The COP9 signalosome of *Aspergillus nidulans* : Regulation of protein degradation and transcriptional pathways in sexual development

PhD Thesis

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Dla mojej Matki

Declaration

I hereby confirm that this thesis has been written independently and with no other sources and aids than quoted.

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Summary

The COP9 signalosome (CSN) is a conserved eukaryotic multiprotein complex, playing a multifaceted role in the regulation of protein degradation by the ubiquitin system. The primary function of CSN is the removal of the ubiquitin-like Nedd8 moiety from the cullin subunits of SCF ubiquitin ligases, termed deneddylation. A complete conserved CSN was previously isolated in the model filamentous fungus *Aspergillus nidulans*, where it is necessary for sexual fruit body formation and establishment of developmental balance, but not essential for survival, providing an opportunity to study the functions of this complex in a genetically amenable organism.

In the course of this work I have investigated the mechanism of CSN action during *A. nidulans* development using two complementary approaches: mutagenic analysis of the deneddylase-bearing CsnE subunit and transcriptome profiling of a *csnE* deletion strain.

Mutagenesis of the JAMM metalloprotease motif reveals that it is primarily responsible for the *Δcsn* phenotype and therefore the deneddylase activity is probably essential for *A. nidulans* sexual development. Mutated CsnE is still able to interact with CsnF, another CSN subunit, indicating that the phenotype is not caused by impaired complex formation. We additionally show that *rubA*, a close homologue of mammalian Nedd8 is essential for *A. nidulans* growth.

The results of the genome-wide transcriptome profiling of a *csnE* deletion strain show that the fungal CSN affects the regulation of many groups of genes across development. Differentially expressed genes include oxidoreductases, genes involved in cell wall degradation and membrane transport as well as a putative cluster of secondary metabolite biosynthesis genes. CSN is required for developmentally induced expression of *brlA*, the central regulator of asexual sporulation and the hormone synthesis oxygenase *ppoC* involved in regulation of developmental balance. It is also necessary for developmentally induced β-glucanase activity, which might be prerequisite for fruit body formation. Analysis of cis-regulating sequences upstream of regulated genes suggests a possibility of crosstalk between the sexual and asexual signalling pathways. The results obtained in this work provide a basis for further elucidating transcriptional networks acting in *A. nidulans* developmental signalling and afford for insights into downstream targets of CSN in eukaryotes.

Abbreviations

4-NQO	4-nitroquinoline 1-oxide
APC	anaphase-promoting complex
aRNA	antisense amplified aminoallyl-RNA
ATP	adenosine tri-phosphate
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
bp	base pair
CDK	cyclin-dependent kinase
cDNA	complementary DNA
COP9	constitutive photomorphogenesis
CRL	cullin-RING-finger ligase
CSN	COP9 signalosome
DHN	dihydroxynaphthalene
DMF	dimethyl fluoride
EDTA	ethylenediamine tetraacetic acid
eIF3	eukaryotic translation initiation factor 3
EST	expressed sequence tag
GPCR	G-protein coupled receptor
GPI	glycosyl-phosphatidylinositol
HECT	homologous to E6-associated protein C-terminus
JAMM	Jab1/MPN domain
kbp	kilobasepair
kDa	kilodalton
LB	lysogeny broth / Luria Bertani medium
MAPK	mitogen-activated protein kinase

MFS	major facilitator superfamily
MPN	Mpr1p, Pad1 N-terminal
mRNA	messenger RNA
NADP	Nicotinamide adenine dinucleotide phosphate
NHS	N-hydroxysuccinimidyl
ORF	open reