores budding directly from the vesicle (Miller *et al.* 1991). Concerning sexual development, these mutants are acleistothecial but do produce Hülle cells (Dutton *et al.* 1997; Vallim *et al.* 2000). On the molecular level, STUA is required for the correct spatial distribution of BRLA and ABAA (Miller *et al.* 1992). Contrary, MEDA is responsible for proper temporal expression of *brlA* transcripts and also functions as co-activator of *abaA* expression (Busby *et al.* 1996). Strains with *medA* defects also show disorganised asexual structures, with branching metulae and secondary conidiophores. In regard to sexual development they are acleistothecial but do produce Hülle cells (Clutterbuck 1969). These developmental modifiers demonstrate that, beneath the complex regulation of specific asexual and sexual reproduction, a sophisticated cross-talk must exist between the two developmental pathways.

The physiological and morphological versatility of *A. nidulans*, as described in this chapter, implies interconnected control mechanisms that conduct the orchestra of molecular processes in the fungal organism. This work shows how regulatory networks that were originally described to control primary metabolism (cross-pathway control) or development (COP9 signalosome-dependent network) additionally impact other circuits. Chapter 2 reveals a cross-connection between regulation of histidine biosynthesis by the cross-pathway control and sexual development of *A. nidulans*. Chapter 3 describes how the cross-pathway control contributes to the regulation of biosynthesis of the secondary metabolite penicillin. Last but not least, Chapter 4 reveals a link of the COP9 signalosome-dependent network to sexual development as well as production of a red pigment as secondary metabolite.

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Chapter 2

Regulation of the *Aspergillus nidulans hisB* gene by histidine starvation

2.1 Abstract

The *hisB* gene of the filamentous fungus *A. nidulans* encodes the imidazole glycerol-phosphate dehydratase (IGPD; E.C. 4.2.1.19) catalysing the seventh enzymatic step of histidine biosynthesis. The gene was isolated and it's deduced peptide sequence of 247 amino acids showed up to 54% identities to IGPD enzymes of organisms comprising all three kingdoms. Expression of *hisB* cDNA in a *Saccharomyces cerevisiae his3*Δ mutant strain functionally complemented the growth phenotype under histidine limitation. Addition of histidine did not affect *hisB* mRNA levels in *A. nidulans* wild type cells. Histidine starvation conditions increased the *hisB* transcript level fourfold, suggesting regulation by the cross-pathway regulatory network. Deletion of the complete *hisB* open reading frame in the *A. nidulans* strain A234 resulted in histidine auxotrophy. Additionally, *hisB* deletion strains were blocked in sexual fruit body formation on medium containing low histidine concentrations. This developmental phenotype of the *hisB* deletion mutant strain correlated with the induction of the cross-pathway control system.

2.2 Introduction

The filamentous fungus *Aspergillus nidulans* is used as a simple model organism for molecular analysis of interrelated processes in eukaryotes. The ascomycete is particularly advantageous for studies on metabolic multiplicity, genetic networks and multicellular differentiation. Proceeding from multinucleate hyphal growth, complex differentiation processes of asexual and sexual development are induced by environmental signals (Adams *et al.* 1998). To date several lines of evidence indicate a connection between development and metabolism in *A. nidulans* (Käfer 1977a; Serlupi-Crescenzi *et al.* 1983; Eckert *et al.* 1999).

We aimed to completely delete a histidine biosynthetic gene to investigate whether there is a connection between this amino acid biosynthesis and development in *A. nidulans*. We chose the histidine biosynthetic pathway to discriminate an amino acid specific effect from a broad amino acid effect as supposed by the studies on tryptophan auxotrophs (Eckert *et al.* 1999). The latter effect is mediated by the cross-pathway control which constitutes a global regulatory network that ensures an increased transcription of a variety of amino acid biosynthetic genes when supply of a single amino acid is limited. This system is common to several filamentous fungi like *Neurospora crassa, A. nidulans* and

Aspergillus niger (Carsiotis *et al.* 1974; Piotrowska 1980) but studied best in *Saccharomyces cerevisiae* where it is called general control of amino acid biosynthesis. The central transcriptional activator (Gcn4p) of the general control is activated under amino acid starvation conditions via a complex regulatory cascade. Gcn4p binds to the *cis*-acting palindromic GCRE (general control response element: 5'-ATGA (C/G) TCAT-3') in the promoters of the amino acid biosynthetic target genes and thus enhances their transcription (Hinnebusch 1997). For the filamentous fungi *N. crassa* and *A. niger*, homologues of the yeast Gcn4p have already been described to act on similar motifs, now designated CPREs for cross-pathway response elements (Paluh *et al.* 1988; Wanke *et al.* 1997).

None of the genes belonging to the histidine biosynthetic pathway in *A. nidulans* have been isolated so far. Histidine is synthesised in 10 enzymatic steps from PRPP (phosphoribosyl pyrophosphate), ATP and glutamine in a complex biosynthetic pathway which is biochemically the same in all microorganisms investigated (Alifano *et al.* 1996). Enforced histidine starvation is commonly used for induction of the cross-pathway control: The false histidine feedback inhibitor 3-amino-1,2,4-triazole (3AT) competitively inhibits the enzyme imidazole glycerol-phosphate dehydratase (IGPD) which results in histidine starvation and subsequent induction of the cross-pathway control (Kanazawa *et al.* 1988). In the seventh step of histidine biosynthesis, IGPD catalyses the dehydration of imidazole glycerol-phosphate (IGP) to imidazole acetol-phosphate (IAP) (Alifano *et al.* 1996). Corresponding genes have been isolated from several other organisms. The yeast IGPD encoding gene (*HIS3*) constitutes a marker commonly used for molecular techniques. In some γ-proteobacteria the IGPD is a bifunctional enzyme with additional histidinol phosphatase (HPase) activity (Carlomagno *et al.* 1988; Clark *et al.* 1998). In none of these organisms deletion of the IGPD encoding genes lead to additional phenotypes except histidine auxotrophy.

In this work we present the isolation of the *A. nidulans hisB* gene which encodes the imidazole glycerol-phosphate dehydratase of the histidine biosynthetic pathway. The transcriptional regulation was characterised under various conditions. By deletion of the *hisB* gene a defined loss-of-function mutation of an amino acid biosynthetic gene in *A. nidulans* was constructed. Investigations of the mutant's sexual reproductive cycle identified a strong correlation between an induced cross-pathway control and a block in sexual development at the level of micro-cleistothecia. In contrast, overproduction of HISB did not show any effect on the development of the fungus.

2.3 Experimental procedures

2.3.1 Strains and media

Yeast strains H1515 (*leu2*, *-112 ura3-52 trp1 GAL2*; obtained from A. Hinnebusch; Bethesda, USA) and RH2037 (*ura3-1*, *trp1-1*, *ade2-1*, *leu2-3,112*, *his3-11,15*, *rna15-2, GAL2*) (Minvielle-Sebastia *et al.* 1991) were cultivated on minimal medium with 2% glucose or galactose as sole carbon source (Miozzari *et al.* 1978).

The *A. nidulans* strain FGSC A234 (*yA2*, *pabaA1*; *veA1*) was provided by the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, KS, USA). Strain GR5 (*wA3*; *pyrG89*; *pyroA4*; *veA1*) was obtained from G. May (Houston, USA). *A. nidulans* strains were cultivated at 30°C on minimal medium supplemented as described (Käfer 1977b; Bennett and Lasure 1991). The standard concentration of histidine in the medium was 0.3 mM except where noted. For amino acid starvation, mycelia of liquid overnight precultures were adapted for 1 h in fresh medium before 20 mM 3-amino-1,2,4-triazole (3AT) was added. On solid medium supplemented with 2 mM 3AT, conidia were inoculated on sterile miracloth filters to enable the transfer to fresh 3AT plates in two-day intervals. For enhanced cleistothecia development, plates were incubated under oxygen-limiting conditions by wrapping petri dishes with tape (Clutterbuck 1974). For construction of the *hisB* deletion mutant, the *Bam*HI/*Kpn*I-fragment of plasmid pME1657 was transformed into *A. nidulans* strain A234 as described earlier (Eckert *et al.* 2000). 14 transformants were obtained upon selection for the presence of the *ble* gene of *Streptoalloteichus hindustanus* on minimal medium containing 10 µg/ml phleomycin (Cayla, France). Single homologous recombination events were proven by PCR of the transformant's genomic DNA and by Southern hybridisation experiments (Fig. 2.2). Two transformants contained exclusively the *hisB* deletion and both strains showed exactly the same phenotypic effects in all respects. One of them was strain AGB40. For construction of the *hisB* overexpression strain, plasmid pME1764 was transformed into *A. nidulans* GR5 and selected for the plasmid's *pyrG* marker on minimal medium lacking uridine. Eight of the transformants contained additional *hisB* copies in the genome as detected by Southern hybridisation experiments. *A. nidulans* strain AGB75 contained two additional copies of the *hisB* gene. Increased *hisB* transcript levels were verified by Northern experiments under ethanol induced conditions.

2.3.2 Isolation of *hisB* and plasmid construction

Full length cDNA clones of *hisB* were isolated from an *A. nidulans* inducible *GAL1*-driven cDNA expression library as described (Krappmann *et al.* 1999; Hoffmann *et al.* 2000a) via functional complementation of the yeast *his3*Δ mutant strain RH2037 on medium with galactose as sole carbon source but lacking histidine. Yeast cells were transformed as described earlier (Ito *et al.* 1983). In six out of 10,000 yeast transformants histidine prototrophy was restored and the isolated plasmids revealed an identical cDNA sequence resulting in plasmid pME1511. For isolation

of the genomic *hisB* clone, a *Xho*I-digested genomic DNA sublibrary of *A. nidulans* was subcloned into pBluescriptSK$^+$ and transformed into *E. coli* for colony hybridisation using the cDNA fragment as probe which revealed plasmid pME1512. For construction of the deletion cassette pME1512 was amplified via PCR with primers excluding the *hisB* coding region which was replaced by the *Ssp*I-cut phleomycin resistance cassette of plasmid pAN8-1 (Punt and van den Hondel 1992) via blunt end ligation resulting in plasmid pME1657 (Fig. 2.2). The overexpression cassette in plasmid pME1764 was constructed by blunt end ligation of the *hisB* cDNA (*Sal*I/*Not*I fragment of pME1511) to the *alcA*-promoter (*SmaI*) of pME1565 (Hoffmann *et al.* 2000a).

2.3.4 Recombinant DNA techniques and computational analyses

Standard molecular methods for DNA isolation and manipulation were applied according to Sambrook (Sambrook *et al.* 1989). For Northern hybridisation analysis, total RNAs were isolated with TRIZOL Reagent (Gibco BRL). Total RNA (20 µg per lane) were separated on a formaldehyde agarose gel, electroblotted on a nylon membrane (Biodyne, PALL) and hybridised with ^{32}P-labeled DNA probes (Feinberg and Vogelstein 1984). The RNA-ladder of Gibco BRL was used as RNA size standard. Signal intensities were quantified with a bioimager using the program MacBAS v2.5(E) from Fuji.

For DNA sequence analysis with custom oligonucleotides (Gibco BRL) plasmid DNA was labelled with the Big Dye^{TM} Terminator kit of the Cycle Sequencing Reaction Mix from Perkin Elmer. Sequencing was performed by a Perkin Elmer Sequenator 310 (Norwalk, CT, USA) with sequence analysis program ABI EditView 1.0.1. Further DNA analysis was performed with the Lasergene Navigator software from DNASTAR (Madison, WC, USA). IGPD sequences were identified using BLAST search of a non-redundant protein database (Altschul *et al.* 1990). Pairwise alignments and the multiple sequence alignment with hierarchial clustering was performed using the software "CLUSTALW" of F. Corpet (Corpet 1988). Putative transcription factor binding sites were identified with TFSEARCH vs 1.3 (Heinemeyer *et al.* 1998) at the Real World Computing Partnership/Parallel Application TRC Lab. (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). The sequence data of the genomic fragment (4.347 kb of plasmid pME1512) containing the complete *hisB* gene have been submitted to the GenBank database under accession number AF246264.

2.3.5 Microscopic analysis

Growth, conidiation and cleistothecia development of *A. nidulans* were monitored under a Zeiss Stemi 2000-C binocular (Jena, Germany). For detailed microscopical analysis a Zeiss Axiovert S100 microscope (Jena, Germany) with Nomarski-DIC optics was used. For fluorescence microscopy, cleistothecia and micro-cleistothecia were carefully pressed under a cover slide and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) according to Pringle (Pringle *et al.* 1991).

2.4 Results

2.4.1 The *hisB* gene of *Aspergillus nidulans* encodes the imidazole glycerol-phosphate dehydratase of the histidine biosynthetic pathway.

The yeast *HIS3* gene encodes the imidazole glycerol-phosphate dehydratase [IGPD, E.C. 4.2.1.19] essential for histidine biosynthesis. The cDNA of the corresponding *A. nidulans hisB* gene was isolated by functional complementation of the histidine auxotrophy of the *Saccharomyces cerevisiae his3*Δ strain RH2037. Sequence analysis of the 980 bp cDNA revealed an open reading frame of 741 bp. In Southern hybridisation experiments the isolated cDNA used as probe hybridised to a single 6.2 kb *Xho*I genomic fragment. Thus, only a single copy of the cDNA encoding gene is present in the genome of *A. nidulans* (data not shown). The corresponding 6.2 kb genomic fragment was isolated and contained an open reading frame identical to the one of the cDNA clone, flanked by a 2.4 kb 5'- and a 3.0 kb 3'-region. The open reading frame was interrupted 319 bp downstream of the translational start point by one intron of 59 bp showing the conserved 5'-splicing, internal- and 3'-splicing sequences described for introns of *A. nidulans* (May *et al.* 1987). The cDNA clone marked the poly(A) site 150 bp downstream of the UAA stop codon.

From the deduced peptide sequence of 247 amino acids a molecular weight of 26.2 kD was calculated. The polypeptide sequence shows high identities to IGPDs of organisms comprising representatives of all three kingdoms. The multialignment in Fig. 2.1 presents a selection of IGPD amino acid sequences from other fungi, plants, gram positive and negative bacteria as well as archea. All of them, including the deduced protein sequence of *A. nidulans*, contain the well conserved ´dHHxxE´ domains characteristic for imidazole glycerol-phosphate dehydratases (Parker *et al.* 1994). The identities of up to 54% to genes of other IGPDs and the complementation of the yeast *his3*Δ mutant strain characterises the isolated cDNA as imidazole glycerol-phosphate dehydratase encoding gene of *A. nidulans*. Therefore the corresponding gene was named *hisB*.

2.4.2 Construction of an *A. nidulans hisB* deletion strain auxotroph for histidine.

We constructed a *hisB* deletion strain to analyse the function of *hisB* in the filamentous fungus. The complete *hisB* open reading frame of the haploid *A. nidulans* wild-type strain A234 was replaced by a phleomycin-resistance cassette. Our deletion strain AGB40 contained the resistance cassette as single integration at the original genomic *hisB* locus due to homologous integration of the *hisB* flanking DNA regions (Fig. 2.2). In Northern experiments using the *hisB* cDNA as a probe, no *hisB* transcript was detected for strain AGB40 (data not shown). On minimal medium without histidine, germination and growth were totally blocked in the *hisB* deletion strain AGB40. Supplementation of at least 0.3 mM histidine to the growth medium facilitated germination, vegetative growth and conidiation of the

hisB deletion strain alike the wild-type. Transformation of the 6.2 kb genomic fragment containing the *hisB* gene into the *hisB* deletion strain completely restored histidine prototrophy. Even a *Bgl*II / *Kpn*I fragment of the genomic locus, a truncated 5'-version containing only 730 bp of the upstream region, functionally complemented the histidine auxotrophy of AGB40.

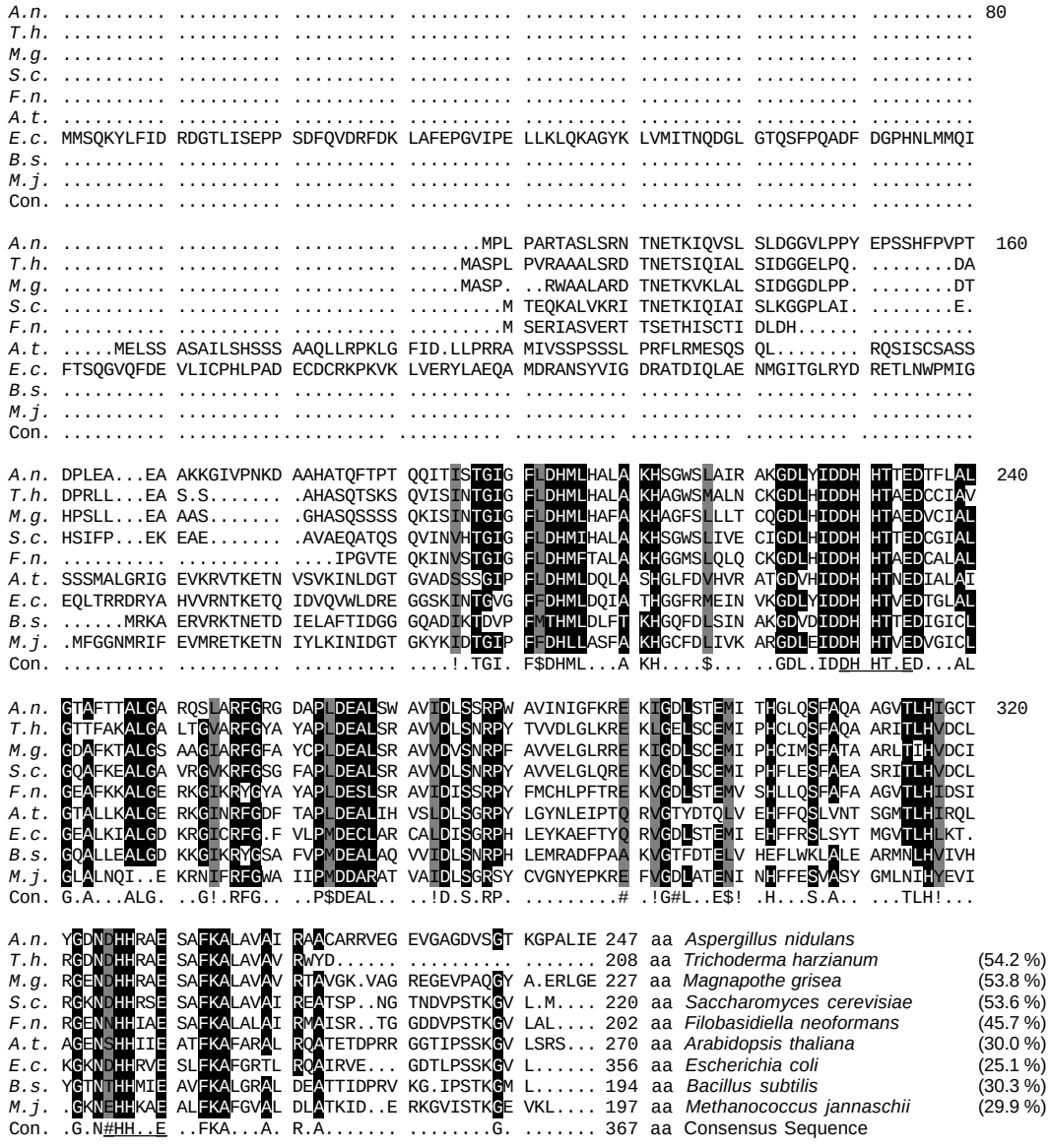
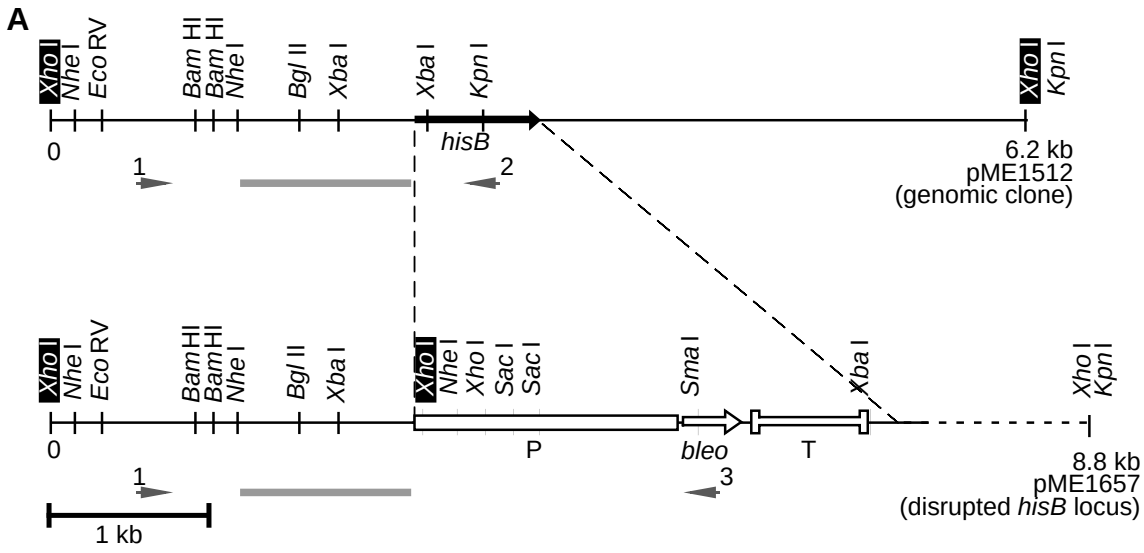


Fig. 2.1: Amino acid comparison of IGPDs. Imidazole glycerol-phosphate dehydratase protein sequences of organisms comprising all three kingdoms were aligned. Accession numbers of the swissprot database are: p34041 (*T. harzianum*); o42621 (*M. grisea*); p06633 (*S. cerevisiae*); p40919 (*F neoformans*); p34047 (*A. thaliana*); p10368 (*E. coli*); o34683 (*B. subtilis*) and q58109 (*M. janaschii*). The percentage of identity of each IGPD sequence to the *A. nidulans* HISB is given in parentheses. Identical amino acids in minimal seven of the sequences are highlighted in black boxes. Grey shading indicates amino acids of high similarity (! = I,V; $ = L,M; % = FY; # = N,D,Q,E,B). The highly conserved "HHxxE" domains are shown underlined in the consensus sequence.



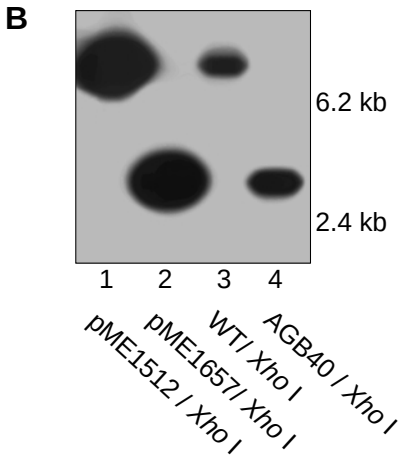


Fig. 2.2: Construction of the *Aspergillus nidulans hisB* deletion strain AGB40. (A) The complete open reading frame of the *A. nidulans hisB* gene on plasmid pME1512 was replaced by a phleomycin resistance cassette (P*gpdA*-promoter, *bleo* phleomycin marker, T*trpC*-terminator). For transformation of *A. nidulans* strain A234, the *Bam*HI/*Kpn*I fragment of the *hisB* deletion cassette was used. Transformants were screened for homologous integration by PCR with three different primers in parallel (grey arrows). **(B)** Integration of the phleomycin resistance cassette was verified by Southern analysis with the *hisB* upstream region as a probe (grey bar). The autoradiography shows *Xho*I-digestion of genomic wild-type and AGB40 DNA (lines 3,4) and of plasmids pME1512 and pME1657 (lines 1,2); and the relevant *Xho*I sites are indicated by black boxes.

2.4.3 The *hisB* gene of *A. nidulans* is regulated on the transcriptional level under amino acid starvation conditions

The promoter sequence of *hisB* of *A. nidulans* contains several putative regulatory elements. Two poly-d(AT) rich regions (5'-TTTTGAAAAAT-3' and 5'-TTTTATTATT-3') at positions -175 and -135 might function as TATA-elements for general transcription (Winter and Varshavsky 1989). Another two represent possible binding sites for developmental and amino acid specific transcription factors: A putative STRE (StuA response element) is located at position -182: 5'-ACGCGGGA-3' and a site similar to yeast's GCRE (general control response element) at position -229: 5'-ATGACGCA-3'.

STREs constitute specific binding sites for the transcription factor STUA and are generally found upstream of genes important for development or cell cycle in *A. nidulans*. Genes under control of this transcription factor have been shown to be regulated during development (Miller *et al.* 1991; Dutton *et al.* 1997). Northern experiments showed equal levels of approximately 1 kb *hisB* mRNAs during vegetative growth, asexual and sexual spore formation (data not shown). This

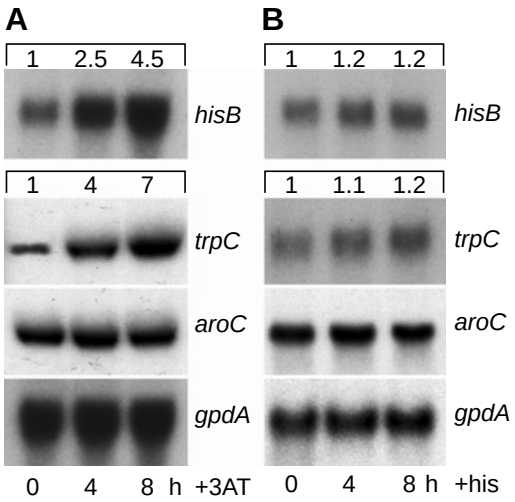


Fig. 2.3: Transcriptional regulation of the *A. nidulans hisB* gene. Mycelia were grown in liquid culture, either **(A)** under induction of the cross-pathway control by 20 mM 3-amino-1,2,4-triazole (3AT) or **(B)** at an excessive histidine (his) concentration of 30 mM. RNA was isolated at the time points indicated and probed with the *hisB* gene in Northern hybridisation experiments. Controls: *trpC* (regulated by the cross-pathway control), *aroC* and *gpdA* (both transcribed constitutively under the conditions tested). For quantification, mRNA amounts were equalised according to the level of *gpdA* transcription and mRNA amounts of *hisB* at the time point 0 were set as 1. Relative *hisB* and *trpC* mRNA levels are given.

suggested that *hisB* transcription is normally not affected during development. Thus, the putative binding site for the STUA protein in the promoter region of *hisB* does not seem to play an important role during development.

The GCRE-like site exhibits high similarity to the binding site of the general control transcriptional activator Gcn4p of yeast (Hinnebusch 1988). The consensus sequences between the Gcn4p protein and its homologue of *A. nidulans* might be similar because the genes *argB*, *trpB* and the *trpC* of *A. nidulans* subjected to this regulatory mechanism show identical sequence motifs in their promoter regions (Mullaney *et al.* 1985; Goc and Weglenski 1988; Eckert *et al.* 2000) which are called CPRE (cross-pathway response element) in filamentous fungi. The CPRE site in the promoter region of *hisB* seems to be important under amino acid starvation conditions. We determined the level of *hisB* transcription upon addition of the histidine analogue 3-amino-1,2,4-triazole (3AT) in Northern hybridisation experiments. 3AT acts as false feedback-inhibitor of the HISB protein and thus causes histidine starvation with subsequent induction of the cross-pathway control. Eight hours after 3AT addition, the *hisB* transcript level increased about fourfold compared to cultures not incubated with 3AT (Fig. 2.3A). Similar results were found for the *trpC* gene [E.C. 2.1.3.3] encoding a trifunctional tryptophan biosynthetic enzyme known to be regulated by the cross-pathway control (Eckert *et al.* 1999). An amino acid biosynthetic gene unaffected by the regulatory network, *aroC*, showed constitutive expression (Krappmann *et al.* 1999). We examined whether the effect of 3AT on *hisB* transcription is reversed by the simultaneous addition of histidine to exclude that the analogue or histidine have a specific effect on *hisB* transcription. *A. nidulans* cells were simultaneously supplemented with 20 mM histidine and 10 mM 3AT, resulting in stable basal *hisB* mRNA levels during cultivation (data not shown). We also tested whether the transcription of *hisB* is affected at excessive histidine supplementation (30 mM) without the analogue. *hisB* mRNA levels of an *A. nidulans* wild-type strain grown on high histidine concentrations after shift from minimal medium without histidine remained on a low level (Fig. 2.3B). These data suggest that on transcriptional level the *hisB* gene of *A. nidulans* is regulated by the cross-pathway control but not by an additional histidine-specific regulatory mechanism.

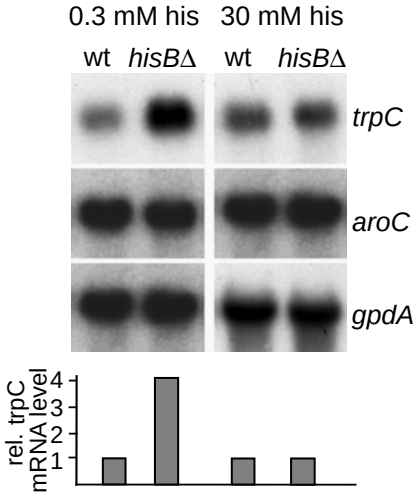


Fig. 2.4: Induction of the cross-pathway control in a *hisB*Δ mutant strain. *A. nidulans* wild-type strain A234 and the *hisB* deletion mutant AGB40 were grown overnight in liquid minimal medium containing 0.3 mM or 30 mM histidine (his). RNA was isolated and the status of the cross-pathway control was monitored using the *trpC* gene as a probe in Northern hybridisation experiments (controls as in Fig. 2.3). mRNA levels were equalised according to the *gpdA* transcriptional level and mRNA amounts of wild-type were set as 1. Relative *trpC* mRNA levels are given.

2.4.4 Growth on low histidine concentration causes induction of the cross-pathway control in the *hisB* deletion mutant.

The histidine analogue 3AT reduced the activity of the HISB protein by false feedback inhibition and therefore induced a shortage of histidine. This starvation for histidine induced the cross-pathway control which subsequently increased *hisB* transcription. Low concentrations of histidine should result in a similar phenotype in an *A. nidulans hisB* mutant strain in the absence of 3AT. Therefore we tested transcription of the cross-pathway control-regulated *trpC* gene (Eckert *et al.* 1999) in the *hisB* mutant AGB40 after incubation in liquid minimal medium which allows exclusively growth of vegetative mycelium. Under low histidine concentrations (0.3 and 3 mM), the mRNA level of *trpC* was increased about fourfold in the mutant compared to the wild-type strain. Under high concentrations of histidine (30 and 60 mM) no difference in mRNA levels was observed between the *hisB* mutant and the wild-type strain (Fig. 2.4). The same results were found for *argB*, a second cross-pathway regulated gene (data not shown). The *aroC* gene which is unaffected by the cross-pathway control showed constant mRNA levels under any condition tested. The same results were obtained in Northern experiments with RNA from cultures grown on solid medium at different developmental time points (data not shown). These data indicate that supplementation of the *hisB* deletion mutant with low concentrations of histidine activates the cross-pathway regulatory network.

2.4.5 Deletion of *hisB* affects sexual development of *A. nidulans*.

A. nidulans strains with point mutations in tryptophan amino acid biosynthetic genes showed an aberrant sexual development dependent on the tryptophan concentration supplied to the medium (Eckert *et al.* 1999). We used our *A. nidulans hisB* deletion strain to analyse whether the sexual development is impaired in this strain with a defined loss-of-function mutation of a different amino acid biosynthetic

gene. Sexual development of the wild-type (strain A234) was independent of the histidine concentration supplied (0.3 mM, 3 mM, 30 mM and 60 mM). After induction of at least 60 h of incubation nests were the first visible sexual structures. Small, soft and pale coloured micro-cleistothecia were formed within the nests after 90 h. The maturation of the sexual fruit bodies was completed another 110 h later resulting in hard, darkly red coloured cleistothecia filled with fertile ascospores. In the *hisB* deletion strain AGB40 sexual development was unaffected for the first 90 h and thus micro-cleistothecia were formed at numbers similar to wild-type at any histidine concentration tested. On medium containing high concentrations of histidine (30 or 60 mM) the *hisB* mutant strain developed wild-type-like mature cleistothecia after 200 h. In contrast, the sexual cycle was blocked under conditions of low histidine concentrations (0.3 and 3 mM) at the reproducible time point of micro-cleistothecia formation without any further maturation even after 200 h (Fig. 2.5A, B). Microscopic analysis showed that the micro-cleistothecia of the *hisB* deletion mutant grown at 0.3 mM histidine formed a small inner cavity, entirely filled with hyphae instead of ascospores after 90 and 200 hours of incubation. In the wild-type, this developmental state of micro-cleistothecia was an intermediate state after 90 hours which then continued to grow to well-rounded mature cleistothecia containing ascospores after an incubation of 200 h (Fig. 2.5C). These observations were confirmed by DAPI staining of hyphal nuclei. After 90 h, the nuclei in the hyphae of wild-type and *hisB* mutant micro-cleistothecia are stained bright blue. Completion of the sexual cycle after 200 h prevents staining by formation of ascospores in the wild-type, but not in the *hisB* mutant strain on low histidine concentrations (data not shown). When the deletion strain AGB40 is complemented with the *hisB* wild type genomic fragment (plasmid pME1512 integrated ectopically in the genome) normal cleistothecia develop at any histidine concentration tested. These data indicate a connection between histidine biosynthesis and sexual development in *A. nidulans*.

Micro-cleistothecia of the *hisB* mutant strain which were blocked in their development by low histidine supplementation were further analysed to distinguish whether the arrest is irreversible or whether development can be completed in a changed environment. Therefore micro-cleistothecia, which were grown for 200 hours on medium containing 0.3 mM histidine, were shifted to media containing 30 and 60 mM histidine and cultivated for additional 120 hours. During this time, micro-cleistothecia released the block and completed development resulting in normal cleistothecia with fertile ascospores (data not shown). Thus, 90 h after induction of the sexual developmental process, a control point exists to decide whether the sexual cycle should be completed.

The effect of blocked sexual reproduction in the *A. nidulans hisB* deletion strain prompted us to check the developmental capacity of a HISB overproducing *A. nidulans* strain. Therefore, *hisB* was fused to the inducible *alcA*-promoter and ectopically integrated into the genome of *A. nidulans* GR5. Strain AGB75 showed up to 30fold increased transcript levels as proven by Northern experiments (data not shown). The functionality of the *hisB* overproduction was checked on solid minimal medium containing 10 mM 3AT. These high 3AT concentration prevents

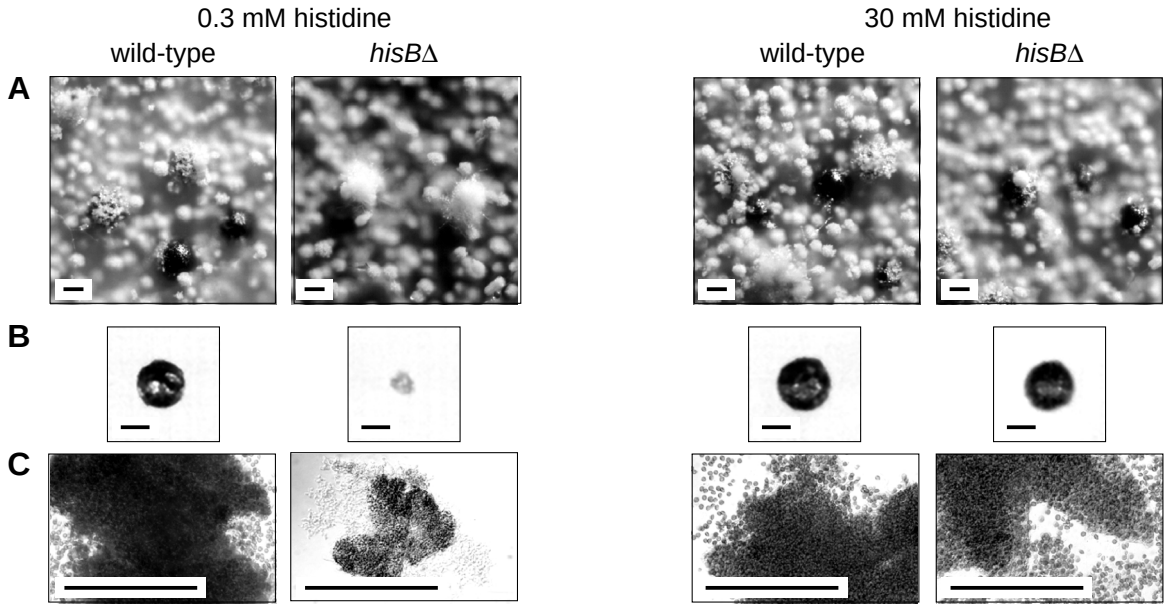


Fig. 2.5: Deletion of the *A. nidulans hisB* gene blocks sexual development under low histidine concentrations. Sexual development of the wild-type strain AGB234 and the *hisB* deletion strain AGB40 were investigated on agar plates containing low (0.3 mM) or high (30 mM) histidine concentrations. The wild-type develops mature cleistothecia under all conditions tested. So does the mutant under high histidine concentrations. At low histidine concentrations, the mutant AGB40 stops the differentiation process after about 90 h development, leading to pale-coloured, soft and small micro-cleistothecia. The morphology of the sexual reproductive structures is shown under a binocular in colony context **(A)** and as isolated structures **(B)**. The structures were slightly pressed under a cover slide and studied under the light microscope: the mature cleistothecia are filled with ascospores whereas the micro-cleistothecium is filled with hyphae **(C)**. Pictures were taken after an incubation time of 200 h. Scale bars approx. 100 µm.

growth of the wild-type but allows growth of strains with a surplus of the analogs' target enzyme HISB. All transformants grew under conditions with 10 mM 3AT. But none of the *hisB* overproducing *A. nidulans* strains did show any significant morphological or developmental aberration during asexual or sexual life cycle when grown under inductive conditions with ethanol as the sole carbon source.

2.5 Discussion

In this work we present the first isolation of a histidine biosynthetic gene of the filamentous fungus *A. nidulans*. Complete deletion of the *hisB* coding region uncovered a block in the sexual development of the mutant strain at low histidine supplementation. Detailed analysis of *hisB* transcript levels showed a correlation of this developmental phenotype with activation of the cross-pathway regulatory system. Overproduction of the HISB protein did not show any effect on the development of the fungus.

We demonstrated that deletion of the complete *hisB* open reading frame results in histidine auxotrophy. Retransformation of the *hisB* gene into the deletion mutant AGB40 completely restored histidine prototrophy. Thus, the *hisB* gene can be easily used as selectable marker in *A. nidulans*. Since *A. nidulans* is highly sensitive for 3AT, the HISB protein as target of this inhibitor might be specifically used to reveal the binding site of 3AT (Kanazawa *et al.* 1988) within the enzyme.

The HISB protein sequence showed up to 54% identity to imidazole glycerol-phosphate dehydratases of other organisms which possibly indicates homology of these proteins. IGPD-encoding genes of archea, bacteria and eukarya have been cloned by complementation of the corresponding *E. coli*, *B. subtilis* or *S. cerevisiae* mutants indicating functional conservation of the IGPD enzymes, even though the IGPD enzymes of γ-proteobacteria are combined with an additional histidinol phosphatase-activity (Alifano *et al.* 1996). This further counts for a common origin of all IGPD encoding genes of histidine biosynthesis.

Computational analysis of the *A. nidulans hisB* promoter region revealed several putative regulatory elements including possible targets for a general transcription factor as well as for developmental and amino acid specific transcription factors. The two poly-d(AT)-stretches are similar to the TATA elements (Tc and Tr) in the *S. cerevisiae HIS3* promoter which direct constitutive and activator-dependent transcription initiation to the sites +1 and +13, respectively (Iyer and Struhl 1995). We showed that the putative STRE site in the *hisB* promoter seems either not functional or has a function not known to date. The transcription factor STUA, site-specific for STREs, is important for regulation of multicellular development in *A. nidulans*. It is involved in asexual development and presumably in the induction of the sexual reproductive cycle in *A. nidulans* (Dutton *et al.* 1997). If the proposed STUA binding site in the *hisB* 5'-regulatory region was functional, *hisB* transcription should be regulated during development. However, under standard growth conditions the *hisB* transcript levels of the *A. nidulans* wild-type A234 were not significantly changed during hyphal growth, the asexual or the sexual phase of *A. nidulans*. We demonstrated that *hisB* is transcriptionally regulated under amino acid starvation conditions which indicates that the CPRE in the promoter region is functional. The corresponding central transcription factor of the cross-pathway control should be homologous to the yeast Gcn4p and the corresponding homologues Cpc-1p and CpcAp of the filamentous fungi *N. crassa* and *A. niger* (Hinnebusch 1988; Paluh *et al.* 1988; Wanke *et al.* 1997). The homologous *HIS3* gene of *S. cerevisiae* contains even three GCREs in the regulatory region and shows similar rates of induction by amino acid starvation (Struhl 1982; Hope and Struhl 1985; Hinnebusch 1988).

When the *hisB* deletion strain AGB40 was grown on low histidine concentrations two phenomena became obvious: the sexual development was blocked on the level of micro-cleistothecia and simultaneously the cross-pathway control was induced. The same strain grown under conditions of high histidine supplementation differentiates mature, fertile cleistothecia and the cross-pathway control is repressed, just like the wild-type on any histidine supplementation tested. These findings indicate a cross-connection between completion of the sexual development and an activated cross-pathway control. This raises the question whether the block of sexual development is a histidine specific effect or a general amino acid deficiency effect mediated by the cross-pathway control. The latter presumption is supported by previous findings: mutations in tryptophan biosynthetic genes of *A. nidulans* resulted in the same block of sexual development and an activated cross-pathway control as shown for our *hisB*-deletion strain

(Eckert *et al.* 1999). Thus, all data confirm that the cross-pathway control is turned on due to amino acid starvation under conditions where no mature cleistothecia are build. Additionally, overexpression of the cross-pathway central transcription factors of *A. niger* and *N. crassa* in an *A. nidulans* wild-type strain resulted in a similar block of sexual development at the level of micro-cleistothecia (Hoffmann *et al.* 2000b). Apart from that, over-supplementation of *A. nidulans* wild-type strains with tryptophan or arginine impaired sexual development (Serlupi-Crescenzi *et al.* 1983; Eckert *et al.* 1999). For histidine, we did not identify any concentration that affects sexual development of the wild-type. Possibly the cross-pathway control is not turned on by over-supplementation with histidine due to a limited uptake of histidine. In contrast to the *A. nidulans trpC* and *argB* mutants, where insufficient tryptophan and arginine supply, respectively, affected asexual development, no effect on conidiation was observed for the *hisB* deletion strain upon supplementation with low levels of histidine. For our *hisB* deletion strain AGB40 the block in sexual development seems to be exclusively caused by induction of the cross-pathway control.

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Chapter 3

Impact of the cross-pathway control on regulation of lysine and penicillin biosynthesis in *Aspergillus nidulans*.

3.1 Abstract

The non-proteinogenic amino acid α-aminoadipate defines the biosynthetic branch point of lysine and penicillin biosynthesis in the filamentous fungus *Aspergillus nidulans*. Regulation of both pathways was analysed in response to amino acid limitation. The *lysF* encoded homoaconitase acts upstream of the α-aminoadipate branch point whereas the *lysA* gene product saccharopine dehydrogenase catalyses the ultimate step of the lysine-specific branch. The *lysA* gene from *A. nidulans* was identified and isolated. Amino acid starvation resulted in significantly increased transcription of *lysA* but not of *lysF*. Starvation dependent changes in transcription levels of *lysA* were dependent on the presence of the central transcriptional activator of the cross-pathway control (CPCA). The effect of amino acid starvation under penicillin production conditions was analysed in *A. nidulans* strains with reporter genes for the penicillin biosynthesis genes *acvA* and *ipnA* and genetically altered activity of the cross-pathway control. Overproduction of CPCA decreased expression of *ipnA* and *acvA* reporter genes and even more drastically reduced penicillin production. This work suggests that, upon amino acid starvation, the cross-pathway control overrules secondary metabolite biosynthesis and favours the metabolic flux towards amino acids instead of penicillin in *A. nidulans*.

3.2 Introduction

Aspergillus nidulans serves as fungal model organism for regulatory networks in eukaryotes. The regulation circuits which direct metabolic fluxes towards either primary or secondary metabolism are scarcely known (Firn and Jones 2000). An immediate connection of primary- and secondary metabolism in β-lactam producing filamentous ascomycetes is represented by the α-aminoadipate branch point of lysine and penicillin biosynthesis. Both metabolites are of biotechnological importance: lysine is an essential amino acid for humans which has to be supplied by the diet, and β-lactam antibiotics are among the most prominent antibiotics used world-wide. *A. nidulans* is due to its sexual cycle more suitable for genetic studies on regulation of penicillin biosynthesis than the commercial production strains of *Penicillium chrysogenum* (Martin 1998). The initial five enzymatic reactions starting from α-ketoglutarate and acetylCoA result in the key intermediate α-aminoadipate (Fig. 3.1). The biosynthesis branches at this point and leads in

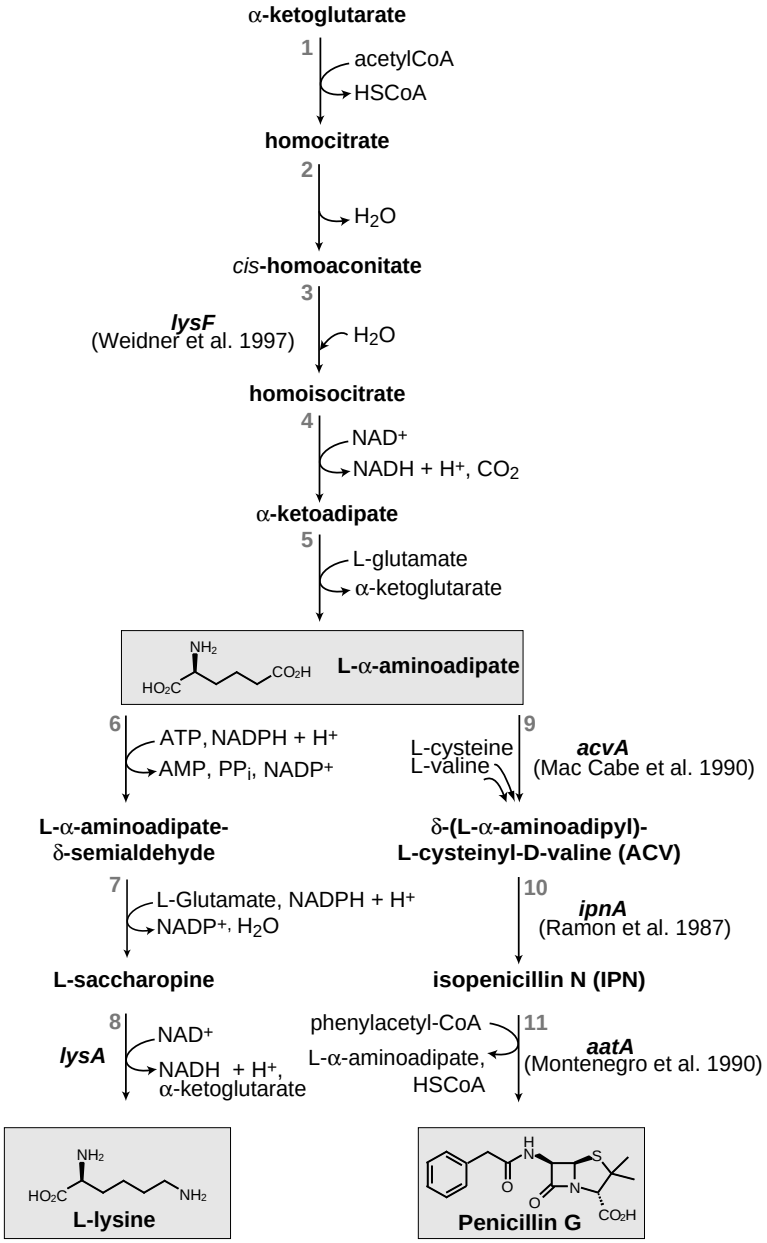


Fig. 3.1: α-aminoadipate constitutes a branch point of lysine and penicillin biosynthesis in β-lactam antibiotic-producing fungi. The α-aminoadipate pathway of lysine biosynthesis is common to fungi and includes eight enzymatic steps: (1) homocitrate synthase EC 4.1.3.21, (2,3) homoaconitase EC 4.2.1.36, (4) homoisocitrate dehydrogenase EC 1.1.1.87, (5) aminoadipate aminotransferase EC 2.6.1.39, (6) aminoadipate reductase EC 1.2.1.31, (7) saccharopine reductase: EC 1.5.1.10 and (8) saccharopine dehydrogenase EC 1.5.1.7 (Zabriskie and Jackson 2000). Penicillin is synthesised via (9) ACV synthetase, (10) IPN synthase and (11) AcylCoA:IPN acyltransferase (Brakhage 1998). Genes isolated from *A. nidulans* encoding enzymes mediating any of the steps mentioned above are shown in bold print.

three enzymatic steps either towards lysine or penicillin (Ramon *et al.* 1987; Zabriskie and Jackson 2000). The regulatory circuits controlling the distribution of the key metabolite α-aminoadipate between the two branches are yet unknown. Taking into account that lysine is needed for translation of proteins but the physiological role of the β-lactam antibiotic for the fungal life is yet unclear, the regulatory potential at this point is of special interest.

The *lysF* gene is the only gene of the lysine/penicillin pathway's common stem which has been isolated and characterised from *A. nidulans*. It encodes

homoaconitase LYSF which catalyses the third and presumably also the second step of lysine biosynthesis (Weidner *et al.* 1997). A negative effect on *lysF* expression is mediated by external supply of lysine and the CCAAT-binding complex AnCF (Weidner *et al.* 1997; Steidl *et al.* 1999; Weidner *et al.* 2001). None of the genes involved in the conversion of α-aminoadipate into L-lysine are yet isolated. By contrast, all genes involved in the penicillin-specific branch of *A. nidulans* are known. The amino acids L-α-aminoadipate, L-cysteine and L-valine are condensed by a non-ribosomal peptide synthetase (ACVS) resulting in the ACV-tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (MacCabe *et al.* 1990). The subsequent oxidative ring closure to isopenicillin N is catalysed by the isopenicillin N synthase IPNS (Schenk 2000). The acylCoA: isopenicillin N acyltransferase (IAT) exchanges the α-aminoadipate side chain with a phenylacetyl moiety resulting in the acylated dipeptide penicillin G (Whiteman *et al.* 1990). In all known penicillin-producing fungi, the penicillin biosynthesis genes are located in a single cluster with the genes *acvA* and *ipnA* being divergently transcribed from a bidirectional promoter region. Expression of the genes is controlled by complex regulatory processes depending on the developmental state of the culture and on growth conditions (Brakhage 1998; Martin 2000). Particularly inclusion of corn steep solids, a by-product of the corn wet-milling industry, to the penicillin fermentation broth significantly stimulates expression of the penicillin biosynthesis genes and increases penicillin production by yet unknown mechanisms (MacCabe *et al.* 1990; Luengo and Penalva 1994).

It is conceivable that a cross-talk exists between regulation of lysine and penicillin biosynthesis. In the bifurcated biosynthesis pathway, amino acids are end product and precursor of the different branches, respectively (Fig. 3.1). Availability of amino acids might thus affect the metabolic flux towards α-aminoadipate, lysine and penicillin. For the genes acting downstream of α-aminoadipate, there are two interesting alternatives which could be pursued by the fungus: penicillin synthesis could be either reduced to favour lysine biosynthesis or increased to kill food competitors as e.g. bacteria and use them as protein or nitrogen source. In *A. nidulans* and other filamentous fungi, limitation of amino acids activates the global regulatory network "cross-pathway control" (Sachs 1996). The transcriptional activator of this system, CPCA (Hoffmann *et al.* 2001), binds to CPCA response elements (CPREs) in the promoter region of the corresponding target genes and increases their transcription. The optimal binding site for Gcn4p, the CPCA homologue in *Saccharomyces cerevisiae*, is the asymmetric sequence 5'-TGA(C/G)TCA-3', though Gcn4p can also bind with lower affinity to sequences that differ from this optimal sequence in one or even two positions (Hinnebusch 1986; Oliphant *et al.* 1989; Mavrothalassitis *et al.* 1990). In *A. nidulans*, the tryptophan biosynthesis gene *trpC* (Eckert *et al.* 2000) and the histidine genes *hisB* (Busch *et al.* 2001) and *hisHF* (Valerius *et al.* 2001) contain CPREs in their 5' regulatory region and are transcriptionally activated upon amino acid starvation. But another amino acid biosynthesis gene, *aroC*, is not affected by this system (Krappmann *et al.* 1999) whereas a gene probably not related to amino acid biosynthesis is (Strittmatter *et al.* 2001), indicating that regulation by the cross-

pathway regulatory network in *A. nidulans* is not restricted to amino acid biosynthesis genes.

Lysine biosynthesis is highly regulated by Gcn4p in yeast (Natarajan *et al.* 2001). But to our knowledge, data on regulation of penicillin biosynthesis by amino acid starvation are limited. External supply of amino acids to *A. nidulans* liquid cultures results in various effects on penicillin reporter gene expression, probably independent of the cross-pathway control system (Brakhage and Turner 1992; Then Bergh and Brakhage 1998). In *P. chrysogenum*, artificially induced histidine starvation differentially affects the α-aminoadipate pool size and penicillin production dependent on the strain used (Hönlinger *et al.* 1988). In this work, the implication of amino acid starvation and corresponding activation of the cross-pathway control on selected genes acting in the different branches of the bifurcated lysine/penicillin biosynthesis pathway in *A. nidulans* was questioned. Two experimental set-ups were applied, artificially induced histidine starvation and genetically altered intracellular CPCA levels. The results of this work suggest that amino acid starvation favours the metabolic flux towards amino acids and diminishes the flux towards penicillin at the α-aminoadipate branch point of *A. nidulans*.

3.3 Experimental procedures

3.3.1 Growth conditions

A. nidulans strains were cultivated at 30°C in 100 ml liquid minimal medium (50 mM glucose, 70 mM $NaNO_3$, 7 mM KCl, 11.2 mM KH_2PO_4 (pH 5.5), 2 mM $MgSO_4$, 1x trace elements) (Bennett and Lasure 1991) or fermentation medium (100 mM lactose, 2% w/v corn steep solids, 100 mM $CaCO_3$, 50 mM KH_2PO_4, 3.3 mM phenoxy acetic acid, pH 6.0) (Brakhage and Turner 1992) supplemented as described (Käfer 1977). For amino acid starvation, mycelia of liquid overnight pre-cultures (10^9 spores in 300 ml medium) were filtrated through sterile miracloth, transferred into fresh minimal/fermentation medium and grown for 1 h/40 h prior to addition of 10 mM/50 mM 3-amino-1,2,4-triazole (3AT), respectively. Expression from the *alcA* promoter in the complex fermentation medium was induced by 10 mM cyclopentanone as described before (Waring *et al.* 1989; Kennedy and Turner 1996).

3.3.2 Isolation procedures

Three independent cultures were used to extract RNAs, DNAs and proteins. *A. nidulans* mycelia were filtered through sterile miracloth, freeze-dried and mechanically pulverised. RNAs were isolated from a volume of 100 µl mycelia mixed with 1 ml Trizol™ (GIBCO BRL) as recommended by the manufacturers. DNAs were isolated from a volume of 1 ml mycelia according to Lee and Taylor (Lee and Taylor 1990). Crude protein extracts were obtained from the supernatant

derived from 50 mg lyophilised mycelia ground with equal volume of sterile sand and mixed with 3.5 ml cold 0.2 M phosphate buffer.

3.3.3 Hybridisation experiments

50 µg and 15 µg of total RNA or 15 µg chromosomal DNA per lane were separated in a 1.4% formaldehyde-containing agarose gel or a 1% agarose gel, respectively. The RNA-ladder and 1 kb-DNA-ladder of Gibco BRL were used as size standards. RNA quantities were standardised according to the 16S and 23S rRNA signals on the ethidium bromide stained gel and quantified via the Image Station 440CF (Kodak, Rochester, NY, USA). For Northern and Southern hybridisation experiments, nucleotides were transferred on nylon membranes by capillary blotting, fixed for 30 min at 65°C (GeneScreen) and crosslinked with UV light. For colony hybridisation experiments, cells were directly transferred from agar plates to the membrane and treated successively with 0.5 M NaOH / 1.5 M NaCl, 1.5 M NaCl / 0.5 M Tris (pH 7.4) and 2 x SSC. Membranes were prehybridised for 2 h in hybridisation mix (7% SDS, 1% BSA, 1 mM EDTA, 250 mM $NaPO_4$, pH 7.2). Specific probes for *acvA*, *aroC*, *cpcA*, *gpdA*, *hisB*, *ipnA*, *lysA* and *lysF* were amplified via PCR with T3 and T7 primers or specific primer pairs (Tab. 3.1) and ^{32}P-labelled with the HexaLabel™ DNA Labelling Kit (MBI Fermentas). Following overnight hybridisation at 65°C, membranes were rinsed twice and washed for 30 min with 0.1 x SSC / 0.1% SDS, dried on air and exposed to imaging plates and x-ray films. Signal intensities were quantified using a bioimager and MacBAS v2.5(E) from Fuji.

3.3.4 Enzymatic assays

100 µl of protein extracts were mixed with 0.9 ml Z-buffer (48 mM Na_2HPO_4, 39.3 mM NaH_2PO_4, 10 mM KCl pH 7.0, 0.1 mM $MgSO_4$, 0.01 mM PMSF, 270 µl / 100 ml β-mercaptoethanol) and adapted to 25°C in a waterbath. For the photometric β-galactosidase assay, the reaction was started with 200 µl ONPG-solution (4 mg o-nitrophenol-β-D-galactoside / ml Z-buffer) and stopped after 1 to 30 min with 500 µl 1 M Na_2CO_3-solution. Absorbance was measured at 420 nm and specific activity determined as (U/mg) = 1.7 x OD_{420} / extinction coefficient x protein concentration x extraction volume x time. The photometric β-glucuronidase assay was started with PNPG (4 mg / ml p-nitrophenol-β-D-glucuronic acid), stopped with 500 µl 1 M Na_2CO_3-solution and absorbance was measured at 415 nm. Protein content was determined via the Bradford assay (Bradford 1976).

3.3.5 Penicillin bioassay

1 ml samples of *A. nidulans* liquid culture supernatants (stored at -20°C) were centrifuged at 16000 g for 30 min, 50, 25 and 10 µl of the supernatant were dropped on sterile antibiotic test filters (∅ 9 mm, Schleicher and Schüll) and dried. Test filters and calibration samples (50 to 0.003 µg penicillin G / ml) were placed on M1 medium agar plates (0.5% w/v peptone, 0.3% w/v meat extract, 1.5% w/v agar) confluently covered with overnight colonies of the penicillin-sensitive strain

Bacillus calidolactis C953 and incubated at 55°C overnight. The penicillin titre was determined according to the bacterial growth as log concentration versus retardation ring in mm.

3.3.6 Plasmid construction

Plasmids and specific primer pairs used in this study are summarised in Table 3.1. The nucleotide sequence of the *S. cerevisiae LYS1* gene was used for identification of a 434 bp fragment in the *A. nidulans* EST library via homology searches. This *lysA* fragment was amplified via PCR and subcloned into pBluescript®IISK+ (Stratagene) resulting in plasmid pME1750. The insert was used as specific probe in a colony hybridisation experiment of a partially *Sau3*A digested *A. nidulans* genome library (obtained from Bruce Miller, Moskow, Idaho, USA) yielding plasmid pME2159 with a 14 kb insert. From this insert, an eight kb *Kpn*I fragment containing the complete *lysA* coding region was subcloned into pBluescript®IISK+ resulting in plasmid pME2162.

Tab. 3.1: Plasmids

plasmid	insert description (primer for PCR amplification)	reference
pAN5-22	1.2 kb genomic *A. nidulans Bam*HI/*Eco*RI-insert containing *gpdA* in pBR322	Punt *et al.* 1988
pBluescript II	2.96 kb cloning vector (pBSK and pBKS)	Stratagene, CA, USA
pLYSF1	3.1 kb genomic *A. nidulans Hin*dIII/*Sph*I-insert containing *lysF* in pUC18	Weidner *et al.* 1997
pME1498	1 kb *aroC* cDNA of *A. nidulans Sal*I/*Not*I in pBKS	Krappmann *et al.* 1999
pME1511	1 kb *hisB* cDNA of *A. nidulans Sal*I/*Not*I in pRS316-GAL1	Busch *et al.* 2001
pME1603	*cpcA* open reading frame fused to the *alcA*-promoter (P_{alcA}-*cpcA*) in pME1565	Hoffmann *et al.* 2001
pME1702	2.2 kb *cpcA* cDNA of *A. nidulans Sal*I/*Not*I in pRS316-GAL1	Hoffmann *et al.* 2001
pME1707	2.7 kb genomic *A. nidulans Xba*I/*Bam*HI-insert containing *cpcA* in pRG3	Hoffmann *et al.* 2001
pME1750	434 bp *lysA* insert in pBSK (5'-taatggactccaagctcatg-3', 5'-tctcttcagatcttaccagc-3')	this work
pME1751	501 bp *acvA* insert in pBSK (5'-gatttaaagaccacctac-3', 5'-ctcccccaattcgatacg-3')	this work
pME1752	503 bp *ipnA* insert in pBSK (5'-caggaccaagtccgtgcc-3', 5'-caagtagccagtattcgtc-3')	this work
pME2159	14 kb genomic *A. nidulans* insert containing *lysA* in pUI1	this work
pME2162	8 kb genomic *A. nidulans Kpn*I-insert containing *lysA* in pBSK	this work

3.3.7 Sequence analyses

For DNA sequence analysis with custom oligonucleotides (Gibco BRL) the Big Dye™ Terminator kit of the Cycle Sequencing Reaction Mix (Perkin Elmer) was used. Sequence analysis was performed on a Perkin Elmer Sequenator 310 with the ABI EditView 1.0.1 software. Further DNA analysis was performed with the Lasergene software from DNASTAR. Saccharopine dehydrogenase peptide sequences were identified using BLAST search of a non-redundant protein

database (Altschul *et al.* 1997). Pairwise alignments and the multiple sequence alignment with hierarchial clustering was performed using the software "CLUSTALW" (Corpet 1988). The nucleotide sequence of the *lysA* coding region has been deposited in the GenBank database under GenBank Accession Number AY057447.

3.3.8 *A. nidulans* strain construction

Isogenic *A. nidulans* strains containing *acvA* and *ipnA* reporter gene fusions and different expression levels of *cpcA* were constructed by crossing experiments. Genotypes and sources of the *A. nidulans* strains are given in Table 3.2. The *cpcA* deletion (Δ*cpcA*::*bleo*) of strain AGB52 was combined with the mutated β-galactosidase allele (*bga0*) of strain WG355 resulting in strain AGB178. The *bga0*-phenotype of *A. nidulans* strains was tested in a β-galactosidase assay on minimal medium agar plates containing either glucose or lactose as carbon source. Cells were permeabilised with toluol, washed and incubated with 4-methylumbelliferyl-β-D-galactosid (4 mg / ml) to visualise the fluorescence at 320 nm. The reporter gene fusions of the penicillin biosynthesis genes (*argB2:*:P*acvA-uidA*; P*ipnA-lacZ; argB*$^+$) from strain AXB4A were crossed into strain AGB178. The resulting *cpcA* deletion strain AGB187 showed β-galactosidase activities solely expressed by the reporter genes. AGB187 was transformed with a wild-type copy of *cpcA* (plasmid pME1707) or a *cpcA* overproduction construct (plasmid pME1603) resulting in strains AGB188 (single integration of *cpcA*) and AGB189 (5-6 copies of P*alcA-cpcA* ectopically integrated at two genome loci). Genotypes were tested by PCR and Southern hybridisation analyses.

Tab. 3.2: *A. nidulans* strains

strain	genotype	reference
WG355	*argB2*; *bgA0*; *biA1*	Brakhage *et al.* 1992
A234	*yA2*, *pabaA1*; *veA1*	FGSC*
AXB4A	*bga0*; *biA1*; *argB2*::P_{acvA}-*uidA* P_{ipnA}-*lacZ*, *argB*$^+$	Brakhage *et al.* 1992
AGB52	Δ*cpcA*::*bleo*; *pabaA1*; *pyrG89*; *pyroA4*; *veA1*; *yA2*	Hoffmann *et al.* 2001
AGB178	*bga0*; Δ*cpcA*::*bleo*; *argB2*; *pyrG89*	this work
AGB187	*bga0*; Δ*cpcA*::*bleo*; *argB2*::P_{acvA}-*uidA* P_{ipnA}-*lacZ*, *argB*$^+$; *pyrG89*	this work
AGB188	*bga0*; Δ*cpcA*::*bleo*; *argB2*::P_{acvA}-*uidA* P_{ipnA}-*lacZ*, *argB*$^+$; *pyrG89*/*pyr-4*$^+$ *cpcA*	this work
AGB189	*bga0*; Δ*cpcA*::*bleo*; *argB2*::P_{acvA}-*uidA* P_{ipnA}-*lacZ*, *argB*$^+$; *pyrG89*/*pyr-4*$^+$ 6xP_{alcA}::*cpcA*	this work

* Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, KS, USA).

3.4 Results

3.4.1 The *lysA* gene of *A. nidulans* shows up to 62% amino acid indentities to lysine-forming saccharopine dehydrogenases

The effect of amino acid starvation on the regulation of genes situated in the different branches of the bifurcated lysine/penicillin synthesis pathway was questioned. The only lysine biosynthesis gene isolated from *A. nidulans* to date encodes the homoaconitase LYSF acting upstream of the α-aminoadipate branch point. The *lysF* gene comprises an open reading frame of 2325 bp interrupted by one intron with a transcript size of about 2.7 kb (Weidner *et al.* 1997). Since no gene of the lysine-specific branch of the pathway was isolated yet, a 7.5 kb genomic *Kpn*I-fragment containing the complete *A. nidulans lysA* locus was isolated from an *A. nidulans* genomic library by colony hybridisation with a *lysA* specific probe. DNA sequencing revealed that the *lysA* coding region spans 1673 bp including eight putative introns of 47 to 171 bp (Fig. 3.2A). Southern hybridisation experiments with genomic *A. nidulans* DNA digested with several restriction endonucleases suggested that *lysA* is a single copy gene in this fungus (data not shown). From the deduced peptide sequence of 375 amino acids (aa) a relative mass of 41 kDa was calculated for the gene product. The deduced polypeptide sequence showed high identities to NAD^+-dependent, L-lysine forming saccharopine dehydrogenases (SDH; EC 1.5.1.7) of fungal origin, like from *S. cerevisiae* (Ogawa *et al.* 1980), *Y. lipolytica* (Xuan *et al.* 1990), *C. albicans* (Garrad *et al.* 1994) and *S. pombe* (Gentles *et al.* 1995) (Fig. 3.2B). The identities of up to 62% strongly suggest that the saccharopine dehydrogenase (LYSA) encoding gene from *A. nidulans* was identified.

3.4.2 Transcript levels are increased for *lysA* but reduced for *lysF* by starvation-dependent activation of CPCA

Implication of the cross-pathway control on *lysF* transcription seemed conceivable since *in silico* analysis revealed one sequence within 250 bp of the promoter region (5'-TGAGTCT-3') that differed in only one position from the optimal Gcn4p binding motif and could thus possibly serve as CPRE. *lysF* transcript levels of mycelia grown under standard and amino acid limited conditions were compared (Fig. 3.3B). Amino acid starvation was induced by addition of the false feedback inhibitor 3-amino-1,2,4-triazole (3AT), which causes histidine starvation with subsequent induction of the cross-pathway control (Sachs 1996). Mycelia of wild-type strain A234 and the *cpcA* deletion strain AGB52 were cultivated in standard minimal medium with and without histidine limitation for 6 hours. Specific transcript levels in Northern hybridisation experiments were quantified and equalised to the rRNA signal intensities. The relative transcription level of the wild-type grown at standard conditions in minimal medium was set as one. As controls, transcription levels of the *hisB* (Busch *et al.* 2001) and *aroC* (Krappmann *et al.* 1999) amino acid biosynthesis genes and of *gpdA* were monitored (data not shown). When cells of

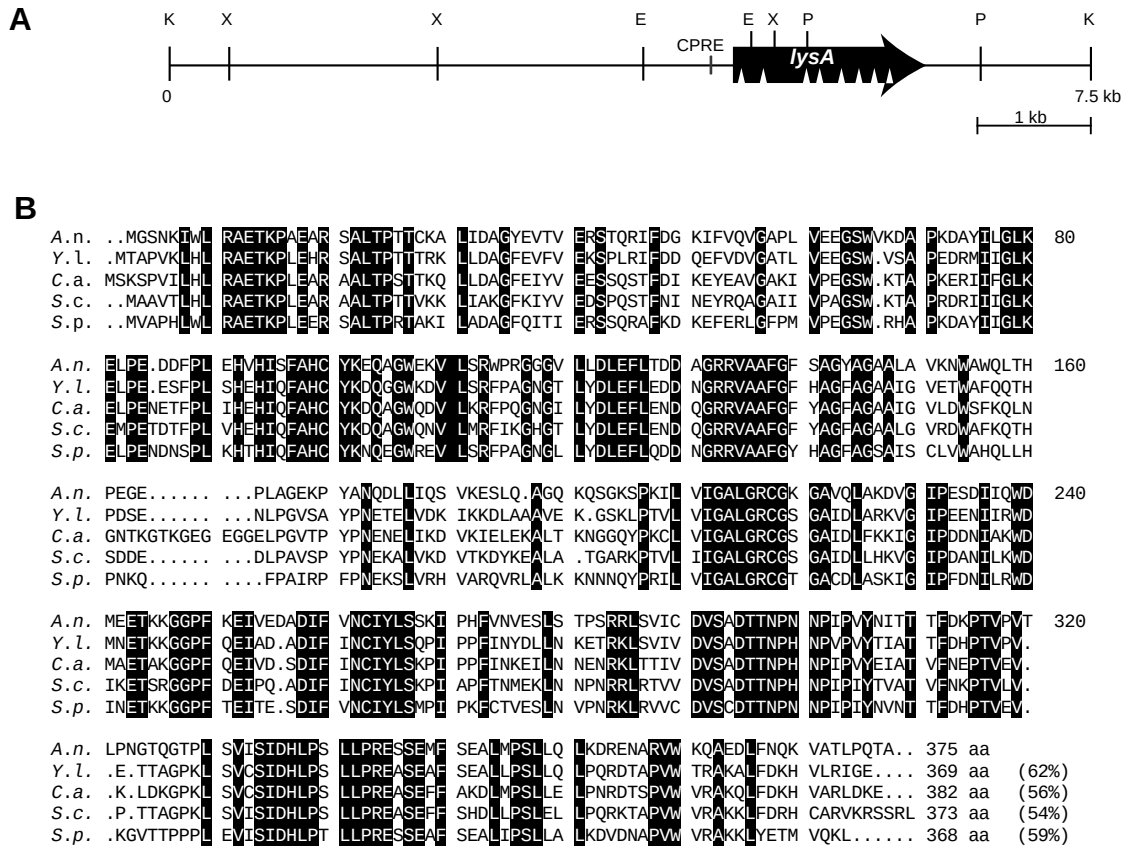


Fig. 3.2: The deduced peptide sequence of the *A. nidulans lysA* gene shows up to 62% amino acid identities to other fungal saccharopine dehydrogenases. (A) Schematic overview of the *lysA* locus in *A. nidulans*. Plasmid pME2162 contains the complete *lysA* coding region within a 7.5 kb genomic DNA fragment of *A. nidulans*. Introns are indicated by white triangles. CPRE indicates the proposed cross-pathway response element. Restriction sites of *Kpn*I (K), *Xba*I (X), *Eco*RI (E) and *Pst*I (P) are indicated. **(B)** The global multialignment of fungal saccharopine dehydratases of *A. nidulans* LYSA (*A.n.*; GenBank Accession Number AY057447), *Yarrowinia lipolytica* (*Y.l.*; P38997), *Candida albicans* (*C.a.;* P43065), *Saccharomyces cerevisiae* (*S.c.;* P38998) and *Schizosaccharomyces pombe* (*S.p.;* Q09694) shows a high conservation level with amino acid identities of 45%. Identical amino acids are indicated by black boxes. The percentage of identity of each single protein sequence to the *A. nidulans* (*A.n.*) *lysA* gene product is given in parentheses.

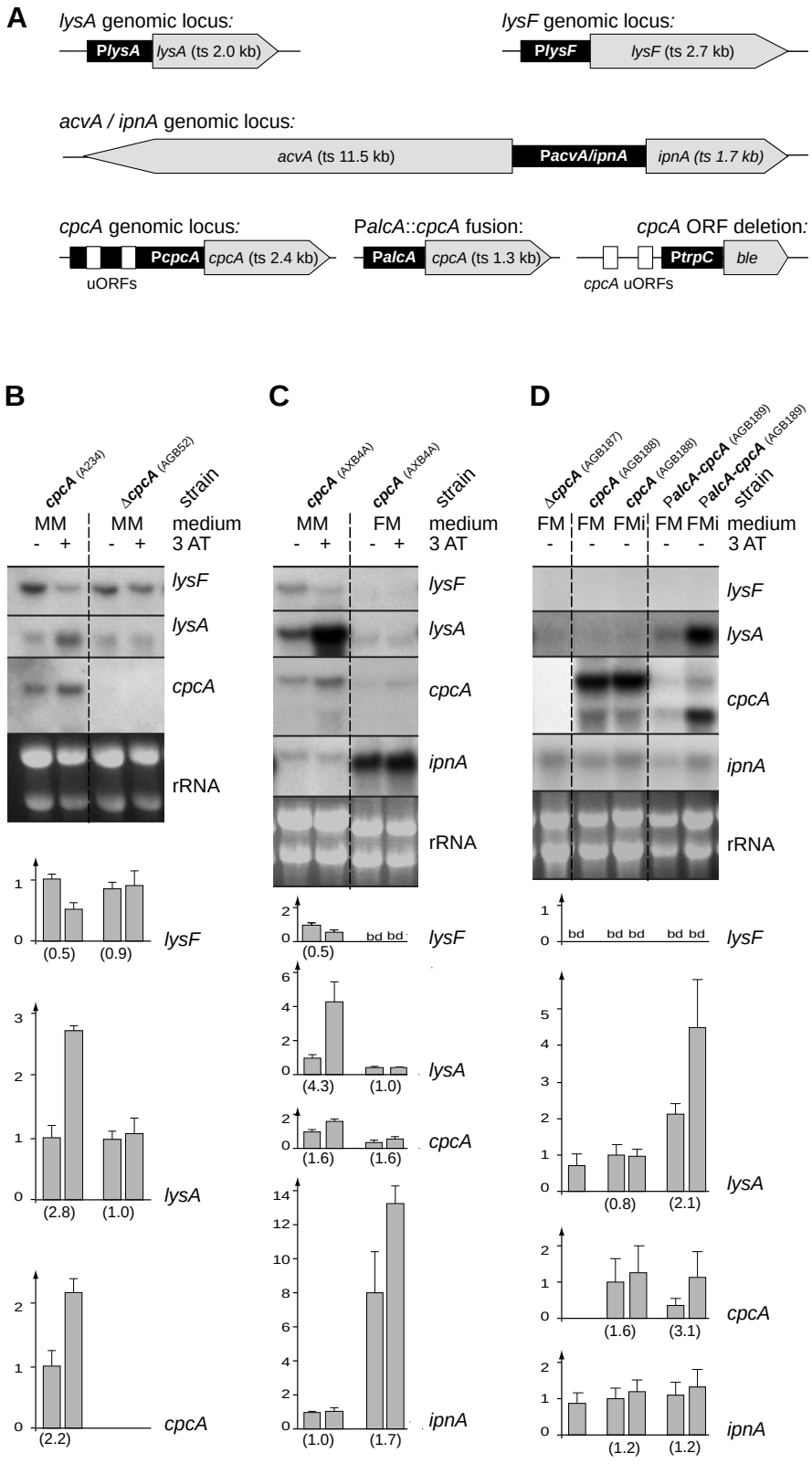
the *cpcA* wild-type strain A234 were starved for histidine, *cpcA* but not *lysF* mRNA levels were elevated (Fig. 3.3B). On the contrary, transcription of *lysF* was nearly halved compared to non-starvation conditions in the wild-type but not in the *cpcA* deletion strain. These data suggest that *lysF* as representative of the common stem of the pathway is not activated by the cross-pathway control system in response to amino acid starvation. The Northern hybridisation analyses revealed a *lysA* transcript size of about 2 kb. In minimal medium, transcription of *lysA* in the wild-type strain A234 is increased about threefold upon amino acid starvation when compared to non-starvation (Fig. 3.3B). Correspondingly, one potential CPRE perfectly matching the Gcn4p consensus motif was identified in the *lysA* promoter region (5'-TGACTCA-3') with the central C at position -174. The increase in *lysA* transcription levels during amino acid limitation seems to be primarily dependent on the transcription factor CPCA, because the *cpcA* deletion strain AGB52 is unable

to elevate *lysA* mRNA levels in response to this stress situation (Fig. 3.2B). Thus, *lysA* is a target gene of the cross-pathway control in *A. nidulans*.

3.4.4 Steady state levels of *lysF*, *lysA* and *cpcA* mRNA are reduced under penicillin production conditions

The two penicillin-specific genes *acvA* (MacCabe *et al.* 1990) and *ipnA* (Ramon *et al.* 1987) are divergently transcribed from a bidirectional promoter region (Fig. 3.3A). Significant expression of these genes and corresponding penicillin production require an appropriate lactose-based complex medium including corn steep solids and a prolonged incubation period of about 48 hours. Mycelia grown in the standard minimal medium with glucose as carbon and $NaNO_3$ as sole nitrogen source hardly express the penicillin biosynthesis genes (MacCabe *et al.* 1990; Then Bergh and Brakhage 1998). For transcriptional analyses, the *cpcA* wild-type strain *A. nidulans* AXB4A that contains reporter gene fusions to the *acvA-ipnA* intergenic region (Fig. 3.4A) was used to allow subsequent analysis of the corresponding penicillin gene expression from the same mycelia batch. In addition to growth under standard minimal conditions, the strain was cultivated under penicillin production conditions in fermentation medium for 48 hours. Transcript levels of specific *lysF*, *lysA* and *ipnA* mRNAs from mycelia derived from fermentation medium and minimal medium, respectively, were compared (Fig. 3.3C). The cultures grown in penicillin production broth showed significantly lower levels of *lysA* and *lysF* mRNAs than cultures grown in minimal medium. Transcript levels were nearly halved for *lysA* and hardly detectable for *lysF*. The

Fig. 3.3: Transcriptional regulation of *lysF*, *lysA* and *ipnA* in response to amino acid supply and functionality of the cross-pathway control. The transcriptional level of specific mRNAs was analysed in Northern hybridisation experiments with RNA isolated from three independent cultures each. Mean signal intensities were equalised with respect to rRNA levels due to the lack of an established internal reference gene constitutively transcribed at all conditions used in this study. Signal intensities were set as one for the *cpcA* wild type strains. The values given in parentheses indicate the relative change in transcriptional levels between the corresponding conditions. The mean standard deviation did not exceed 20%. Growth conditions: Mycelia of overnight cultures were transferred into fresh medium and then grown for 3 h or 40 h in minimal or fermentation medium prior to addition of 10 mM or 50 mM 3-aminotriazole, respectively. Mycelia were harvested 8 h later so total growth time was 11 h or 48 h in minimal or fermentation medium (unless stated otherwise). Abbreviations: 3-aminotriazole (3AT), below detection limit (bd), P*alcA*-induction in fermentation medium (FMi), fermentation medium (FM), minimal medium (MM), phleomycin resistance marker (*ble*), promoter (P), transcript size (ts), upstream open reading frame (uORF). **(A)** The transcriptional units of *lysA*, *lysF* (Weidner *et al.* 1997), *acvA* (MacCabe *et al.* 1990), *ipnA* (Perez-Esteban *et al.* 1993) and *cpcA* (Hoffmann *et al.* 2001) as well as the different *cpcA* constructs are shown. Transcription sizes are given in parentheses. **(B)** Comparison of *lysA* and *lysF* transcript levels at 3AT-induced amino acid starvation (after 6 h) in the wild-type strain A234 and the *cpcA* deletion strain AGB52. **(C)** Comparison of 3AT-induced amino acid starvation on transcriptional levels in minimal and fermentation medium in strain AXB4A which is wild-type in respect to *cpcA*. **(D)** Comparison of transcriptional levels in isogenic *A. nidulans* strains with different internal levels of CPCA. Different transcript sizes of *cpcA* in the overproduction strain result from transcription controlled by the *alcA* promoter.



amount of *cpcA* transcript is reduced in fermentation medium by about 30%. As expected, transcription of *ipnA*, representing one of the two genes for the initial steps in penicillin biosynthesis, is approximately eight times higher in fermentation than in minimal medium.

To study the effect of amino acid starvation on penicillin biosynthesis under penicillin production conditions, the concentration of 3AT which induced histidine

starvation in the complex fermentation medium was defined by determining increased transcription of *cpcA*, encoding the cross-pathway regulator. At 50 mM 3AT an 1.5 fold increase of *cpcA* mRNAs was measured which corresponds to the 1.5 fold increase of the same transcript in minimal medium supplemented with 10 mM 3AT. The *cpcA* wild-type strain AXB4A was cultivated in fermentation medium for 40 hours and further incubated for 8 hours upon 3AT-induced histidine depletion. However, amino acid limitation monitored by *cpcA* mRNAs increased *lysA* transcription in minimal medium fourfold whereas transcription of *lysA* is about equal in fermentation medium regardless of histidine availability (Fig. 3.3C). *ipnA* mRNAs were significantly elevated by 3AT-addition in fermentation but not in minimal medium. These data suggest that in fermentation medium, a more careful analysis of the impact of the cross-pathway control is required with various defined levels of CPCA protein within the cell.

3.4.3 CPCA overproduction decreases expression of the penicillin-specific genes as well as the penicillin titre

To further analyse gene expression of the two penicillin biosynthesis genes, activities of reporter gene fusions of the divergent promoter region of *ipnA* and *acvA* (Fig. 3.4A) in *A. nidulans* strain AXB4A were measured. Crude protein extracts of mycelia grown in minimal and fermentation media with and without 3 AT-addition were isolated for quantification of specific reporter gene activities. In agreement with the results of the Northern hybridisation experiments, *ipnA* reporter gene expression was threefold higher in fermentation than in minimal medium (Fig. 3.4B). Expression of *acvA* was even increased by a factor of eight upon penicillin production conditions. Whereas the Northern hybridisation results suggested that 3AT-addition in fermentation medium slightly increased *ipnA* expression, both penicillin biosynthesis genes fused to reporter constructs showed even slightly reduced activities under these conditions. It remains to be elucidated whether an additional control mechanism besides transcriptional regulation is acting under these circumstances.

3AT-induced histidine starvation results in increased expression of the central transcription factor of the cross-pathway regulatory network, CPCA (Hoffmann *et al.* 2001). Isogenic *A. nidulans* strains were constructed that contain the penicillin reporter genes P*acvA*-*uidA*, P*ipnA*-*lacZ* (AGB188) as well as deletion (AGB187) or overexpression (AGB189) of the cross-pathway transcription factor. In AGB189, *cpcA* was fused to the *alcA* promoter (P*alcA*-*cpcA*) which allowed to modulate CPCA expression independently of the presence or absence of amino acids. P*alcA* is a well characterised filamentous fungal promoter (Waring *et al.* 1989; Mathieu and Felenbok 1994; Felenbok *et al.* 2001), and an expression system had been established for its use in the complex *A. nidulans* penicillin production broth. Without rendering the fermentation medium composition, this promoter shows only modest activity and can be induced by 10 mM cyclopentanone (Kennedy and Turner 1996). In Northern hybridisation experiments (Fig. 3.2D), *lysA* as a CPCA target gene was used to monitor the activity of the cross-pathway control

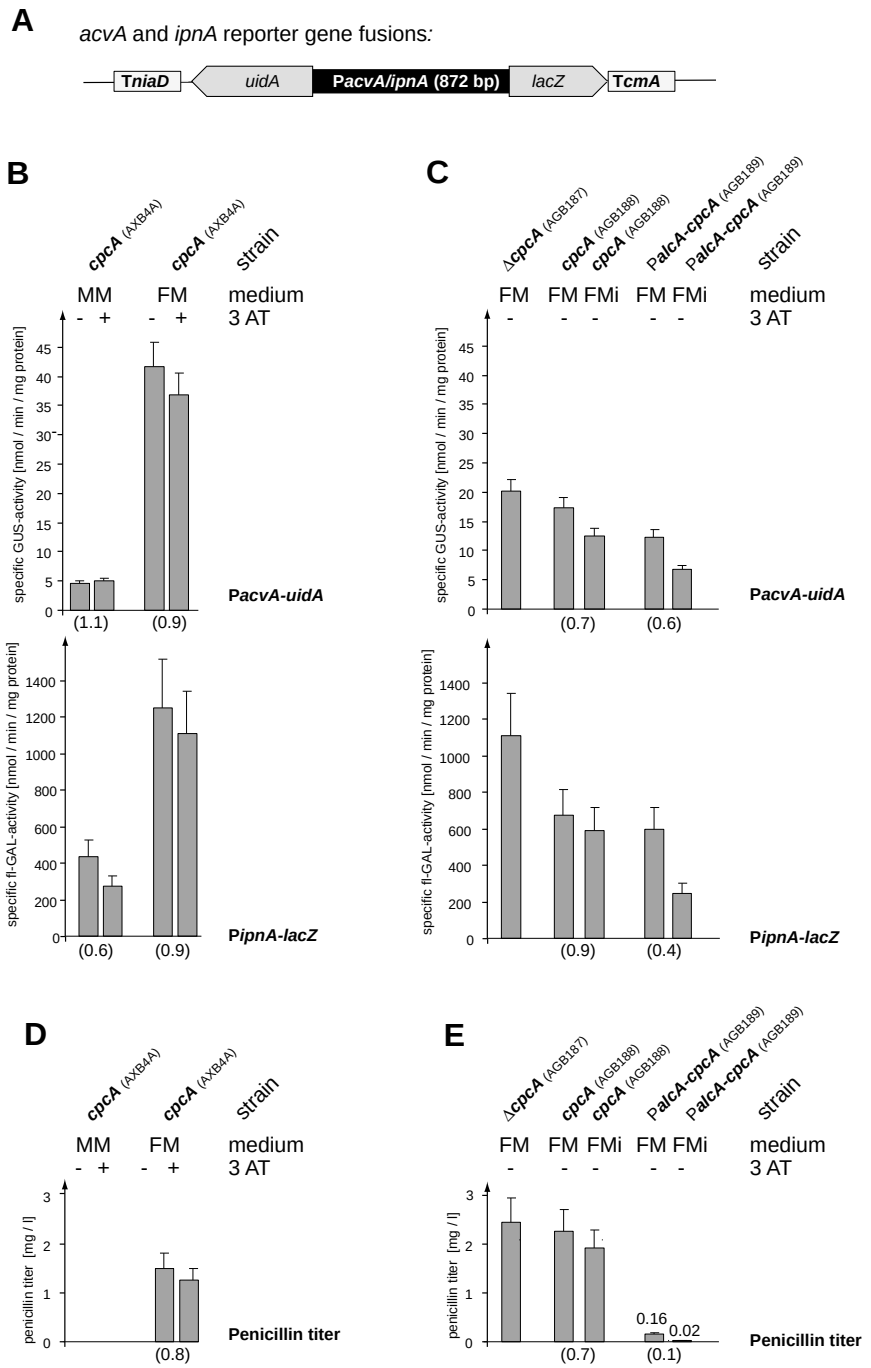


Fig. 3.4: Expression of the penicillin biosynthesis genes and the penicillin titre are decreased at high internal CPCA levels. The specific activity of β-glucuronidase (GUS) and β-galactosidase (β-GAL) as well as the penicillin titre are shown. The values given in parentheses indicate the relative change in reporter gene expression or penicillin production levels between the corresponding conditions. The mean deviation did not exceed 21%. See legend to Fig. 3.2 for growth conditions and abbreviations. **(A)** *A. nidulans* strain AXB4A and the isogenic strains AGB187, AGB188 and AGB189 contain reporter gene fusion for the penicillin genes *acvA* (P*acvA-uidA*) and *ipnA* (P*ipnA-lacZ*) integrated at the *argB* locus (Brakhage et al. 1992). **(B)** Specific activities of the reporter genes are shown with respect to 3AT-induced amino acid starvation **(C)** and internal CPCA level. Additionally, the supernatant of the cultures was tested in a penicillin bioassay for antibiotic activity against the penicillin sensitive strain *B. stearothermophilus* **(D)** with respect to 3AT-induced amino acid starvation **(E)** and internal CPCA level.

since *cpcA* expression is subject to strong post-transcriptional regulation (Hoffmann *et al.* 2001), The *cpcA* deletion strain AGB187 showed lowest *lysA* mRNA levels and no *cpcA* transcript (Fig. 3.3D). Strain AGB188 showed low levels

of *cpcA* transcript corresponding to the single wild-type copy and slightly increased *lysA* mRNA levels. Strain AGB189 exhibited low P*alcA*-driven *cpcA* mRNA levels but threefold increased *cpcA* transcript levels when the *alcA* promoter was induced. Accordingly, *lysA* transcript levels were increased. Thus, the strains AGB187, AGB188, AGB189 and AGB189 grown under cyclopentanone-induced conditions show increasing activity of the cross-pathway control which becomes prominent in successively increased transcription of *lysA* (Fig. 3.3D). The specific *ipnA* mRNA levels are hardly affected by changes in the CPCA concentration of the cell suggesting that the cross-pathway control does not regulate this penicillin biosynthesis gene on transcriptional level. In contrast to the mRNA levels, expression of the *ipnA-lacZ* reporter gene varied dependent on CPCA in the cell. The highest expression was found in the *cpcA* deletion strain AGB187 (Fig. 3.4C). Overexpression of CPCA reduced *ipnA-lacZ* activity by more than twofold in AGB189. For the other penicillin biosynthesis gene, *acvA*, a similar CPCA dependent expression pattern was found when measured as activity of an *acvA-uidA* gene fusion.

The discrepancies between *ipnA* transcript levels which were even slightly increased in penicillin producing fermentation medium during 3AT-induced amino acid starvation and *ipnA-lacZ* expression which was reduced when CPCA was overexpressed, prompted us to analyse the penicillin titre under various conditions. In minimal medium, penicillin production is repressed by glucose whereas the penicillin titre in fermentation medium is significant (Brakhage *et al.* 1992) and only slightly reduced when 3AT is added (Fig. 3.4D). Increased internal CPCA levels resulted in significantly decreased penicillin production (Fig. 3.4E). The CPCA overproduction strain AGB189 produces an order of magnitude less penicillin in comparison to *A. nidulans* strains with low or no CPCA levels. Though we cannot rule out side effects of the P*alcA*:*cpcA* ectopic integration in strain AGB189, the significant decrease of penicillin production upon P*alcA*:*cpcA* induction compared to non-induction counts for a real effect. Therefore, these data demonstrate that histidine starvation and accordingly an activated cross-pathway control increased transcription of *lysA*, a lysine biosynthesis gene acting downstream of the α-aminoadipate branch point, but does not activate the penicillin specific genes which channel this intermediate towards antibiotic biosynthesis. On the contrary, an even reduced expression of the penicillin biosynthesis genes and an even lower penicillin titre was found.

3.5 Discussion

After sharing a common pathway, lysine and penicillin biosyntheses separate at α-aminoadipate and therefore have to compete for this intermediate. This work addressed the question whether stress induced by amino acid starvation impacts the regulation of the two branches. In synthetic *A. nidulans* minimal medium, amino acid starvation and subsequent activation of the cross-pathway control can be artificially induced by addition of the amino acid analogue 3AT (Sachs 1996).

Corresponding transcriptional analyses in this work revealed *lysA* as an activated target gene of the cross-pathway control, but not *lysF* and *ipnA*. The 5'-regulatory region of *lysA* contained one 7 bp sequence that perfectly matched the Gcn4p target motif (Oliphant *et al.* 1989; Mavrothalassitis *et al.* 1990). By contrast, in the promoter region of *lysF* and in the *acvA-ipnA* intergenic region only nucleotide stretches differing with 1 or 2 bases from the consensus were identified. These findings indicate a direct regulation of *lysA* transcription by CPCA in response to amino acid starvation and suggest that at least the lysine-specific branch of the pathway is part of the cross-pathway network. Thus, elevated *lysA* transcription levels seem necessary to provide lysine when amino acids are limited. Similar results were obtained for *P. chrysogenum*, where the last two steps of the lysine-specific branch are subjected to the cross-pathway control, and α-aminoadipate reductase is additionally included in this regulatory process in high production strains (Hönlinger *et al.* 1988).

It was shown here that artificial induction of the cross-pathway control by 3AT in penicillin production broth caused several side effects and thus this method seems restricted for use in fermentation medium. In this complex medium, corn steep solids mediate increased expression of the penicillin specific genes and finally a higher penicillin titre (MacCabe *et al.* 1990). Transcription levels of *lysA* and, astonishingly, also of *lysF* are significantly reduced in this medium. Thus, penicillin production seems not dependent on elevated *lysF* transcription, though its gene product is involved in the biosynthesis of α-aminoadipate. There are several possible explanations for this. Since the final reaction of penicillin biosynthesis recycles α-aminoadipate, this compound might be re-utilized for another round of penicillin biosynthesis. Alternatively, lysine might be degraded to α-aminoadipate to refill the pool required for the antibiotic biosynthesis. The α-aminoadipate pool size which is critical for the rate of penicillin formation in *P. chrysogenum* (Jaklitsch *et al.* 1986; Hönlinger and Kubicek 1989; Lu *et al.* 1992) might be less important for *A. nidulans* penicillin biosynthesis (Brakhage and Turner 1992). Consistent with our findings for *lysF* regulation in *A. nidulans*, expression of the first gene of the pathway's common stem in *P. chrysogenum* is reduced during the penicillin production phase. Additionally, overexpression of the corresponding gene did neither result in an increased α-aminoadipate pool nor in higher penicillin production though homocitrate synthase is thought to be the rate-limiting step (Banuelos *et al.* 1999; Banuelos *et al.* 2000).

This work revealed a significantly lowered transcriptional level of *cpcA* in the penicillin production medium. The effect of the cross-pathway transcriptional activator in complex broth was analysed in *A. nidulans* strains with genetically altered *cpcA* expression where the activity of the cross-pathway control can be easily monitored by the transcriptional level of the target gene *lysA*. Significantly, a change in *lysA* transcriptional levels by factor two is accompanied with a decrease of the penicillin titre by about an order of magnitude. In contrast, mRNA levels of *ipnA* remain fairly constant irrespective of the cross-pathway activity. The corresponding *ipnA* reporter gene activities show a significant increase at highest cross-pathway activity only. These results suggest that there are differential effects

of an activated cross-pathway system on penicillin biosynthesis in the penicillin production broth. Since transcription and reporter gene activity of penicillin genes and the corresponding penicillin titre do not correlate, these effects seem primarily mediated by post-transcriptional and post-translational events. Such effects have also been suggested for other aspects concerning regulation of β-lactam biosynthesis in filamentous fungi where discrepancies between gene expression and corresponding enzyme activity were observed (Brakhage 1998).

The reducing effects of an activated cross-pathway control on penicillin production could be mediated by different ways. Assuming that this effect is not due to a problem in penicillin export, two major explanations are conceivable. **(I)** CPCA probably increases lysine biosynthesis which could result in shortage of the α-aminoadipate pool available for penicillin biosynthesis which cannot be compensated by the activation of α-aminoadipate biosynthetic genes as *lysF*. This hypothesis is in agreement with findings in *P. chrysogenum* where high penicillin producing strains show lower turnover from α-aminoadipate to lysine than low producing strains (Jaklitsch *et al.* 1986; Hönlinger and Kubicek 1989). Additionally, deletion of the aminoadipate reductase encoding gene, acting directly downstream of the α-aminoadipate branch point, doubles the penicillin titre via the increased α–aminoadipate pool (Casqueiro *et al.* 1999). On the basis of a doubled transcription rate of *lysA* at *cpcA* overexpression, and assuming that the whole lysine specific branch downstream of the α-aminoadipate branch point might be increased, the reduction of the penicillin titre might be due to the reduction of the α-aminoadipate pool. Though in contrast to *P. chrysogenum*, the significance of the intermediate's pool size is not yet known since external supply of α-aminoadipate cannot compensate the repressing effect of lysine on penicillin biosynthesis in *A. nidulans* (Brakhage and Turner 1992). However, this might be due to poor uptake of external α-aminoadipate by *A. nidulans* (Brakhage 1998). **(II)** The expression of CPCA could mediate an indirect effect on penicillin biosynthesis genes by interfering with other regulatory proteins. It seems possible that CPCA activates another regulator which in turn acts negatively on penicillin biosynthesis enzymes. In yeast it was recently shown that 3AT-induced amino acid starvation which acts through the CPCA homologue Gcn4p modulates the activity of at least 26 different transcription factors and 11 protein kinases including regulators of amino acid biosynthesis, nitrogen utilisation and TCA cycle intermediates (Natarajan *et al.* 2001). It was shown that 3AT-induced starvation and CPCA overexpression also impacts the sexual development of *A. nidulans*, indicating a complex range of action of CPCA not only restricted to amino acid metabolism (Eckert *et al.* 2000; Busch *et al.* 2001). It is thus likely that CPCA does not directly act on the penicillin biosynthesis genes but on a yet unknown mediator.

In summary, a cross-talk was identified between the regulation of primary- and secondary metabolism mediated at least partly by the cross-pathway control. CPCA does not increase the penicillin biosynthesis pathway of *A. nidulans*. Moreover, the results suggest that under conditions resulting in an activated cross-pathway control, penicillin synthesis is reduced and the lysine specific branch is favoured over the penicillin specific branch at the α-aminoadipate branch point.

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Chapter 4

The COP9 signalosome is an essential regulator of development in *Aspergillus nidulans*

4.1 Abstract

The COP9 signalosome (CSN) was identified as an essential regulator of development resulting in cleistothecia, the sexual fruit bodies of the filamentous fungus *Aspergillus nidulans*. The deduced amino acid sequences of two genes, *csnD* and *csnE*, show up to 38% and 53% identities to the fourth and fifth CSN subunits of higher eukaryotes. The *A. nidulans* CSND protein is accumulated in the nucleus and interacts with CSNE in a yeast two-hybrid assay. Deletion of either *csn* open reading frame resulted in viable strains with identical developmental phenotypes which could be complemented by the according wild-type genes. When grown on an air-medium interface, vegetative cells of *A. nidulans csn* deletion strains were impaired in cell polarity and showed changes in secondary metabolism resulting in the accumulation of a red pigment. The *csn* deletion strains showed disturbed light-dependence of developmental initiation processes, including constitutive repression of the onset of the asexual reproductive cycle in developmentally synchronised and competent mycelia. The Δ*csn* mutants were capable to initiate the sexual cycle and develop primordia of fruit bodies. However, the further maturation and morphogenesis of primordia to sexual fruit bodies was blocked. This developmental arrest could not be overcome by overexpression of the sexual activator velvet (VEA). We conclude that the COP9 signalosome, which is functionally located at the interface of signal-transduction and ubiquitin-dependent proteolysis, is a key regulator in *A. nidulans* which is essential for light-dependent signalling and subsequent sexual development.

4.2 Introduction

Prerequisites for development of multicellular eukaryotes are specific control mechanisms for the cell division cycle, cell specialisation, intercellular communication and numerous modifications of metabolic activities. Co-ordination of such diverse cellular processes requires multiple molecular cross-talk mechanisms. One important regulatory system is targeted protein degradation. The main machinery for controlled proteolysis in eukaryotes is the 26S proteasome, which exclusively degrades ubiquitinylated proteins (Tyers and Jorgensen 2000). Ubiquitinylation of the target proteins is mediated in an enzymatic cascade including E3 ubiquitin ligase complexes. The activity of E3 ubiquitin ligases towards its substrate proteins depends on the reversible conjugation of the

small ubiquitin-like protein Rub1/Nedd8 to their cullin subunit, a process termed neddylation (Ohh *et al.* 2002), and on the phosphorylation status of the substrate. A proposed modulator of E3 ubiquitin ligase activity is the constitutive photomorphogenesis complex 9 (COP9), termed COP9 signalosome (CSN). Several studies suggest that the CSN directly interacts with E3 ubiquitin ligases (Schwechheimer *et al.* 2001; Suzuki *et al.* 2002) and modulates E3 ubiquitinylation activity by at least two distinct enzymatic activities which are probably associated (Bech-Otschir *et al.* 2002): a deneddylation function contributes to deconjugation of Rub1/Nedd8 from the E3 ligase cullin subunit (Lyapina *et al.* 2001; Zhou *et al.* 2001; Yang *et al.* 2002), and a kinase function can phosphorylate E3 substrate proteins (Seeger *et al.* 1998; Bech-Otschir *et al.* 2001).

The COP9 signalosome, the lid of the 26S proteasome (LID) and the eukaryotic translation initiation factor 3 (eIF3), are regulatory multiprotein complexes whose components are characterised through the specific PCI (proteasome, COP9, eIF3) or MPN (Mpr1p, Pad1 N-terminal) protein domains (Glickman *et al.* 1998; Kapelari *et al.* 2000; Kim *et al.* 2001). The eight subunits of the COP9 signalosome are highly conserved in higher eukaryotes. In mammalian cells, the CSN is involved in several cellular processes, for example the control of hormone signalling and tumor growth, by regulation of c-Jun and p53 protein levels (Li *et al.* 2000; Pollmann *et al.* 2001). In insects and plants, the COP9 signalosome is an essential regulator of development and malfunction of the CSN results in a developmental block that leads to post-embryonic lethality: *Arabidopsis thaliana* seedlings with defect in the CSN show a constitutive photomorphogenic phenotype and die after several days (Wei *et al.* 1994), and mutant larvae of *Drosophila melangolaster* pupate but never undergo morphogenesis (Wei *et al.* 1994; Freilich *et al.* 1999). To date, six subunits of the COP9 signalosome have been identified in *Schizosaccharomyces pombe*, though the role of this complex in fission yeast is not essential and seems restricted to the time control of the cell cycle and sensitivity towards ultraviolet light (Mundt *et al.* 1999; Mundt *et al.* 2002). In *Saccharomyces cerevisiae* the CSN seems not to exist in this form, only a protein with vague similarity to subunit 5 was found (Wei and Deng 1999).

The filamentous ascomycete *Aspergillus nidulans* is a model organism for the study of regulatory networks that control metabolic pathways, differentiation and development in eukaryotes. In passing through different phases of growth and development, this fungus establishes highly specialised cell types and produces various secondary metabolites. After germination, multinucleate hyphae are formed. They require a minimum time of 16 to 20 h of vegetative growth to achieve developmental competence, influenced by different physical factors as temperature and cell density. From this time point on, the mycelium is susceptible to environmental signals and subsequent induction of two propagation cycles (Axelrod *et al.* 1973; Yager *et al.* 1982). The asexual reproductive unit is the conidiophore, which forms green-pigmented conidiospores about 24 h after induction (Adams *et al.* 1998). The reproductive structures of the sexual cycle, the cleistothecia, require about 80 h post-induction time for maturation and harbour red-pigmented ascospores. Cleistothecia constitute the most complex

developmental architecture of *A. nidulans*, and since mutant strains defective in their sexual reproduction are still viable, *A. nidulans* is particularly suited for studies on sexual development (Braus *et al.* 2002). The first morphological evidence for sexual propagation are small hyphal aggregates, termed nests. These include specialised hyphae producing globose Hülle-cells, probably serving as nurse cells. Other specialised hyphae form spherical structures within the nests, the cleistothecial primordia. One step beyond, a first separation of surrounding hyphae embracing an inner hyphal mass becomes evident in the micro-cleistothecia. Several layers of the surrounding hyphae, flat and highly branched, merge to a continuous and hard, cleisthin-glued envelope, the cleistothecial wall. Within this protecting shell, specialised ascogenous hyphae eventually give rise to ascospores. To our knowledge, the precise timing of the single steps that finally lead to production of ascospores it is not known to date. However, specialised ascogenous hyphae fuse. Subsequent crozier formation and synchronised mitosis result in dikaryotic, binucleate ascus mother cells. These develop after karyogamy, meiosis and two rounds of mitosis into asci that enclose eight binuclear ascospores. A mature *A. nidulans* cleistothecium is up to 200 µm in diameter and contains approximately 80000 red-pigmented ascospores (Hermann *et al.* 1983; Kirk and Morris 1991; Champe *et al.* 1994; Wu and Miller 1997).

Studies on molecular regulation of the reproductive cycles in *A. nidulans* revealed several transcription factors specific for development. Regulation of asexual propagation (Adams *et al.* 1998) is primarily driven by a cascade of transcriptional activators, with two of them as the main players: BRLA is essential for a developmental switch from apical growth to swelling of the vesicle and ABAA is required for the switch from sterigmata budding to formation of conidiospores. They trigger the expression of downstream structural genes required for asexual development. By contrast, sexual development seems not to be attributed to a single sequential regulatory pathway (Braus *et al.* 2002). Several acleistothecial *A. nidulans* strains defective in distinct developmental steps could be isolated. So far, four transcription factors essential for sexual development have been characterised. No visible sexual structures are produced in *nsdD* (never in sexual development) (Han *et al.* 2001), or *stuA* (stunted) mutant strains (Wu and Miller 1997), whereas mutants of *steA* (sterile) or *medA* (medusa) are acleistothecial but do produce the auxiliary Hülle cells (Clutterbuck 1969; Vallim *et al.* 2000). Notably, *stuA* and *medA* mutant strains additionally produce aberrant conidiophores due to altered spatiotemporal expression of *abaA* and/or *brlA* (Miller *et al.* 1992; Busby *et al.* 1996). Several *A. nidulans* strains defective in regulation of amino acid biosynthesis exhibit a reversible block at the level of micro-cleistothecia due to elevated levels of the cross-pathway transcriptional activator CPCA (Eckert *et al.* 2000; Hoffmann *et al.* 2000; Busch *et al.* 2001). Furthermore, several mutants with regard to karyogamy, meiosis and ascosporogenesis can build cleistothecial shells which are empty (Swart *et al.* 2001).

Knowledge about the regulatory system that drives the initial developmental decisions in *A. nidulans* is scarce. The *veA* gene product connects light as external induction signal with development. Light in combination with aeration as another

external signal direct the differentiation towards the asexual cycle, whereas absence of light and increased partial pressure of carbon dioxide favour sexual propagation (Han *et al.* 1994; Timberlake and Clutterbuck 1994). Strains carrying the mutant *veA1* allele (many commonly used laboratory strains) abolish light-dependence of conidiation and constitutively produce 20fold more conidia and 5fold less cleistothecia. The time course of sexual development is about doubled in *veA1* mutant strains (Mooney and Yager 1990; Champe *et al.* 1994). Additionally, hormone-like signal molecules like the Psi factors (precocious sexual inducer) (Champe *et al.* 1987; Champe and el-Zayat 1989; Lewis and Champe 1995), the FluG factor (Yager *et al.* 1998) and auxin (Eckert *et al.* 1999) affect the developmental capabilities of the fungus.

With *csnD* and *csnE*, two components of the COP9 signalosome of *A. nidulans* were identified in this study. Deletion of either *csn* gene resulted in viable strains impaired in induction of development and maturation of primordia to cleistothecia. This first description of the COP9 complex in a filamentous fungus can serve as a basis in studying the regulatory function in development of this conserved multiprotein complex in an easily amenable, eukaryotic microorganism.

4.3 Experimental Procedures

4.3.1 Growth conditions

A. nidulans strains were cultivated at 37°C in or on minimal medium (50 mM glucose, 70 mM $NaNO_3$, 7 mM KCl, 11.2 mM KH_2PO_4 (pH 5.5), 2 mM $MgSO_4$, trace elements) (Bennett and Lasure 1991) supplemented as described (Käfer 1977). Vegetative mycelia were obtained from submerged liquid culture. Development tests were performed on agar plates with either confluent or point-inoculated ascospores or with mycelia that were pre-grown in submerged culture to synchronise the cell material at the stage of developmental competence. Development was allowed by an medium-air interface. We induced asexual sporulation by incubation on non-sealed plates in continuous white light and cleistothecia formation via oxygen-limiting conditions on tape-sealed plates in the dark (Clutterbuck 1974). Strains of *Saccharomyces cerevisiae* were grown at 30°C under non-selective conditions in YPD or in selective YNB media supplemented as described (Guthrie and Fink 1991).

4.3.2 *A. nidulans* physiological studies

Radial growth tests were performed with about 500 conidiospores centred on an agar plate. Colony growth was recorded as colony diameter. For conidiospore quantification (Bussink and Osmani 1998), 4 ml warm minimal medium containing 0.6% agar and about 1×10^6 spores were poured on solid medium. After 48 h, the top layer was excised with the end of a 1 ml tip (about 1 cm^2), placed in 0.5 ml saline and vortexed for 30 min before spores were counted in a hemacytometer.

4.3.3 Molecular methods

E. coli, *S. cerevisiae* and *A. nidulans* transformations were performed as described (Inoue *et al.* 1990; Elble 1992; Eckert *et al.* 2000). To obtain homogenised cell material, *A. nidulans* mycelia were filtered through sterile miracloth (Calbiochem, LaJolla, CA USA), frozen in liquid nitrogen and pulverised with a mortar and pestle. RNA was isolated from a volume of 100 µl ground mycelia mixed with 1 ml Trizol™ (Gibco BRL Life Technologies, Gaithersburg, MD, USA) as recommended by the manufacturer. Genomic DNA was isolated from a volume of 1 ml mycelia (Lee and Taylor 1990). Standard techniques were applied for Northern and Southern hybridisation experiments (Southern 1975; Rave *et al.* 1979) with ^{32}P-labelled probes employing the HexaLabel™ DNA Labelling Kit (MBI Fermentas). DNA for the *csnD* and *veA* probes was amplified by PCR of plasmids pME2343 and pME2352 with standard primers (T3/T7) and specific primers (SB85: 5'-*Xho*I-TCA TTG AAT TGA ACA TCT TCG-3' and SB86: 5'-*Hin*dIII-ATC GCG CCG GAT ACG GAC ACG-3'). DNA for the *gpdA* probe was cut from plasmid pAN5-22 (*Eco*RI/*Bam*HI). RNA quantities were standardised according to the 16S and 23S rRNA signals on the ethidium bromide-stained gel, quantified via the Image Station 440CF (Kodak, Rochester, NY, USA). Signal intensities of autoradiographies were quantified using a bioimager and MacBAS v2.5(E) from Fuji (Tokyo, Japan).

4.3.4 REMI mutagenesis

Restriction-enzyme mediated integration (REMI) (Schiestl and Petes 1991; Sanchez *et al.* 1998) of *A. nidulans* strain R99 was performed with 5 µg of vector pME1510. pME1510 was constructed from pAN8-1 (Punt and van den Hondel 1992) with the PCR amplified multiple cloning site of pBluescriptSK+ inserted into the blunt-ended *Eco*RI restriction site. It confers phleomycin resistance mediated by the *ble* gene from *Streptoalloteichus hindustanus*, which is expressed by the *gpdA* promoter and the *trpC* terminator. Selection for the *ble* gene was achieved by 20 mg phleomycin per l in the medium. About 4200 phleomycin-resistant colonies were generated with 5 to 20 U of the different restriction endonucleases *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII and *Kpn*I. Transformation efficiencies reached 150 transformants per µg of vector used. 50 mutants with fluffy, acleistothecial or hyper-cleistothecial phenotypes were isolated. Strain AGB37 contains a single integration of the vector in the genome. Backcrossing with the cleistothecial wild-type strain R99-6 (*pabaA1*) showed that the acleistothecial phenotype was linked to the phleomycin resistance. For plasmid rescue, 10 µg restriction enzyme-cut genomic DNA was religated and transformed into *E. co*li SURE cells (Stratagene, LaJolla, CA, USA). Plasmid pME1661 was rescued from AGB37 containing an insert of genomic DNA of 560 bp.

4.3.5 Isolation of genomic and cDNA of *csnD* and *csnE*

The REMI-rescued fragment of pME1661 was used to identify a 9 kb *Xho*I genomic fragment which was subcloned into pBKS. It contains the complete *csnD* open

reading frame in plasmid pME2338. A genomic fragment as probe for *csnE* was generated by PCR with primer pair SB102 (5'-*Xba*I-GAG CTA GAG AAT GCT GTT ACC CTG-3') and SB103 (5'-*Xba*I-CAA AGT CCT CGG CTT TGT TAA GCG-3') deduced from searches in the Monsanto Microbial Sequence Database for *Aspergillus nidulans* (http://microbial.cereon.com) and cloned into the vector pBSK as plasmid pME2234 via *Xba*I. Colony hybridisation of a genomic *Eco*RI sublibrary in pBSK with the pME2234 insert as a probe revealed a 6 kb genomic *Eco*RI fragment containing the complete *csnE* coding region in plasmid pME2237. cDNAs were generated with RT-PCR with a polyT-primer and specific primers (SB57: 5'-TAT GAA TCT AAT AGG TAC CAG AGA-3' and SB58: 5'-CAG GAA GAG AAT ATA GTT ACG AGT-3', SB123: 5'-TAT TCT CCA AAT CGA TAA TTA GC-3' and SB124: 5'-CCA TCC AAA TAG ATC TAT ACG G-3') for *csnD* and *csnE*, and ligated via TA-cloning into pBSK resulting in plasmids pME2364 and pME2363, respectively.

4.3.6 Construction of plasmids for *A. nidulans* manipulation

Plasmids used and constructed in this study are summarised in Table 4.1. For deletion of *csnD*, the 3.5 kb *Eco*RI/*Hin*dIII fragment of pME2338 was subcloned into pBluescript II. From the resulting vector pME1762 the flanking region was amplified via PCR with a specific primer pair (SB140: 5'-CGC AGA CGG AAC AAC AGT-3' and SB139: 5'-TTG AAT CAC AGC AGT GCA-3') and blunt end ligated to the 2.6 kb *Eco*RI/*Ssp*I *pyr-4* expression cassette of vector pRG3 to obtain the deletion cassette vector pME2342. For disruption of *csnE*, the 6 kb *Bam*HI/*Eco*RI fragment of pME2237 was subcloned into pUC19, the 5' part of the coding region was removed via *Cla*I/*Kpn*I and substituted by the 2.6 kb *Eco*RI/*Ssp*I *pyr-4* expression cassette of vector pRG3 via blunt end ligation resulting in vector pME2369. For complementation of Δ*csnD*, the 3 kb *Bam*HI/*Sal*I fragment of pME2338 was subcloned into pBSK, opened with *Bam*HI/*Xba*I and ligated with the 3.3 kb *Bgl*II/*Xba*I phleomycin-resistance cassette of pME1510, resulting in plasmid pME2345. Accordingly, for complementation of Δ*csnE*, the phleomycin-resistance cassette of pME1510 was subcloned with *Xba*I and *Xho*I into pBSK, opened with *Xba*I and blunt end ligated with the pME2237 *Eco*RI fragment resulting in plasmid pME2423. For overexpression from the *A. nidulans alcA* promoter, the *csnD* open reading frame was PCR-amplified from pME2338 with a specific primer pair containing *Kpn*I and *Bam*HI restriction sites (SB87: 5'-*Kpn*I-ATG CCA TCC CAA AAG ATA ATC TCC-3' and SB89: 5'-*Bam*HI-TCA ACG TAC CAG ATG GCC C-3') and cloned into pME1565 as plasmid pME2354 (P*alcA*:*csnD*:T*his2B*; *pyr-4*). Additionally, the *alcA* promoter of pME1565 (P*alcA*:MCS:T*his2B*; *pyr-4*) was substituted with the *niiA* promoter from pME2341 via *Eco*RI and opened with *Kpn*I to insert the *csnD*-PCR fragment with *Kpn*I sites in the specific primer pair SB87 (5'-*Kpn*I-ATG CCA TCC CAA AAG ATA ATC TCC-3') and SB125 (5'-*Kpn*I-TCA ACG TAC CAG ATG GCC C-3') resulting in plasmid pME2361 (P*niiA*:*csnD*; *pyr-4*). The complete *csnD* open reading frame was PCR-amplified from pME2338 (SB87: 5'-*Kpn*I- ATG CCA TCC CAA AAG ATA ATC TCC-3' and SB88: 5'-*Kpn*I- ACG TAC CAG ATG GCC CTC G-3') and fused in frame to the GFP into plasmid pMCB32

(Fernandez-Abalos *et al.* 1998) by using the inserted *Kpn*I sites downstream of the *alcA* promoter resulting in plasmid pME2353.

Tab. 4.1: Plasmids

plasmid	description	reference
pAN8-1	*bleo* resistance cassette (*amp*^{R}; P*gpdA*:*ble*:T*trpC*)	Punt and v.d. Hondel 1992
pBSK	pBluescript®II: cloning vector (*amp*^{R}; MCS)	Stratagene
pEG202	2-hybrid BD (*amp*^{R}; P*ADH*:*lexA*:T*ADH*, *HIS3*, 2µm)	Golemis and Brent 1996
pJG4-5	2-hybrid AD (*amp*^{R}; P*GAL1*:*B42*:T*ADH*, *TRP1*, 2µm)	Gyuris *et al*. 1993
pMCB32	GFP vector (*amp*^{R}; P*alcA*:*gfp*)	Fernandez-Abalos *et al*. 1998
pME1510	REMI vector (*amp*^{R}; pBSK-MCS; P*gpdA*:*ble*:T*trpC*)	this work
pME1565	overexpression (*amp*^{R}; *pyr-4*; P*alcA*-MCS-T*his2*)	this work
pME1661	REMI-rescue AGB37 (pME1510:500 bp insert)	this work
pME1762	*csnD* genomic (*Eco*RI/*Hin*dIII fragment of pME2338)	this work
pME2234	*csnE* probe (609 bp *csnE* PCR-fragment in pBSK)	this work
pME2237	*csnE* genomic (6 kb fragment *Eco*RI in pBSK)	this work
pME2338	*csnD* genomic (10 kb fragment *Xho*I in pBKS)	this work
pME2341	*niiA*/*niaD* promoter (*amp*^{R}; P*niia*-P*niaD*)	this work
pME2342	*csnD* deletion (*amp*^{R}; P_{csnD}:*pyr-4*:T_{csnD} in pME1762)	this work
pME2343	*csnD* probe (*Cla*I/*Not*I fragment of pME2338)	this work
pME2345	*csnD* complementation (P*gpdA*:*ble*:T*trpC*; *csnD*)	this work
pME2352	*veA* probe (500 bp *veA* PCR fragment in pBSK)	this work
pME2353	GFP fusion for *csnD* (P*alcA*::*csnD*::GFP)	this work
pME2354	*csnD*-overexpression (*amp*^{R}; *pyr-4*; P*alcA*-*csnD*-T*his2*)	this work
pME2355	*csnD*-bait (*amp*^{R}; P*ADH*:*lexA*:T*ADH*, *HIS3*, 2µm)	this work
pME2357	*csnD*-prey (*amp*^{R}; P*GAL1*:*B42*:T*ADH*, *TRP1*, 2µm)	this work
pME2361	*csnD*-overexpression (*amp*^{R}; *pyr-4*; P*niiA*-*csnD*-T*his2*)	this work
pME2363	*csnE* cDNA (in pBSK)	this work
pME2364	*csnD* cDNA (in pBSK)	this work
pME2369	*csnE* partial deletion (*pyr-4* in pSB50)	this work
pME2370	*csnE*-bait (*csnE*-cDNA *Eco*RI in pEG202)	this work
pME2371	*csnE*-prey (*csnE*-cDNA *Eco*RI in pJG4-5	this work
pME2423	*csnE* complementation (P*gpdA*:*ble*:T*trpC*; *csnE*)	this work
pRG3	*pyr-4* marker (*amp*^{R}; *pyr-4*)	Waring *et al*. 1989
pUC19	cloning vector (*amp*^{R}; MCS)	Vieira and Messing 1982

4.3.7 *A. nidulans* strain construction

Strains used and constructed in this study are summarised in Table 4.2. As wild-type strains, *A. nidulans* AGB152 and AGB10 were used for a *veA* and *veA1* genetic background, respectively. Both strains were transformed with vector pRG3 resulting in control strains AGB160 and AGB162. Deletion of *csnD* was achieved with the deletion cassette of the *Xba*I-linearised plasmid pME2342 in *A. nidulans* strains AGB152 and AGB10, resulting in Δ*csnD* strains AGB195 and AGB192, respectively. For deletion of *csnE*, the *Xba*I-linearised vector pME2369 was transformed into AGB152 resulting in strain AGB209. For complementation of the *csn* deletions, pME2345 was transformed into AGB195, AGB192 and AGB209, resulting in strains AGB203, AGB193 and AGB211, respectively. The *csnD* deletion was combined with the *veA* overexpression by transformation of plasmid pME2342 into strain AGB221, the latter one was constructed by a series of crossing experiments between OVAR5, DVAR1, A4 and GR5 (Tab. 4.2.). The *csnD* overexpression vectors pME2354 and pME2361 were ectopically integrated into *A. nidulans* wild-type strains AGB152 and AGB10 via the *pyr-4* marker resulting in strains AGB205 and AGB206, respectively. For localisation studies, ectopic integration of the GFP-

plasmid pME2353 in *A. nidulans* strain AGB152 resulted in strain AGB197. All mutant strains were back-crossed to the genetic wild-type strain for purification. Homologous integration of the marker expression cassettes and ectopic integration of plasmids were verified by diagnostic PCR and Southern hybridisation analyses.

Tab. 4.2: *A. nidulans* strains

strain	genotype	reference: construction
A4	glasgow wild-type	FGSC*
AGB10	*pyrG98*, *pyroA4*; *veA1*	(Eckert et al. 2000)
AGB37	*nsd*::*bleo*	this work: R99 + pME1510
AGB152	*pyroA4*, *pyrG98*	this work: A4 x GR5
AGB160	*pyroA4*, *pyrG98*/*pyr-4*$^+$	this work: AGB152 + pRG3
AGB162	*pyroA4*, *pyrG98*/*pyr-4*$^+$; *veA1*	this work: AGB10 + pRG3
AGB192	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnD*; *veA1*	this work: AGB10 + pME2342
AGB193	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnD*/*csnD*::*bleo*; *veA1*	this work: AGB192 + pME2345
AGB195	*pyroA4*, *pyrG98*/*pyr-4*$^+$ Δ*csnD*	this work: AGB152 + pME2342
AGB197	*pyroA4*; *pyrG98*/*pyr-4*$^+$ P*alcA*::*csnD*::*gfp*	this work: AGB152 + pME2353
AGB203	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnD*/*csnD*::*bleo*	this work: AGB195 + pME2345
AGB205	*pyroA4*; *pyrG98*/*pyr-4*$^+$ P*niiA*::*csnD*	this work: AGB152 + pME2361
AGB206	*pyroA4*; *pyrG98*/*pyr-4*$^+$ P*niiA*::*csnD*; *veA1*	this work: AGB10 + pME2361
AGB209	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnE*	this work: AGB152 + pME2369
AGB211	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnE*/*csnE*::*bleo*	this work: AGB209 + pME2423
AGB220	*pyrG98*/*pyr-4*$^+$ Δ*csnD*; Δ*argB*::*trpC*ΔB/*argB*$^+$ P*niiA*::*veA*; *trpC801*	this work: AGB222 + pME2342
AGB221	*pyrG98*; Δ*argB*::*trpC*ΔB; *trpC801*	this work: AGB152 x DVAR1
AGB222	*pyrG98*; Δ*argB*::*trpC*ΔB/*argB*$^+$ P*niiA*::*veA*; *trpC801*	this work: AGB221 x OVAR5
DVAR1	*yA2*, *pabaA1*; Δ*argB*::*trpC*ΔB/*argB*$^+$ Δ*veA*; *trpC801*	R. Fischer*
GR5	*pyrG98*, *pyroA4*; *veA1*; *wA3*	G. May*
OVAR5	*yA2*, *pabaA1*; Δ*argB*::*trpC*ΔB/*argB*$^+$ P*niiA*::*veA*; *trpC801*; *veA1*	R. Fischer*
R99	wild-type "arizona"	D. Geiser*
R99-6	*pabaA1*	D. Geiser*

* Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, KS, USA); Dave Geiser (Penn State University, University Park, PA, USA); Gregory May (University of Texas, Houston, TX, USA); Reinhard Fischer (Phillips-Universität Marburg, Germany).

4.3.8 Microscopy

Standard morphologic analyses were performed with a Stemi 2000-6 ZEISS binocular and an Axiolab ZEISS light microscope, using a KAPPA digital camera (DX30 ProgressiveScan) and the KAPPA ImageBase software for photography and calibration of magnifications (KAPPA opto-electronics). For GFP localisation, *A. nidulans* was grown over night in liquid P*alcA*-inducing medium and hyphae were microscoped by either differential interference contrast (DIC) or fluorescence microscopy using a GFP filter set (AHF Analysetechnik AG) under a Axiovert S100 ZEISS microscope. Standard DAPI filter sets were used for visualisation of nuclei stained with 4',6-Diamino-2-phenylindole (DAPI). Photographs were taken using a Xillix Microimager digital camera and the Improvision Openlab software (Improvision).

4.3.9 Two-hybrid analysis

The transcriptional activation of a reporter system with plasmids pEG202 and pJG4-5 was used to identify interacting proteins (Gyuris *et al.* 1993; Golemis and Brent 1996). The complete *csnD* open reading frame was PCR-amplified from pME2338 with specific primers containing flanking *Xho*I site (SB96: 5'-*Xho*I- ATG CCA TCC CAA AAG ATA ATC TCC-3' and SB97: 5'-*Xho*I- TCA ACG TAC CAG ATG GCC CTC-3'). Accordingly, the *csnE* cDNA sequence of plasmid pME2363 was amplified with primers flanked by an *Eco*RI site (SB135: 5'-*Eco*RI- ATG CAA GCT GCT CAA CTA TCC-3' and SB136: 5'-*Eco*RI-CTA AGT AGA CTC TAC CGT CTG TTT TC-3'). All PCR fragments were cloned into pEG202 and pJG4-5, resulting plasmids pME2355, pME2370, as 'baits' and pME2357, pME2371 as 'preys'. The constructs were transformed into the yeast two-hybrid reporter strain EGY48-p1840 (MATα, *his3*, *trp1*, *ura3-52*, *leu2*::*pLEU2-LexA*op6, *URA3*::*lacZ-LexA*op2) (Golemis and Brent 1996). For interaction tests, 1.5 ml of overnight yeast cultures were washed twice with saline, resuspended in 1 ml saline and 10 µl of an appropriate dilution was dropped on SC-plates containing 2% galactose as carbon source for the growth test. For β-galactosidase tests, the medium was supplemented with leucine (0.2 g/l) and covered with a filter paper. Cells were grown for 48 h at 30°C. The filter was lifted, shock frozen in liquid nitrogen and applied to a filter soaked with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) supplemented with 40 µl XGal (1% in DMF). The filters were incubated at 30°C and the reaction was stopped with Na_2CO_3 (1 M) after 1 to 24 h.

4.3.10 Sequence analyses

For DNA sequence analysis with custom oligonucleotides (Gibco BRL) the Big Dye™ Terminator kit of the Cycle Sequencing Reaction Mix from Perkin Elmer was used. Sequence analysis was performed on a Perkin Elmer Sequenator 310 with the ABI EditView 1.0.1 software. Further DNA analysis was performed with the Lasergene software from DNASTAR. Saccharopine dehydrogenase peptide sequences were identified using BLAST search of a non-redundant protein database (Altschul *et al.* 1990). Pairwise alignments and the multiple sequence alignment with hierarchial clustering was performed using the software "CLUSTALW" (Corpet 1988). Identification of protein motifs was performed *in silico* with PROSITE SCAN at http://hits.isb-sib.ch/cgi-bin/PFSCAN_parser. The nucleotide sequence of the genomic inserts of plasmids pME2338 and pME2237 containing the complete *csnD and csnE* coding regions have been deposited in the GenBank database under GenBank Accession Numbers AF236662 and AY126455, respectively.

4.4 Results

4.4.1 The *A. nidulans csnD* gene encodes a PCI domain protein similar to the fourth subunit of the COP9 signalosome

We have mutagenised the *A. nidulans* wild-type strain R99 by the restriction-enzyme mediated integration technique (REMI) to screen for phenotypic defects in the formation of cleistothecia, the sexual fruit bodies of this fungus. REMI is a tagging mutagenesis method aiming to disrupt genes by inserting a marker, which was in this case the *ble* gene that mediates resistance to phleomycin, into genomic restriction sites (Sanchez *et al.* 1998). Genetic analysis of one of the mutant strains, AGB37, showed linkage between the single vector integration event conferring phleomycin resistance and the acleistothecial phenotype as proven by Southern and backcrossing experiments. The corresponding plasmid rescued from this strain contained a 560 bp genomic insert. Using this insert as a probe in colony hybridisation experiments, we isolated a genomic 9 kb *Xho*I fragment containing an open reading frame of 1227 bp (Fig. 4.1A). Southern hybridisation

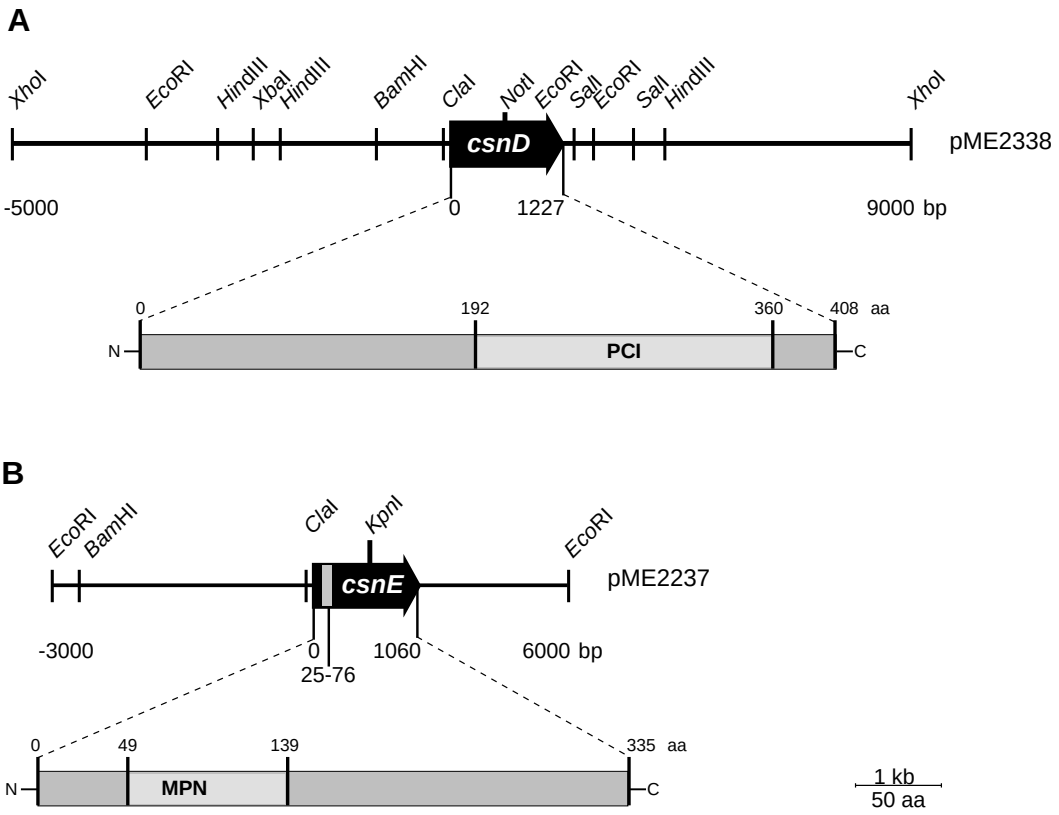

Fig. 4.1: The *A. nidulans* genes *csnD* and *csnE* encode PCI and MPN proteins typical for subunits of the COP9 signalosome. Schemes of the genomic loci of *csnD* (GenBank accession number AF236662) **(A)** and *csnE* (GenBank accession number AY126455) **(B)** are shown. The intron of *csnE* is indicated by a grey box. Predicted PCI (proteasome, COP9, eIF3) and MPN (Mpr1p, Pad1 N-terminal) motifs were identified from the deduced amino acid (aa) sequence by Prosite Scan.

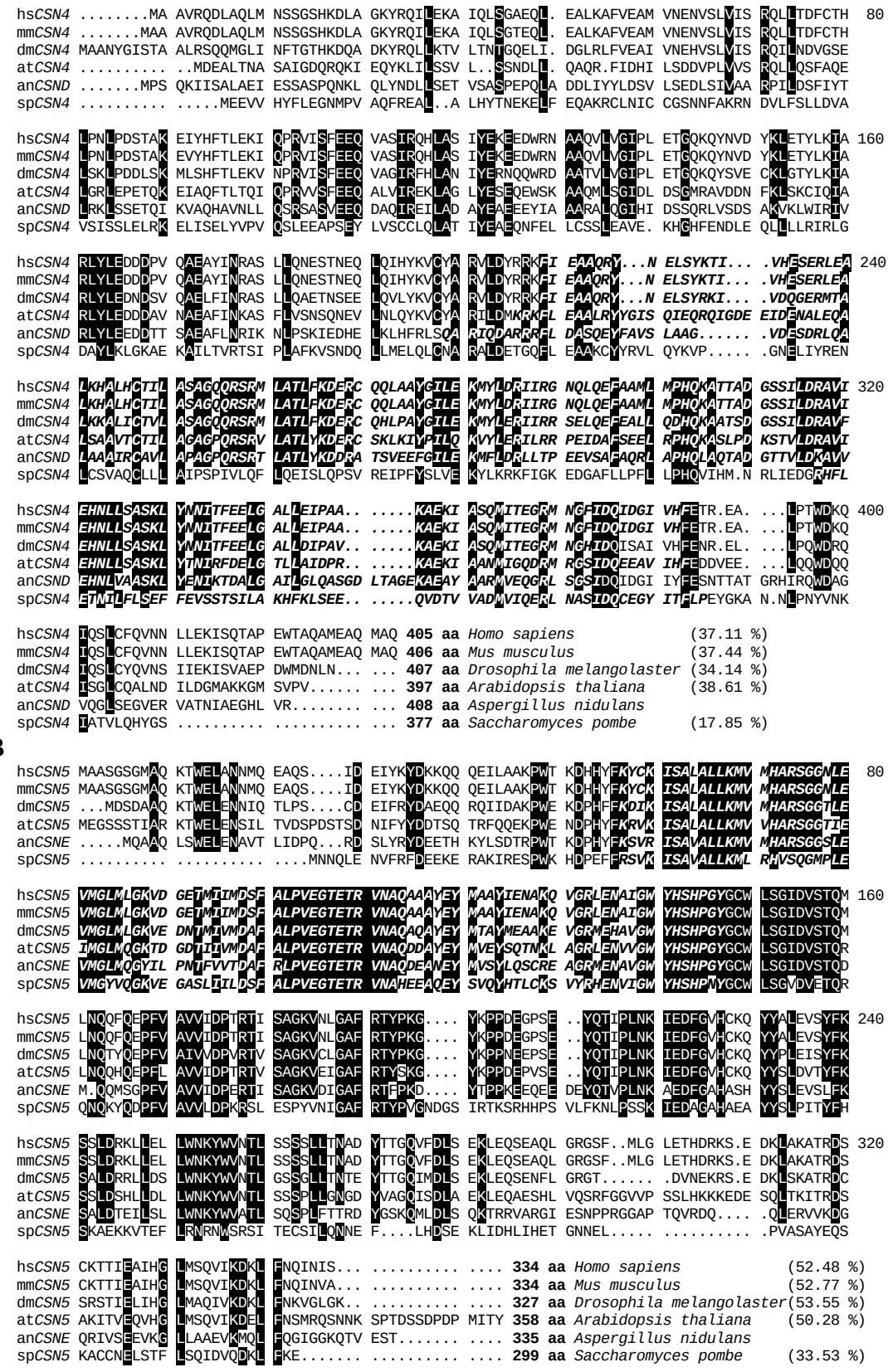

Fig. 4.2: CSND and CSNE show high amino acid identities to corresponding CSN subunits of higher eukaryotes. The multialignments of deduced amino acid sequences of *csnD* **(A)** and *csnE* **(B)** to the corresponding sequences *of Homo sapiens*, *Mus musculus*, *Drosophila melangolaster*, *Arabidopsis thaliana* and *Schizosaccharomyces pombe* were performed *in silico* using ClustalW. Residues identical in five of the sequences are highlighted. The predicted PCI and MPN domains as identified by Prosite Scan are indicated for each sequence by bold italic print. Percentage of single amino acid identities to the *A. nidulans* sequence is given in parentheses.

experiments suggest that the respective locus is present in a single copy in the *A. nidulans* genome (data not shown). A corresponding cDNA was isolated via RT-PCR and comparison of both genomic and cDNA nucleotide sequences revealed that the coding region is not interrupted by an intron. From the deduced peptide sequence of 408 amino acids a molecular mass of 44.9 kDa was calculated. The putative protein contained a predicted PCI domain (proteasome, COP9, eIF) and displayed identities of up to 38% to proteins described as the fourth subunit of the COP9 signalosome (CSN) from various organisms (Fig. 4.2A). According to the revised signalosome subunit nomenclature (Deng *et al.* 2000) the gene was named *csnD* (GenBank accession number AF236662). Identities were higher when CSND is compared to mammalia, vertebrates and plants than to the ascomycete *S. pombe*. The complete genome sequence of *S. cerevisiae* includes no orthologs of this subunit.

4.4.2 The *csnD* gene is constitutively transcribed through the entire life cycle of *A. nidulans* and its gene product is enriched in the nucleus

The COP9 signalosome is described as a regulator of developmental transitions in higher eukaryotes (Wei *et al.* 1994; Freilich *et al.* 1999). We thus analysed the presence of *csnD* transcript in *A. nidulans* in vegetative and differentiated cell material. Strain AGB162, which is wild-type for *csnD*, was grown in liquid minimal until developmental competence, transferred to solid minimal medium and induced either asexually on unsealed plates or sexually on tape-sealed plates. At harvest, the asexual cultures showed immature and mature conidiophores, respectively. Sexual cultures additionally differentiated Hülle cells, nests and mature cleistothecia. Northern experiments with RNA size standard revealed distinct *csnD* transcript signals of about 1.4 kb in length (Fig. 4.3). Equal amounts of RNA were loaded for each developmental state and *csnD* transcript levels were compared to the amount of rRNAs. Specific *csnD* mRNA overall signal intensities were comparably low but the ratios relative to the rRNA signals persist in the developing mycelia during all stages. As control, transcriptional levels of the metabolic house-keeping gene *gpdA*, encoding glyceraldehyde-3-phosphate dehydrogenase (Punt *et al.* 1990), were monitored which significantly decreased as soon as development was induced.

Further attention was drawn to the localisation of the CSND protein within the hyphal cell compartments by use of the *gfp*-encoded green fluorescent protein (Fernandez-Abalos *et al.* 1998). The complete *csnD* open reading frame driven by the strong inducible *alcA* promoter was fused in-frame to the *gfp* gene. This construct was ectopically integrated into the *A. nidulans* wild-type strain AGB152. Upon either ethanol or cyclopentanone induction of the *alcA* promoter on solid medium, the resulting strain AGB197 grew and differentiated both asexually and sexually like the wild-type (not shown). When mycelia were grown in submerged liquid culture under inductive conditions, bright signals were detected under the fluorescence microscope, which were absent in the wild-type strain (Fig. 4.4). Thus, this fusion protein consisting of the CSND subunit of the COP9 signalosome and

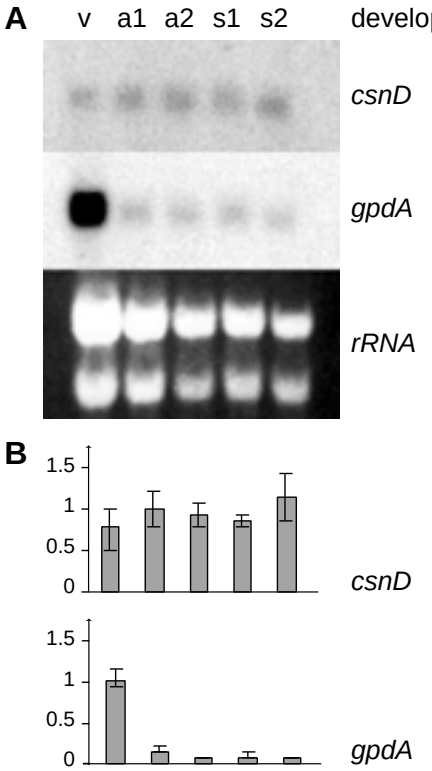

Fig. 4.3: Transcripts of *csnD* are expressed in vegetative and differentiated mycelia of *A. nidulans*. Mycelia of the *A. nidulans* wild-type strain AGB160 were developmentally synchronised by 18 h vegetative growth (v) in liquid culture. Mycelia were transferred to solid medium for harvest of synchronised asexual (a1 for immature conidiophores; a2 for mature conidia) and sexual (s1 for nests including H¸lle cells; s2 for mature cleistothecia) tissue types. RNA was isolated and applied to Northern hybridisation analysis. Specific mRNA signals for *csnD* and *gpdA* **(A)** were quantified relative to rRNA signal intensities and the values determined from vegetative mycelia were set as one **(B)**.

the green fluorescent protein was expressed in *A. nidulans*. In the majority of the hyphae, the fluorescence was ubiquitous but accumulated as distinct spots. Nuclei were stained with DAPI and *in silico* merge of the resulting images revealed that the spots obtained from nuclear stain and GFP matched. Such enrichment of CSN subunits in the nucleus, accompanied by a cytoplasmatic subcomplex of the CSN, was also observed in higher eukaryotes (Tomoda *et al.* 2002). This analogy in localisation additionally accounts for the identity of CSND as a component of a COP9 signalosome in *A. nidulans*.

4.4.3 Deletion of the *csnD* gene blocks sexual development and causes altered pigmentation and cell morphologies in *A. nidulans*

A transgenic *A. nidulans* strain carrying a targeted deletion of *csnD* was constructed to verify the acleistothecial phenotype of the REMI mutant strain AGB37. The complete open reading frame of *csnD* was replaced by a *pyr-4* expression cassette in the *A. nidulans* wild-type strain AGB152. The resulting transformants were viable. Homologous integration of the *csnD* flanking regions into the genome was verified by Southern analysis. After backcrossing of the transformants to the wild-type strain, the resulting deletion strain AGB195 produced no specific *csnD* transcripts as proven in Northern hybridisation experiments. Radial colony expansion from a point spore inoculum on an agar surface resulted in similar growth rates with an average of about 0.37 mm radial vegetative colony growth per hour for the *csnD* deletion strain compared to 0.41 mm/h for the wild-type at 37°C. In submerged liquid culture, where *A. nidulans* generally does not induce any development for lack of an air-medium interface (Axelrod *et al.* 1973), the *csnD* deletion strain was viable and did not show obvious anomalies in the morphology of vegetative hyphae.

However, when the *csnD* deletion strain was grown at an air-medium interface for a minimum of 48 hours, two distinct peculiarities of hyphae became obvious: cellular abnormalities and red colouring (Fig. 4.5). When the surface material *of A. nidulans* colonies was removed by washing the agar plates under the tab, an aberrant reddish compact hyphal mat became visible that partly penetrated the agar surface. To facilitate microscopic analysis, cultures were grown on the surface of liquid medium which revealed the same hyphal traits. The *csnD* deletion strain produces, beneath apparently normal filaments, highly branched hyphae consisting of very short cells, indicating a polarity defect. Single hyphae showed a brownish-red pigmentation that was completely absent in mycelia from the wild-type strain which indicates a problem in secondary metabolism. The red colouring appeared in a majority of the aberrant cells, but to a minor extent also in some morphologically wild-type like hyphae and conidiophore stalks. More careful observations of this phenomenon revealed that the centre of a *csnD* mutant colony discoloured earliest 48 h after inoculation. This colouring pattern spread towards the edge of the colony within time. We could not detect major secretion of a dye into the agar medium.

Conidiophore morphology of the *csnD* deletion strain resembled that of the wild-type. Single colonies or a point inoculum of this mutant differentiated conidiospores predominantly, but not exclusively, in the centre of the colony. At confluent inoculation, but not in pregrown developmentally competent mycelia (as described below), the number of conidiospores produced after two days was about the same range in both mutant and wild-type strain. Thus, the process of conidiation seemed not generally affected in the *csnD* deletion mutant AGB195. In contrast to that, the *A. nidulans csnD* deletion strain showed a severe defect in the sexual propagation cycle (Fig. 4.5). The first morphologically visible stages of the sexual cycle, including formation of Hülle cells and nests, were not affected. Within the nests cleistothecial primordia developed, which morphologically resembled that of the wild-type. The next developmental step, formation of micro-cleistothecia, was never observed in the *csnD* deletion strain. Consequently, the mutant strain failed to produce mature cleistothecia with a hard shell and red pigmented ascospores as seen in the wild-type. This specific block in sexual development, together with the polarity and colouring defects, were complemented in strain AGB203 where a genomic wild-type *csnD* fragment was ectopically integrated in the *csnD* deletion mutant (Fig. 4.6). Thus, all mutant phenotypes were dependent on the *csnD* gene. The connection between the red colouring, the morphologically different hyphae and the block in sexual development in the *csnD* deletion strain remains to be elucidated.

4.4.4 An *A. nidulans* strain lacking the fifth CSN subunit (CSNE) shows a Δ*csnD*-like phenotype

A second component of the proposed multiprotein complex was isolated and deleted to verify the existence of the COP9 signalosome in *A. nidulans*. A nucleotide stretch with similarity to CSN subunit 5 from *A. thaliana* was identified in the *A. nidulans* genome of the Cereon database. The corresponding genomic DNA

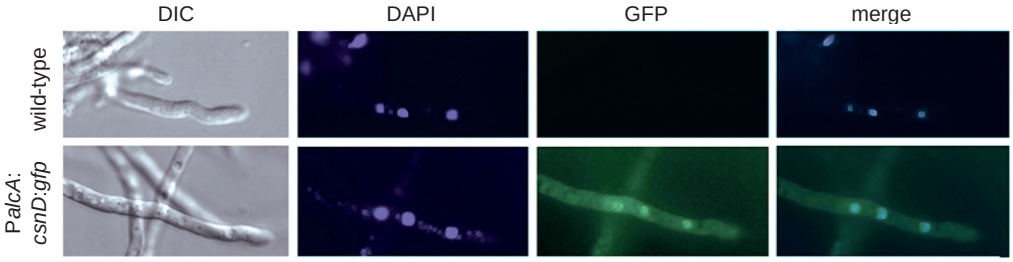

Fig. 4.4: CSND is enriched in the nuclei of *A. nidulans*. *A. nidulans* wild-type strain AGB160 and a strain carrying a P*alcA*-driven overproduction of a CSND::GFP fusion protein (AGB197) were grown over night in liquid submerged culture under inductive conditions. The CSND:GFP fusion accumulated in the nucleus. Nuclei of liquid mycelia were stained by DAPI and pictures of microscopy were taken with DIC, DAPI fluorescence and GFP fluorescence.

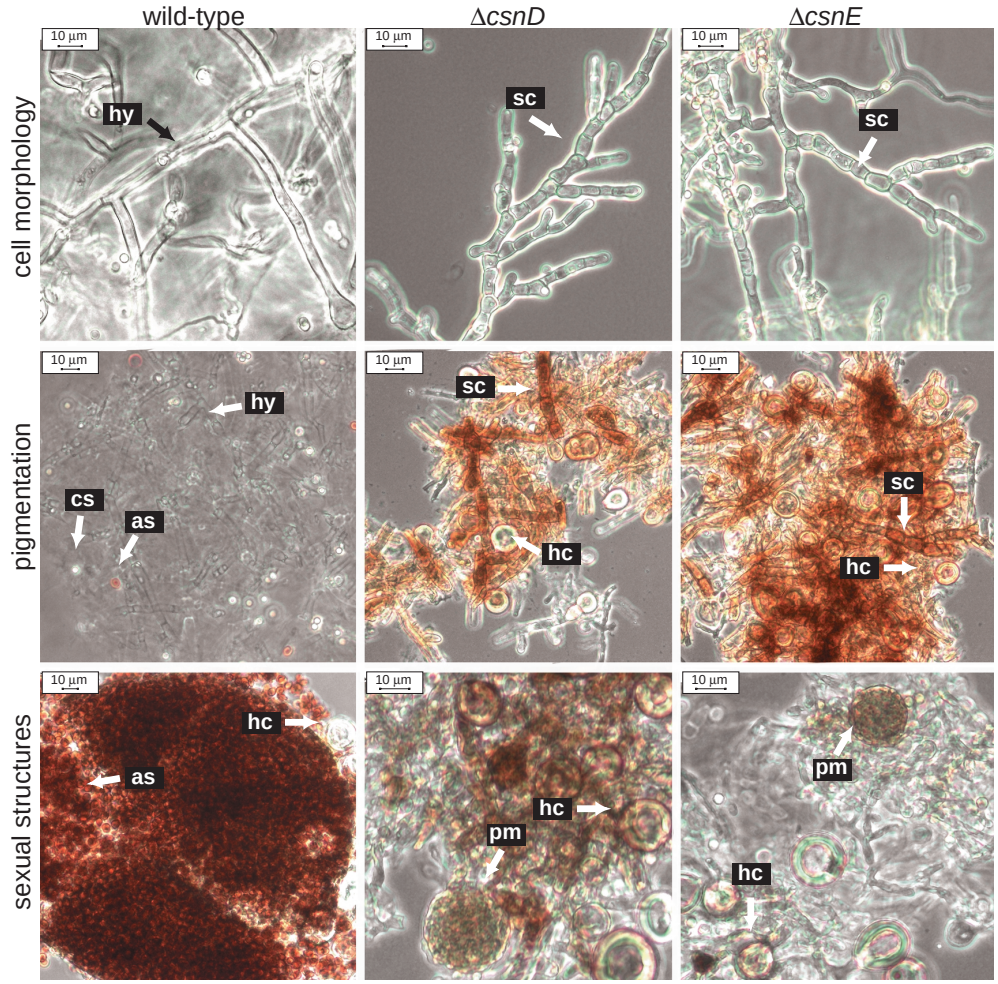

Fig. 4.5: *A. nidulans* strains that lack CSN subunits exhibit pleiotrophic mutant phenotypes. Deletion of *csnD* (strain AGB195) or *csnE* (strain AGB209) result in identical mutant phenotypes as depicted by microscopic analysis. As control, the wild-type strain AGB160 is shown. (Row 1) Wild-type and mutant strains were grown on a surface of liquid medium to obtain isolated hyphal structures, since hyphae scratched from an agar surface tend to tightly aggregate. The cell morphologies in the *csn* mutant strains include wild-type vegetative hyphae (hy) and hyperbranched filaments consisting of aberrant short cells (sc), in some cases including a red pigment as seen in the next row. (Row 2) The surface material of the A. nidulans colonies was carefully removed under the water-tap and the hyphal material was the scratched from surface agar layer. Single hyphae in the *csn* mutant strains, including normal filaments and short cells, synthesise an intensive red coloured pigment. (Row 3) Sexual structures of the strains were collected from the colony surface material. Sexual development of the wild-type strain results in mature cleistothecia with ascospores (as) whereas the *csn* mutant strains develop only Huelle cells (hc) and primordia (pr).

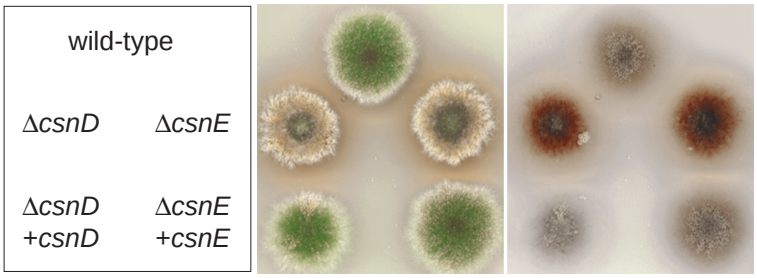

Fig. 4.6: The *A. nidulans csnD and csnE* deletion strains can be complemented by ectopic integration of the corresponding genomic loci. The *A. nidulans csnD* and *csnE* deletion strains and AGB195 and AGB209, respectively, were complemented with the corresponding wild-type *csnD* and *csnE* genes in strains AGB203 and AGB211, respectively. The wild-type strain AGB160 is shown as control. The strains were point inoculated on solid medium and incubated at conditions that allow both asexual and sexual development (left). The *csn* deletion strains failed to develop mature cleistothecia and produce a compact hyphal mat beneath the surface structures containing aberrant pigmentation. The surface cell material was removed by carefully washing the plates with a cotton ball under the water-tap (right) which uncovered the red pigmentation.

was amplified by PCR using a specific primer pair deduced from this sequence information. This way, we isolated and sequenced a 609 bp fragment with deduced amino acid similarities to CSN subunit 5. This fragment was used as probe in colony hybridisation experiments which revealed a 6 kb genomic *Eco*RI fragment containing a coding region of 1060 bp (Fig. 4.1B). Southern hybridisation experiments indicate that the coding region is present with one single copy in the genome (data not shown). The corresponding cDNA was isolated via RT-PCR. Comparison of genomic and cDNA sequences revealed one intron of 51 bp. From the deduced peptide sequence of 335 amino acids a molecular mass of 37.8 kDa was calculated. The putative protein showed up to 53% identity to the fifth subunits of the COP9 complex from various organisms (Fig. 4.2B). Subunit five is the only component of the COP9 signalosome which has, with Rri1p, a putative counterpart in the yeast genome. Rri1p of *S. cerevisiae* exhibits only 20,2% identities to the *A. nidulans* CSNE (not shown). Analysis of the amino acid sequence revealed a conserved MPN domain characteristic for subunit 5 of the signalosome. The gene was named *csnE* (GenBank accession number AY126455) according to the unified COP9 signalosome nomenclature (Deng *et al.* 2000).

We substituted the 5'-terminal part of the *csnE* coding sequence with the *pyr-4* marker cassette. The resulting mutant strain AGB209 showed homologous integration of the marker in Southern experiments and no *csnE* transcripts in Northern hybridisation analysis. Similarly to the *csnD* deletion strain, the *csnE* mutant was acleistothecial but developed Hülle cells and primordia (Fig. 4.5). This strain also produced highly branched and red hyphae when grown at an air-interface (not shown). All mutant phenotypes were abolished in strain AGB211 where a genomic wild-type copy of *csnE* was ectopically integrated in the deletion strain. (Fig. 4.6). Thus, absence of either subunit 4 or subunit 5 of the COP9 signalosome in *A. nidulans* mediated identical pleiotrophic mutant phenotypes. This indicates that both, CSND and CSNE, are involved in the same function that is probably similar to the function of the COP9 signalosome in higher eukaryotes.

4.4.5 The *csnE* and *csnD* gene products of *A. nidulans* interact in a two-hybrid assay.

All subunits of the COP9 signalosome contain either a PCI or a MPN domain that probably confer to protein-protein interactions (Kapelari *et al.* 2000). The corresponding regions were identified *in silico* from the deduced CSND and CSNE peptide sequences (Fig. 4.2). Protein interactions for the *A. nidulans* proposed CSN subunits were analysed in the yeast two-hybrid system in which interaction of two test partners activates transcription of the *LEU* and *lacZ* genes in the yeast two-hybrid reporter strain EGY48-p1840 (MATα, *his3*, *trp1*, *ura3-52*, *leu2*::*pLEU2-LexA*op6, *URA3*::*lacZ-LexA*op2). Correspondingly, growth and expression of the β-galactosidase reporter were analysed on yeast SC medium with galactose and raffinose as carbon source and lacking leucine. *csnD* was cloned as fusion to both the LexA DNA binding-domain and the transcriptional activation domain. *csnE* was fused to the transcriptional activation domain only, since fusion of the corresponding mammalian CSN5 orthologue to the GAL1p binding domain showed strong background activity (Nordgard *et al.* 2001). CSND and CSNE fusion proteins were combined with the empty two hybrid vectors and with each other in the *S. cerevisiae* reporter strain. Both CSND fusions showed no background activity whereas the CSNE fusion protein showed slight intrinsic transcriptional activity. We identified protein-protein interaction between CSND and CSNE as well as self-association of CSND (Fig. 4.7). Both are in agreement with findings of subunit-subunit interactions of the COP9 signalosome in other organisms (Kapelari *et al.* 2000; Tsuge *et al.* 2001). We thus assume that CSND and CSNE function within a protein complex and the phenotypic effects discussed in this work resulted from malfunction of this complex in *A. nidulans*. All our data suggest that this protein complex is the fungal equivalent of the COP9 signalosome of higher eukaryotes.

4.4.6 Light-dependence of development is disturbed in the *csnD* deletion strain.

Our results suggest that the COP9 signalosome of *A. nidulans* is involved in molecular control of several physiological and morphological processes. We further questioned whether the regulatory role of this complex includes integration of environmental signals. In *A. nidulans*, knowledge about developmental response to physical parameters on the molecular level is scarce. One important parameter for initiation of development in *A. nidulans* is illumination: Light generally induces asexual development and represses sexual development. In the plant *Arabidopsis thaliana*, the COP9 signalosome was initially described as a regulator of light-induced development (Wei *et al.* 1994). We were thus interested in the developmental response of the *A. nidulans csnD* deletion strain to light induction. The only component of a proposed light-regulatory pathway in *A. nidulans* is the *veA* gene product (Mooney and Yager 1990). VEA (GenBank accession number AF109316) was described as mediator of light-signalling, though the molecular function of this regulatory protein is still obscure. Overexpression of *veA* in strain OVAR5 mediates increased cleistothecial development and low conidiophore

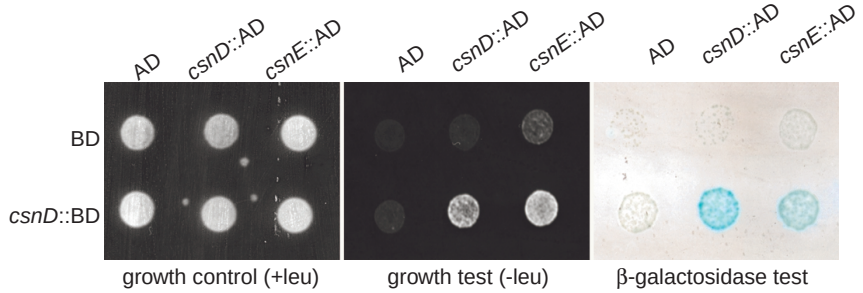

Fig. 4.7: *A. nidulans* CSND interacts with CSNE in a yeast two-hybrid assay. *csnD* was fused to the LexA DNA-binding domain (BD) and the yeast GAL1p transcriptional activation domain (AD), respectively, and tested in combination with itself and *csnE* fused to the AD domain. Protein-protein interaction between the two test partners allows growth of the leu-auxotrophic *S cerevisiae* strain EGY48-p1840 in the absence of leucine in the medium due to the LexA-DNA binding site in the deficient *LEU2* promoter. In addition, the heterologous *lacZ* gene is activated by a functional hybrid.

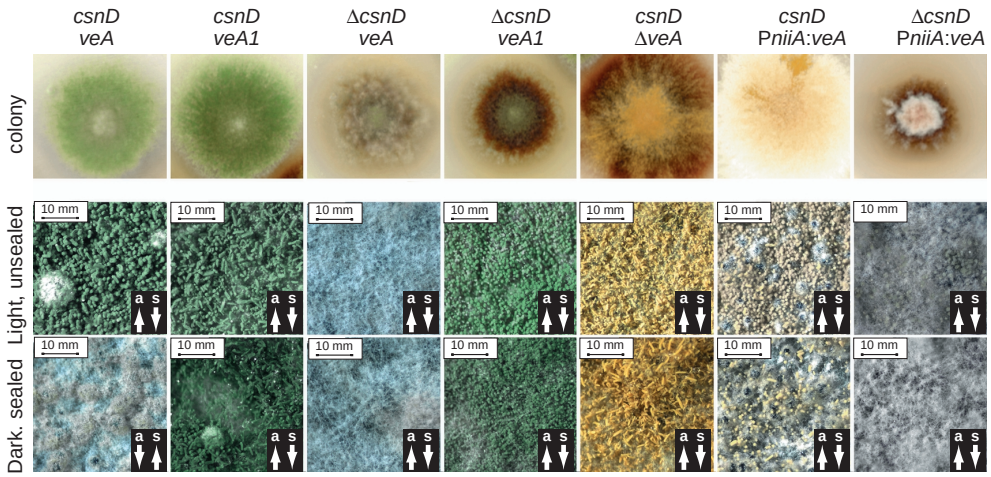

Fig. 4.8: The COP9 signalosome of *A. nidulans* is involved in light-dependent initiation of development. The *csnD* deletion was combined with the truncated *veA1* allele (strain AGB192) and a *veA* overexpression (AGB220). As controls, the *A. nidulans* wild-type strain AGB160, the *csnD* deletion strain AGB195, the *veA1* mutant strain AGB162, the *veA* deletion strain DVAR1 and the *veA* overexpression strain OVAR5 are shown. The *veA* overexpression is driven by the *A. nidulans* P*niiA* promoter which is repressed by weak nitrogen sources. Notably, this promoter may become repressed on older plates when nitrogen sources become exhausted. The overall phenotype of the strains on solid medium incubated in the light is shown in photographs of the colonies (upper row). The *csnD* deletion strains produced conidiospores predominantly in the centre of the colony. The *veA* deletion strain produced exclusively conidiophores and the *veA* overexpression strain predominantly cleistothecia (few conidiophores show up in the *veA* overproduction strain at longer incubation time). Red colouring beneath the surface material was evident in all *csnD* deletion strains and in the Δ*veA* mutant. Light response of development was analysed with pre-grown, developmentally competent mycelia that were transferred to solid medium and induced either asexually in the light (middle row) or sexually on sealed plates in the dark (lower row). Pictures were taken under the binocular. Occurrence of any sexual tissue types was valued as induced sexual reproduction cycle. Induction or repression of asexual (a) or the sexual (s) reproduction is indicated by arrows.

production. In contrast to that, the *veA* deletion strain DVAR1 produces conidiophores but is acleistothecial, additionally producing a red hyphal pigment reminiscent to the one observed in the *csnD* deletion strains (Fig. 4.8). Notably, most frequently used *A. nidulans* laboratory strains carry the *veA1* allele (GenBank accession number AF109317), a partial deletion of the open reading frame, which abolishes light-dependence of conidiation and reduces production of cleistothecia and thus resembles a weak *veA* deletion phenotype (Champe *et al.* 1994).

Strains with alterations in the *csnD* and *veA* locus were constructed. The *csnD* gene was deleted in the *A. nidulans veA1* background of strain AGB162, resulting after backcross in the *csnD* deletion strain AGB192. In *A. nidulans* strain AGB220, the *csnD* deletion was combined with the P*niiA*-driven *veA* overexpression of strain OVAR5. To analyse light-dependence of development, we concentrated on the initiation of the two reproduction pathways in developmentally synchronised, competent mycelia transferred to agar plates. The differential induction of the two reproductive cycles is generally not absolute, and for clarity, occurrence of less than about 25% conidiophores or cleistothecia compared to the wild-type situation is not taken into account. An *A. nidulans* wild-type strain predominantly induced the sexual cycle in the dark and the asexual cycle in the light (Fig. 4.8). In the strains with altered cellular VEA levels or the *veA1* mutant allele, illumination made no difference: the *veA1* partial deletion and *veA* knock-out strains predominantly induced the asexual cycle whereas *veA* overexpression resulted in sexual induction. Comparably, deletion of *csnD* in wild-type background (AGB195) predominantly caused induction of the sexual cycle independent of the light signal. Thus, a deletion of *csnD* abolishes light dependence of development in *A. nidulans* indicating that the COP9 signalosome of *A. nidulans* is essential for correct light-dependent signalling. Combination of Δ*csnD* with different *veA* alleles, results in strains lacking light dependence of development. Combined with the *veA1* allele, the *csnD* deletion strain (AGB192) preferentially induces the asexual cycle, like its parent *veA1* strain. And in combination with *veA* overproduction, a *csnD* deletion results in induction of the sexual cycle (AGB220), like a pure Δ*csnD* or *veA* overproduction strain. Thus, with respect to developmental induction, the *csnD* deletion did not change the *veA* mutant phenotypes. This indicates an additional function of the COP9 signalosome in *A. nidulans* in a light-dependent signalling pathway that acts genetically upstream or at the level of VEA - or in a *veA*-independent pathway.

4.4.7 The velvet gene product does not release the sexual block of *csnD* deletion strains

We investigated a molecular connection between *csnD* and *veA*. A dependency of *csnD* and *veA* transcripts was analysed in Northern hybridisation experiments. Wild-type, *csnD* deletion and *veA1* strains were grown in liquid overnight culture for 18 h to synchronise the mycelia at the stage of developmental competence. This cell material was transferred to solid medium and induced asexually or sexually in light or in the dark on sealed plates. On the transcriptional level (Fig. 4.9), specific

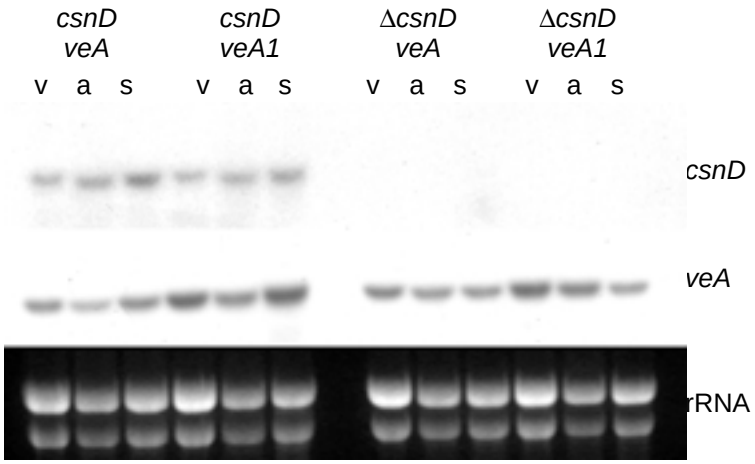

Fig. 4.9: Transcription of *csnD* and *veA* during development. *A. nidulans* strains AGB160 (*csnD, veA*), AGB162 (*csnD, veA1*), AGB195 (Δ*csnD, veA*) and AGB192 (Δ*csnD, veA1*) were pre-grown in submerged liquid culture, the developmentally competent mycelia were transferred to solid medium and induced either asexually in the light or sexually in the dark on sealed plates. Vegetative (v), asexual (a) and sexual (s) tissue types were harvested for RNA isolation for Northern hybridisation experiments using *csnD* and *veA* as specific probes. Levels of specific *csnD* and *veA* mRNAs were independent of the strain and the developmental conditions.

csnD and *veA* RNA signals from this cell material, compared to rRNA, were not significantly altered after shift from vegetative to differentiating cultures in the wild-type strain and the *veA1* strain. Apparently, the mean *veA* transcript levels were generally higher in the *veA1* strain. *csnD* transcripts were present independent of the *veA* allelic state. Vice versa, the quantity of *veA* transcript was similar in *csnD* wild-type, deletion and overexpression strains. Taken together, transcription of *csnD* and *veA* proceeds independently, irrespective of the developmental state of the culture.

The phenotypic consequence of the *veA1* deletion and the overproduction of the *veA* wild-type gene in a *csnD* deletion strain was analysed to test their genetic relationship. The Δ*csnD*/*veA1* mutant (AGB192) showed both red pigmentation and aberrant cell forms like the *csnD*/*veA*+ strain (AGB195). AGB192 also produced Hülle cells and primordia that never matured to cleistothecia, though apparently less than the corresponding *csnD*/*veA*+ strain. (Fig. 4.8). Thus, the *veA1* mutant is unable to suppress the block in development caused by *csnD* deletion. All phenotypes of the deletion mutant AGB192 were complemented by ectopic integration of the corresponding *csnD* genomic fragment in strain AGB193. The *A. nidulans* Δ*csnD*/P*niiA*:*veA* strain (AGB220) showed red colouring, highly branched hyphae with aberrant cells were readily visible in this mutant, and also sexual development was blocked at the level of cleistothecial primordia (Fig. 4.8). This indicates that even overproduction of VEA, which normally leads to enhanced cleistothecia production, does not lead to development of mature cleistothecia in a *csnD* deletion strain. Thus, neither high VEA levels nor the changed *veA* gene product are able to overrule the developmental block in *csnD* deletion strains. Consequently, *csnD* is epistatic to the *veA1* loss of function and *veA* gain of function mutations, which places this function of CSND in respect to specific sexual development genetically downstream of VEA.

4.5 Discussion

This study identifies the existence of the COP9 signalosome in filamentous fungi and describes it as key regulator of fungal development. The *csnD* and *csnE* genes encode the fourth and fifth CSN subunits of *A. nidulans*. The deduced peptide sequences for CSND and CSNE contain PCI and MPN motifs, respectively, which are characteristic for proteins of the 26S proteasome lid, eIF3 and the COP9 signalosome multiprotein complexes (Kim *et al.* 2001). Protein-protein interactions between CSND and CSNE are comparable to that described for subunits 4 and 5 of the COP9 signalosome in other organisms (Kapelari *et al.* 2000; Tsuge *et al.* 2001). At the transcriptional level, *csnD* mRNAs are abundant in both vegetative and developing cultures. In analogy to this, specific mRNAs of CSN subunits were detected in all mouse embryonic and adult tissue tested (Bounpheng *et al.* 2000). A fusion of CSND to the green fluorescent protein is dispersed in the cytoplasm and clearly enriched in the nuclei. This is in agreement with observations in other organisms, where subunits of the CSN are predominantly localised in the nucleus as multiprotein complex, and subunits 4 to 8 were additionally found in the cytoplasm probably forming a smaller subcomplex (Chamovitz *et al.* 1996; Kwok *et al.* 1998; Tsuge *et al.* 2001; Tomoda *et al.* 2002). All our data suggest that the products of the two identified genes represent the first members of the COP9 signalosome in filamentous fungi.

In higher eukaryotes, defects in subunits of the COP9 signalosome result in severe developmental phenotypes and post-embryonic lethality (Wei *et al.* 1994; Freilich *et al.* 1999), whereas malfunction of the complex in *S. pombe* is not lethal and leads to minor mutant phenotypes like delayed progression through the cell cycle and increased sensitivity to ultraviolet light (Mundt *et al.* 1999; Mundt *et al.* 2002). This work identified the COP9 signalosome of *A. nidulans* as a key regulator in the development of the organism, essential for proper regulation of metabolism, cell morphology, hyphal polarity, light-regulation and sexual reproduction (Fig. 4.10). The study of the COP9 signalosome in the model organism *A. nidulans* has three major advantages: it is easily accessible to molecular manipulations. In contrast to COP9 signalosome defects in higher eukaryotes, an *A. nidulans* strain defective in its sexual cycle is still viable and can propagate via its asexual cycle. Last but not least, *A. nidulans* is evolutionary closer related to humans than plants are.

Malfunction of the COP9 signalosome in *A. nidulans* results in changes of secondary metabolism which is visible with the naked eye: overproduction of a red pigment. To date, we have no indication about the origin of this red substance. In *A. nidulans*, knowledge about regulation of secondary metabolism, and especially about red pigments, is rather restricted. Wild-type strains deposit brownish melanin in walls of older hyphae when mycelia are grown in submerged liquid culture (Pirt and Rowley 1969). This phenomenon is not altered in the *csn* deletion mutants compared to the wild-type (not shown). Notably, absence of melanin is correlated with defective sexual reproduction *in A. nidulans*, indicating a cross-connection between secondary metabolism and sexual development (Champe *et*

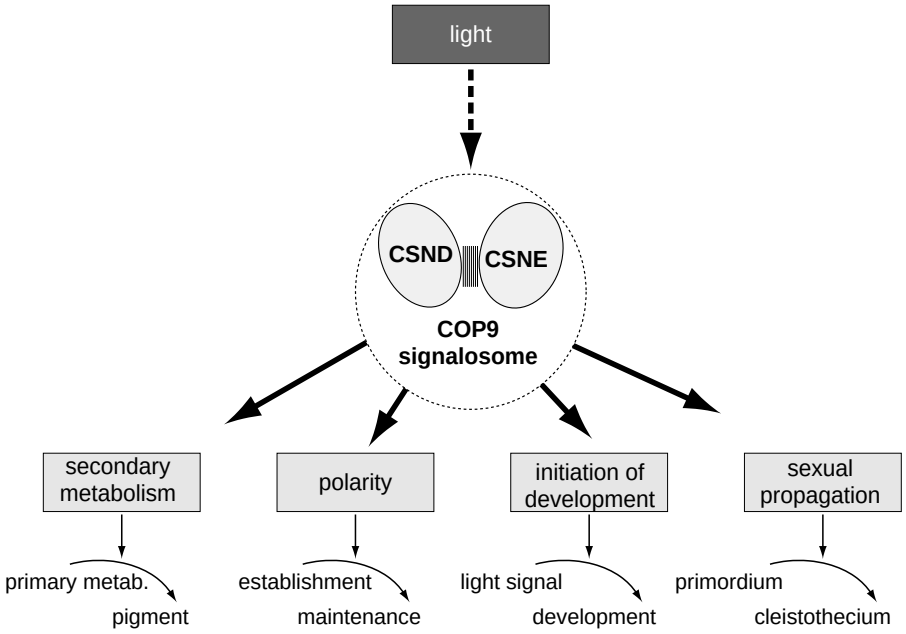

Fig. 4.10: Multifaceted role of the COP9 signalosome (CSN) in *A. nidulans*. Protein-protein interaction between the two CSN subunits CSND and CSNE are indicated by paralleled bars. The impact of the COP9 signalosome in a light-dependent signalling pathway is indicated by a broken arrow line and the function of the CSN in several downstream target pathways by full arrow lines.

al. 1994). The second known red pigment produced by *A. nidulans* is responsible for the colour of the ascospores: the anthraquinone asperthecin, which is difficult to isolate (Howard and Raistrick 1955). It seems striking that the red colouring in the *csnD* mutant becomes visible after two-three days of growth which is the time scale where also the first structures of sexual development become visible. Notably, CSN mutants of the plant *A. thaliana* overproduce anthocyan, the flowering colour (Misera *et al.* 1994). This raises the question whether the red pigment produced in the *csn* deletion strains is related to the one that dyes the ascospores. Preliminary tests using 0.5 M $NaHCO_3$ and Na_2CO_3 according to Howard and Raistrick (1955) did not reveal whether the red colour of the *csn* deletion mutants was asperthecin (data not shown). The aberrant colouring of hyphae in the *csn* deletion strains appears independent of developmental induction by light and the allelic *veA* state. Regulation of the production of the putative red pigment thus seems to be mainly mediated by time and/or growth phase. Thus, an impact of the COP9 signalosome in the internal regulation of onset of secondary metabolism and/or development is conceivable. Astonishingly, red coloured hyphae are also seen in a *veA* deletion strain, but it is not clear whether the red pigment produced by Δ*csn* and Δ*veA* strains is identical.

The COP9 signalosome of *A. nidulans* is involved in the control of polar apical growth and lateral branching in surface-grown cultures. Generally, establishment of polar growth after germination and its later maintenance seem independent processes in *A. nidulans*, with a proposed persistent signal for ongoing apical extension (Momany *et al.* 1999). Young hyphae of surface-grown *csn* deletion colonies as well as hyphae grown in submerged liquid culture show

no obvious aberrance. Thus, the establishment of polarity seems not generally disturbed in the *csn* deletion strains. But maintenance of apical extension in elderly, surface-grown cell material seems to be a target of the COP9 signalosome. In *A. nidulans* wild-type strains, polarised apical growth is generally turned off during developmental programs. In the asexual propagation cycle, a switch from polarised growth to a bud-like growth form is seen during sterigmata formation and conidiation (Adams *et al.* 1998). As for propagation of sexual spores, ascogenous hyphae are formed that can be seen in young immature cleistothecia as branched filaments with knobby cells (Braus *et al.* 2002). *A. nidulans* strains with overproduction of the transcription factor STEA block vegetative growth and produce highly branched hyphae with small, knobby cells very similar to ascogenous tissue, though a direct relation has not been proven yet (Vallim *et al.* 2000). Nevertheless, the short and highly branched hyphae in elderly *csn* deletion strains do not morphologically resemble the phenotype described for the STEA overproduction strain and young ascogenous tissue. Similar to the polarity defect of *A. nidulans csn* deletion strains, malfunction of the *A. thaliana* COP9 signalosome causes aberrant cell morphologies. Transgenic plants with reduced CSN levels show a general increase in secondary inflorescences and a reduction of internode length and cell size. These phenomena are primarily due to a loss of apical dominance, which in turn is driven by the phytohormone auxin. In *A. thaliana*, the auxin-response is controlled by the COP9 signalosome, probably by degradation of the AUX/IAA transcriptional repressors (Schwechheimer *et al.* 2001). Auxin, a tryptophan-related hormone-like signal molecule, is product of secondary metabolism. We have recently reported a role of auxin for development in *A. nidulans* (Eckert *et al.* 2000). Strains auxotrophic for tryptophan arrest sexual development at the level of micro-cleistothecia, which is one step beyond the arrest at primordia seen in *csn* deletion strains. External supply of high amounts of tryptophan or traces of auxin released this developmental block. Future studies will focus on a possible co-ordination of sexual development and hormone signalling by the *A. nidulans* COP9 signalosome.

The severe mutant phenotype of *csnD* or *csnE* deletion in *A. nidulans* is a block in sexual development at the level of cleistothecial primordia. To our knowledge, a specific developmental arrest at this stage was not described before in *A. nidulans*. Initiation of the sexual cycle and differentiation processes leading to the general architecture of primordia are not impaired in the *csn* deletion strains. But further differentiation and maturation of wall and ascospores is blocked. This suggests that after successful formation of the primordial structure, a regulatory process exists that is expendable for the first steps in sexual development leading to cleistothecial primordia but essential for completion of the sexual cycle. The COP9 signalosome seems to be an essential player in this regulatory process mediating maturation of primordia in *A. nidulans*. A similar developmental block at a level of metamorphosis of a primordial to mature stage can also be observed in homozygous CSN mutants of the fruit fly *D. melangolaster*. The mutant embryos hatch and develop normally until the middle of the third instar and frequently pupate, but then cease to develop and die (Freilich *et al.* 1999). This block in sexual

development seems to be the most severe phenotype of a defect in COP9 signalosome function in *A. nidulans*. And as stated above, the additional phenotypes of cell polarity and red pigmentation may also be related to developmental processes. It is thus conceivable that the COP9 signalosome in *A. nidulans* is dispensible for growth and housekeeping functions but essential for correct regulation of development.

Due to its impact on secondary metabolism, polarity and sexual development, the COP9 signalosome probably has several different downstream targets, summarised in Figure 4.10. This raises the question which upstream factors regulate CSN activity. An external signal important for development in *A. nidulans* is light, with the *veA* gene product as a proposed part of a corresponding signal transduction pathway. A *csnD* deletion strain is "blind" to light-regulation, like strains with constitutively low or high *veA* expression. Thus, the COP9 signalosome of *A. nidulans* is involved in light-dependent signalling and may even be connected with the same signal transduction pathway as VEA. Notably, in the plant *A. thaliana*, the CSN is involved in the repression of photomorphogenesis in the dark. The proposed E3 ubiquitin ligase COP1 accumulates in the nucleus in the dark where it mediates, assisted among others by the COP9 signalosome, ubiquitinylation of an transcriptional activator of light-regulated genes (Osterlund *et al.* 1999; Osterlund *et al.* 2000; Schwechheimer and Deng 2001; Suzuki *et al.* 2002). The product of the *A. nidulans veA* gene has a negative influence on initiation on the asexual but a positive on the onset of the sexual cycle, as seen by the corresponding deletion and overproduction strains. It seems striking that the *csnD* deletion strain in a velvet wild-type background acts like a *veA* overproduction strain: a constitutive induction of the sexual cycle. In analogy to the findings in *A. thaliana*, it is thus conceivable that the COP9 signalosome of *A. nidulans* mediates a negative post-transcriptional effect on VEA, resulting in increased VEA protein levels in a *csnD* deletion strain. The function of the COP9 signalosome in light signalling might thus be genetically placed upstream or at the level of VEA, though this question should be addressed in future studies.

In summary, we present the first report of components of the COP9 signalosome in filamentous fungi and present strong evidence of its key regulatory function of development of the mold *Aspergillus nidulans*. The COP9 signalosome of *A. nidulans* is involved in several cellular processes including pigment synthesis, cell morphology, light-dependent signalling and specific sexual development. The function of the COP9 signalosome in filamentous fungi resembles in some respects that of higher eukaryotes. Because mutant strains are viable and can be propagated, this study represents an attractive basis to deliver new insights of the functions of the COP9 signalosome in eukaryotes.

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Chapter 5

Conclusions and outlook

5.1 Amino acid biosynthesis and the COP9 signalosome in *Aspergillus nidulans*

This study focuses on two molecular networks that mediate 'genetic supervision' in eukaryotes, the cross-pathway control of amino acid biosynthesis (CPC) and the COP9 signalosome-dependent network of development (CSN). In this study, components of both regulation circuits of *Aspergillus nidulans* have been isolated and characterised. The CPC and the CSN-dependent system are essential for proper response to external signals, amino acids and light, respectively. Additionally, both molecular networks are involved in the regulation of metabolism as well as development (Fig. 5.1). The CPC not only transcriptionally activates specific target genes of primary metabolism but also significantly impacts sexual development (Chapter 2) and penicillin biosynthesis (Chapter 3). Similarly, function of the CSN is not restricted to cleistothecia formation but additionally affects secondary metabolism (Chapter 4). The results thus imply that cross-connections between physiology and morphology in *A. nidulans* are partly mediated by the cross-pathway control and the COP9 signalosome.

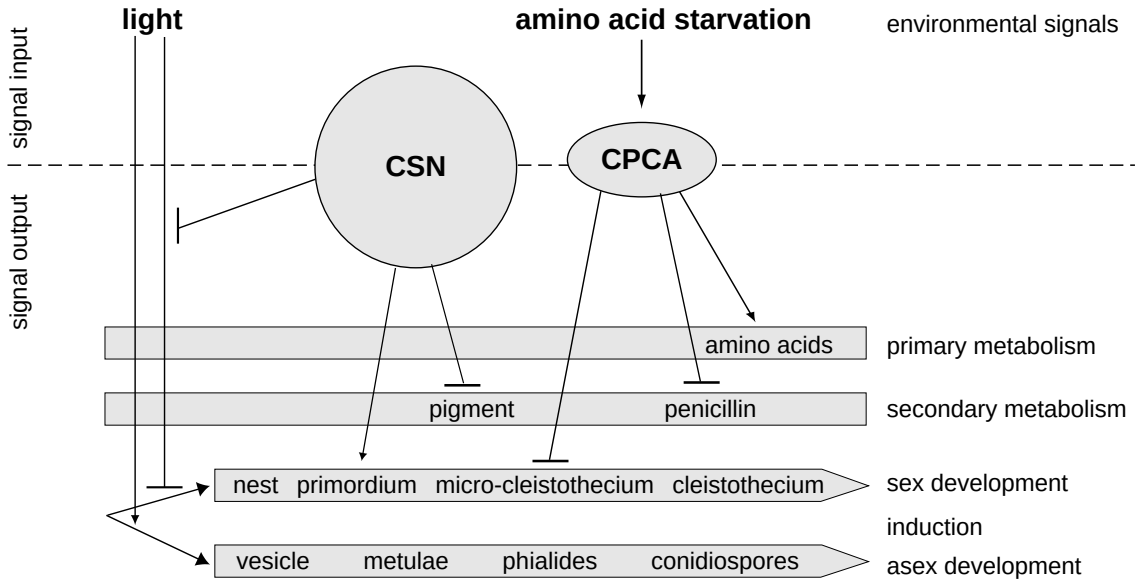

Fig. 5.1: Multifaceted role of cross-pathway control and COP9 signalosome. Both molecular regulators are involved in the control of physiological and metabolic processes in *A. nidulans*.

5.1.1 Signalling of the cross-pathway control

The impact of the cross-pathway control on physiology as well as morphology implies that various cellular targets are affected. Target selection of this system is thoroughly studied in yeast (see 2.2). It is mediated by the 5'-TGA C/G TCA-3' *cis*-acting nucleotide consensus sequence in the 5' regulatory region of the target genes, that is recognised by the central transcriptional activator Gcn4p, though aberrations of one or two bases from the consensus can eventually be tolerated (Mavrothalassitis *et al.* 1990). The *A. nidulans* CPCA, counterpart of yeast Gcn4p, was recently isolated (Hoffmann *et al.* 2001). Functional complementation of a yeast *GCN4* deletion strain with the *A. nidulans cpcA* gene (Hoffmann *et al.* 2001) strongly suggests that in this filamentous fungus the same consensus accounts for transcriptional activation upon amino acid starvation. First evidence for such functional cross-pathway response elements (CPREs) in *A. nidulans* resulted from intensive mutant promoter complementation studies of *argB*, the gene encoding ornithine carbamoyltransferase of arginine biosynthesis (Goc and Weglenski 1988). Both CPCA target genes identified in this work, *hisB* and *lysA*, contain putative CPREs in the proximal 400 bp of their promoter regions, which is comparable to those identified for other *A. nidulans* CPC-regulated amino acid biosynthesis genes *argB*, *hisHF* and *trpB*. From this putative CPCA binding sites, the CPRE consensus 5'-TGA C/G TSW-3' can be deduced for *A. nidulans*, which closely resembles that of the yeast consensus (Tab. 5.1). Strikingly, most of the aberrations from the proposed optimal binding site are located within the 3' halfsite. An asymmetry of the two halfsites concerning the tolerance of base substitutions from the consensus was also reported from studies on the yeast system, leading to the hypothesis that the two sites are probably not equivalent (Mavrothalassitis *et al.* 1990).

Tab. 5.1: Proposed CPREs in *A. nidulans* cross-pathway target genes.

gene	regulatory circuit	sequence (5'-3')	pos.[1]	reference
	yeast consensus:	TGA C/G TCA		Oliphant *et al.* 1989
argB	arginine biosynthesis	TAA C TCT	-127	Goc and Weglenski 1988
		TGA G TCA	-79	
		TTA C TAA[2]	-66	
cpcA	cross-pathway control	TCT C AGT	-1085	Hoffmann *et al.* 2001
		TGA C TCA	-1070	
hisB	histidine biosynthesis	TGA C GCA	-225	this work
hisHF	histidine biosynthesis	TGA C TGT	-396	Valerius *et al.* 2001
		TGA G TCA	-154	
jlbA	DNA binding protein	TGA G TCA	-776	Strittmatter *et al.* 2001
		TGA C TCC	-491	
		TGA C TCA	-308	
lysA	lysine biosynthesis	TGA C TCA	-174	this work
trpB	tryptophan biosynthesis	TGA C TCA	-182	Eckert *et al.* 2000
	deduced Aspergillus consensus [3]*:*	TGA C/G TSW		

[1] Position of the central G/C base relative to the AUG translational start.
[2] Site 3'-5'
[3] Only bases occurring more than once were taken into account. S=C,G ; W=A,T

The strong indications about functional conservation between yeast Gcn4p and *A. nidulans* CPCA suggest an evolutionary conservation of the complete cross-pathway system in fungi. The second key player of the corresponding signal transduction cascade is the sensor kinase Gcn2p (Wek *et al.* 1995) that perceives the external signal 'amino acid starvation' (see 1.2.1.2). A homologue of yeast *GCN2* was isolated from the filamentous fungus *Neurospora crassa*, *cpc-3* (Sattlegger *et al*. 1998). But the corresponding *A. nidulans* sensor kinase is not yet identified. During this work, functional complementation of a yeast *GCN2* deletion strain with an *A. nidulans* cDNA library revealed no positive clones, though this might be due to a lack of the comparably long proposed cDNA (probably about 1.6 kb) in the preparation. Also, application of degenerate primer pairs resembling regions conserved between the deduced amino acid sequences of yeast *GCN2* and *N. crassa cpc-3* were not successful. However, completion of the *A. nidulans* genome sequencing project will probably reveal the cross-pathway sensor kinase of this fungus.

5.1.2 COP9 signalosome-dependent signalling

Knowledge about the informational flux from environmental signals to CSN-mediated cellular responses is scarce. This work revealed an impact of the COP9 signalosome on light-dependent signalling in *A. nidulans* (Chapter 4). Two major scenarios seem conceivable: an impact of light on the activity of the COP9 signalosome or an effect of the CSN on a light-dependent regulator. The *veA* gene product is strongly involved in light-dependent regulation of development (see 1.4.2.1), but this study shows at least on transcriptional level no evidence of a direct interdependency between *veA* and *csnD*. Whether the CSN acts in the same light-dependent signalling pathway as VEA and, if so, whether CSN and VEA interact post-transcriptionally remains to be elucidated.

Nothing is known about other incoming signals yet. During the course of growth and development, the level of *csnD* transcripts remained fairly constant as shown in Chapter 4. Nevertheless, it cannot be excluded that transcription of *csn* genes is increased within a narrow time window during acquisition of competence or in the course of development. It is noteworthy that the 5'-regulatory regions of both *A. nidulans csn* genes include putative binding sites for several transcription factors of *A. nidulans* (see Tab. 1.2 for references). The *csnD* and *csnE* promoters both contain two putative response elements for the AREA transcription factor that controls nitrogen metabolism (5'-A/T/C GATA G/A-3'). Several additional 5'-GATA-3' stretches might contribute to regulation by other GATA-like transcription factors like AREB, SREA and NSDD. The *csnE* regulatory region contains two putative CPCA response elements and one possible STUA binding site (5'A/T CGCG A/T N A/C-3'), whereas the *csnD* promoter includes only imperfect CPCA and STUA consensus sequences. Additionally, a putative CREA response element (5'-C/G C/T GG G/A G-3') is found in the *csnE* promoter as well as putative ANCF (5'-CCAAT-3') and PACC (5'-GCCA G/A G-3') binding sites in the *csnD* promoter. It seems promising to

monitor expression of the CSN subunits in respect to the regulatory pathways affected by these diverse transcription factors. Overall, such a broad range of putative transcriptional control might indicate that expression of the COP9 signalosome is probably controlled by a variety of regulatory pathways, including signal transductions that finally control metabolism and development.

One important part of signal transduction in eukaryotes essential to respond to external stimuli are heterotrimeric G proteins. A link of the COP9 signalosome with G-protein mediated signal transduction is conceivable. The human CSN subunit 1 (former called Sgn1, Gps1) suppresses a constitutively active Gβγ heterodimer in the corresponding yeast Gα-deletion strain in respect to the pheromone response pathway (Spain *et al.* 1996). With FADA, SFAD and FLBA (see Tab. 1.2 for references), components of a heterotrimeric G protein signalling pathway have been identified in *A. nidulans* and cross-connection of this regulatory path to the CSN-dependent network might be questioned in future studies.

The COP9 signalosome functions as modulator of E3 ubiquitin ligases by the associated enzyme activities of a deneddylase and a protein kinase (see 1.2.2.2). An analogous function of the multiprotein complex may be predicted for the *A. nidulans* COP9 signalosome - but remains to be proven by appropriate phosphorylation and neddylation assays. A first insight into CSN activities in filamentous fungi might be gained from studies concerning functional complementation of *A. nidulans csn* deletion strains with the corresponding heterologous counterparts. Strikingly, deletion of *csn* genes is lethal in the plant *A. thaliana* and the fruit fly *D. melangolaster*, but not in the filamentous fungus *A. nidulans*. Functional complementation of the *A. nidulans csnD* deletion strain with the cDNA of the *A. thaliana* CSN4 subunit (Serino *et al*. 1999) will at least answer whether the function of this particular subunit is conserved between higher and lower eukaryotes. To date, six subunits of the *S. pombe* COP9 signalosome have been identified, whereas the yeast genome contains only one open reading frame with significant identities to a CSN subunit (see 1.2.2.4). In the *A. nidulans* genome sequence of the Cereon database (http://microbial.cereon.com), which is not yet complete, putative homologues to other CSN subunits seem present but only vague similarities are found for subunits seven and eight. This raises the question whether the multiprotein complex might be of different size in lower and higher eukaryotes. In this work, the *csnD* gene was cloned in a yeast expression vector (pME2359) and overexpressed in yeast, which was at least not lethal. Isolation of the *A. nidulans* CSN subunit 5 (CSNE) and the corresponding deletion strain (Chapter 4) provide an important tool to study the significance of the putative CSN5 homologue of yeast. Further studies might show whether the proposed yeast CSN5 functionally complements the mutant phenotypes of the *A. nidulans csnE* deletion strain.

Since malfunction of the COP9 signalosome results in multifaceted mutant phenotypes in *A. nidulans*, the CSN-dependent network seems to be involved in the regulation of several downstream circuits. Two hybrid interaction screens or pull-down assays might identify putative target proteins. If a similar function of the CSN in *A. nidulans* compared to the higher eukaryotic systems is assumed, a potential

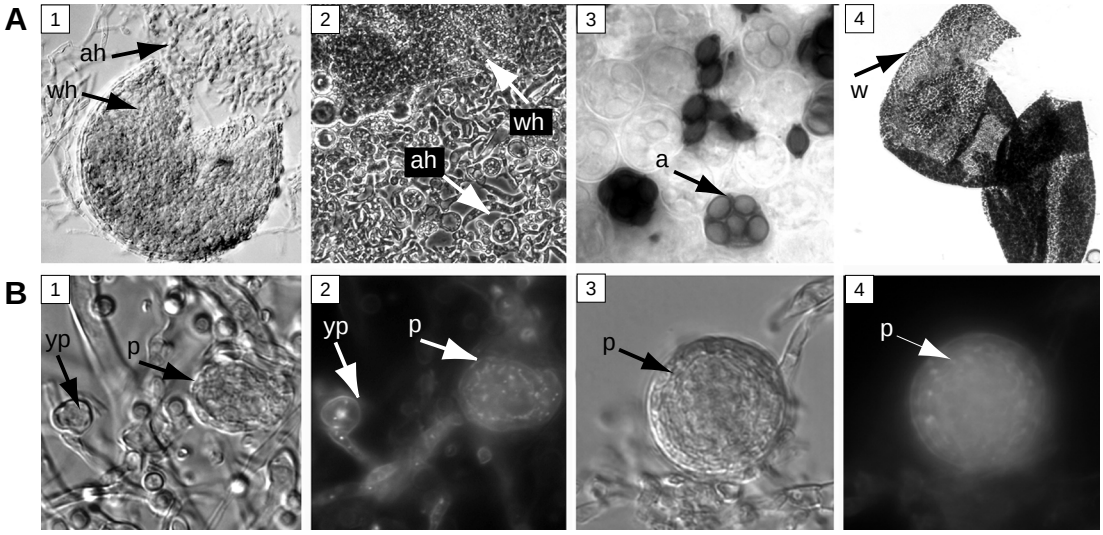

Fig. 5.2: Maturation sexual tissue types in *A. nidulans*. (A) Sexual tissue types in an *A. nidulans wild-type strain*. A very young micro-cleitothecium shows two separate specialised types of hyphae: wall hyphae (wh) and ascogenous hyphae (ah) (1). Both hyphae further differentiate, the wall hyphae get flattened and glued and the the ascogenous hyphae show rounded cells (2). In the mature cleistothecium, the ascogenous hyphae have fully differentiated into asci (a) containing eight ascospores each and the wall hyphae into a hard wall (w) (4). **(B)** Autofluoresce might be a suiteable tool to recognise early sexual developmental structures in *A. nidulans*. In a *csn* deletion strain, several putative stages of young (yp) and older primordia (p) were recogniseable upon light (1,3) and fluorescence (2,4) microscopy.

target protein of the COP9 signalosome might have already been cloned from *A. nidulans* recently, the proposed *culA*-encoded cullin (Eckert 2000). However, up to now a connection between this cullin and the CSN is only hinted at the shared acleistothecial phenotype and the knowledge of a CSN-associated cullin-deneddylation function in other organisms (see 1.2.2.2).

Strikingly, the CPCA homologue of mammalian cells, c-Jun, is among the CSN target proteins in higher eukaryotes (Claret *et al.* 1996). A direct interaction was shown for this oncogene with subunit 5 (CSN5) that was thus formerly termed Jun activation domain binding protein (JAB1). The c-Jun protein is phosphorylated by the CSN which prevents its ubiquitinylation and subsequent degradation (Naumann *et al.* 1999; Chamovitz and Segal 2001). Thus, it seems promising for future studies to question a link between the cross-pathway control and the COP9 signalosome in *A. nidulans*. A first approach should examine the response of the *csn* deletion strains to amino acid starvation conditions, accompanied by monitoring of CPCA protein stability upon starvation and non-starvation conditions in these strains.

5.2 Metabolism and development of *A. nidulans*

Little is known about the molecular processes that drive secondary metabolism and sexual development in *A. nidulans*. Thus, the multifaceted impact of cross-pathway control and the COP9 signalosome-dependent network on physiological as well as morphological traits constitutes a promising starting point for future

analyses. Especially the developmental block at distinct intermediate stages during cleistothecia formation and a simultaneous dis-regulation of secondary metabolite production are of interest and may contribute to new insights about cross-connections between secondary metabolites and sexual development in *A. nidulans*.

5.2.1 Morphogenesis of sexual fruit bodies

An *A. nidulans* cleistothecium is an organised multi-cellular tissue. Nothing is known about the underlying morphogenetic events that lead to maturation of cleistothecial primordia into the characteristic shape of the sexual fruit body. The formation of cleistothecia includes two major maturation processes: from specialised sterile hyphae to a wall and from specialised fertile hyphae to ascospores (Fig. 5.2A). The first conception of the sexual reproductive structure is the cleistothecial primordium, in which at least on the visual level no separation between future wall and ascospores can be discriminated. Future microscopic analyses with isolated primordial structures should further examine this observation. Micro-cleistothecia represent an intermediate developmental stage in which the initial determination processes that control cell fate must have been successfully passed, since in isolated micro-cleistothecia a soft and pale coloured wall encloses ascogenous tissue. Acleistothecial *A. nidulans* strains have been described, but the defects in sexual development are restricted to either the absence of any type of sexual tissue or to production of solely Hülle cells (see 1.4.4.2). This work demonstrates that malfunction of the COP9 signalosome or activation of the cross-pathway control by impaired amino acid biosynthesis lead to a developmental arrest at the sexual stages of primordia or micro-cleistothecia, respectively. These regulatory networks are thus particularly suited to study morphogenesis of the sexual reproductive structures of *A. nidulans*.

Recently, CPCA of *A. nidulans* was characterised in more detail which revealed that, in contrast to the developmental arrest of a *cpcA*-overproduction strain, deletion of *cpcA* results in no obvious developmental mutant phenotypes (Hoffmann *et al.* 2001). Additionally, preliminary experiments show that intermediate levels of CPCA might be the cause of an impressive phenomenon: eventual differentiation of mature ascospores that are not surrounded by a cleistothecial wall (Hoffmann 2000). Vice versa, *A. nidulans* mutant strains that produce empty cleistothecia consisting of a mature wall but lacking ascospores have been described (Swart *et al.* 2001). Thus, the maturation processes of wall and ascospores can probably proceed independently. Whether the phenotype of "open cleistothecia" is indeed due to the reduced level of CPCA remains to be proven. It seems of major concern for future studies to exactly analyse why this phenotype is instable and whether such open cleistothecia produce no wall at all - or whether a first wall conception is build, like in micro-cleistothecia, that is not enlarged and modified during further differentiation and thus bursts.

If genesis of wall and ascospores are unlinked processes, it must be questioned whether the circular structure of the cleistothecial primordium

represents a yet fully potent tissue or whether its cells fate is already determined. The *csn* deletion strains, arrested at the developmental stage of primordia, are thus useful for experimental setups that address questions of developmental biology. During the course of this work, autofluorescense has been helpful to identify early sexual stages in *A. nidulans*, particularly in the *csn* deletion strains (Fig. 5.2B). Generally, *Aspergillus* species produce autofluorescing substances (Mann 1983), but it was not yet examined whether quantity and quality of luminescence under the fluorescence microscope is identical in wild-type and *csn* mutant strains.

The results of this study show that the cross-pathway control negatively controls maturation processes whereas the COP9 signalosome is essential for either the onset or the progression of maturation. It was discussed above that a link between cross-pathway control and COP9 signalosome is conceivable. If a conserved function of the CSN in *A. nidulans* is proposed and an analogy to the mammalian system is taken into account, a stabilisation effect of COP9 signalosome towards CPCA should be examined (1.2.2.3.). An interplay of both regulatory networks would be of special interest regarding the control of sexual development. During the course of this work, an *A. nidulans csnD*/*cpcA* double deletion strain (AGB204) was constructed by crossing experiments (AGB192 x AGB52), that completely resembled the Δ*csnD* mutant phenotype. It should be questioned whether an *A. nidulans* strain deleted for a CSN subunit but with overproduction of CPCA still blocks sexual development at the level of primordia like a *csnD* deletion strain or proceeds until formation of micro-cleistothecia like a CPCA overproduction strain. Additionally, it would be interesting to question whether a modulated CSN activity in any case leads to aberrant cleistothecia lacking either solely the wall or the ascospores. However, a simple overexpression of single COP9 signalosome subunits is not promising and thus artificial modulation of overall CSN activity seems a challenge *per se*.

5.2.2 Cross-connections of secondary metabolism and sexual development

It is striking that changes in single enzyme activities within two different regulatory networks, the cross-pathway control and the COP9 signalosome-dependent system, impact sexual development as well as production of secondary metabolites in *A. nidulans*. Taken into account that in filamentous prokaryotes like *Streptomyces coelicolor* a biochemical transition from primary to secondary metabolism generally coincides with the onset of morphological differentiation (Vohradsky *et al.* 1997; Onaka *et al.* 1998), it might be worthwhile to question the molecular relationship between metabolism and development in filamentous fungi. Such cross-connections may rely on production of specific signal molecules like the FluG, PsiC and auxin (see 1.3.2.2), or on a co-regulation of metabolic and developmental pathways by a shared regulator. A well-studied example for a correlation between development and secondary metabolite production in *A. nidulans* is the proposed common regulation of the onset of asexual sporulation and biosynthesis of sterigmatocystin (see 1.3.2.1) (Adams and Yu 1998; Guzman-de-Pena *et al.* 1998). It is hypothesised that sterigmatocystin and conidiospore

production share a regulatory signal transduction cascade including FLBA as a mediator of G-protein signalling (Yu *et al.* 1996), the G-protein subunits FADA and SFAD (Hicks *et al.* 1997; Rosen *et al.* 1999; Tag *et al.* 2000) and the cAMP-dependent protein kinase PKAA (Shimizu and Keller 2001). Both, sporulation and mycotoxin production, require inactivation of the FLBA/FADA-dependent signalling pathway (Hicks *et al.* 1997). In *A. nidulans* strains with an activated cross-pathway control, the sexual cycle is arrested (Chapter 2) and penicillin production is neglected (Chapter 3). This raises the important question whether these physiological and morphological phenotypes correlate, similar as described for sterigmatocystin biosynthesis and sporulation. It might be revealing to determine the penicillin titre in other acleistothecial or hypercleistothecial *A. nidulans* strains.

The developmental arrest in *cpcA*-overproduction strains (Chapter 2) might be due to either a specific effect of the CPCA transcriptional activator on a developmental regulator or to a general shortage of primary and secondary metabolites caused by activation of the cross-pathway control. It might be worthwhile trying to release the developmental block in amino acid starved mycelia by known signal molecules. A promising candidate might be auxin: recently, this tryptophan-related secondary metabolite was proven to promote development in *A. nidulans*. External supply of either high amounts of tryptophan or low amounts of auxin restored the developmental block of tryptophan-auxotrophic strains (Eckert *et al.* 1999; Eckert *et al.* 2000). It should now be tested whether auxin also enables maturation of cleistothecia in other amino acid auxotrophic strains, like the *hisB* deletion strain supplemented with low histidine concentrations, or in a *cpcA* overproduction strain.

A. nidulans csn deletion strains not only arrest sexual development, but also produce an aberrant red pigment in some hyphae which indicates that secondary metabolism is affected. It is questionable whether de-repressed pigment synthesis in the *csnD* deletion strains somehow contributes to the block in sexual development. For the tyrosine-related pigment melanin, a correlation between defective hyphal melanin synthesis and defective sexual reproduction was described (Pirt and Rowley 1969; Polacheck and Rosenberger 1977). It might be revealing to delete the *csn* genes in *A. nidulans* strains with defects in its ascospore pigment production. Strains mutated in the *clA*/*clB* or *blA* loci produce colourless or blue cleistothecia, respectively (Apirion, 1963). It is promising to examine whether malfunction of the COP9 signalosome in these strains still results in production of a red pigment in hyphae. Possibly, production of the red pigment is not the only pathway of secondary metabolism that is regulated by the COP9 signalosome-dependent network. Preliminary HPLC analysis of mycelia from the *csnD* deletion strain revealed that the chromatogram of mutant and wild-type strain differ, not in the quantity but in the constituent amount of the metabolites (Dr. H.B. Bode, personal communication). Similarly, for the pre-competence *aco*$^{-}$ mutant strains impaired development as well as overproduction of phenolic substances were described (Butnick *et al.* 1984). Whether these changed metabolic profiles include signal molecules essential for development remains to be elucidated. It might be revealing to supply known developmental signal

molecules, like the PsiC factor or auxin, to surface-grown *csn* deletion strains as a try to restore formation of a rigid wall or ascospores - or even a complete cleistothecia.

system of primary metabolism in yeast, is involved in the regulation of histidine biosynthesis as well as penicillin production and sexual development of the filamentous fungus *Aspergillus nidulans*. The COP9 signalosome-dependent network, known to control development of higher eukaryotes, contributes to regulation of light-dependent signalling, sexual development and pigment production in *A. nidulans*. These simultaneous effects on metabolism and development by both regulatory networks suggest several cross-connections between physiology and morphology. This includes several topics of more general interest, like determination of cell fate during development and the question about the biological role of secondary metabolites. The basic processes of the co-ordinated control of integration of environmental stimuli, physiology and morphology can be studied in the eukaryotic mikroorganism *A. nidulans*, and it will be interesting to transfer the knowledge learned from this model organism to higher eukaryotic systems.

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Curriculum vitae

Silke Busch
Born on March the 12th, 1971
in Vechta, Germany.

Education

1978 - 1984 **Primary education** in Diepholz (GER)

1984 - 1991 **Secondary education** at the Graf-Friedrich-Schule in Diepholz (GER)

Scientific Background

1991 - 1993 **Study** of biology at the Universität Osnabrück (GER)

1993 - 1994 **Study** of biology at the University of Sussex, Brighton (UK)

1994 - 1998 **Study** of biology at the Georg-August Universität Göttingen (GER). **Graduation** in biology with the diploma thesis "Untersuchung des natürlichen DNA-Transformationssystems in *Acinetobacter* sp. BD413: Identifizierung und Charakterisierung der Kompetenzgene *comE* und *comF*" in the Institut für Mikrobiologie und Genetik, Abteilung für Allgemeine Mikrobiologie of Prof. Dr. G. Gottschalk

1998 - 2002 **Scientific assistant** at the Georg-August Universität Göttingen (GER), in the Institut für Mikrobiologie und Genetik, Abteilung für Molekulare Mikrobiologie und Genetik of Prof. Dr. G.H. Braus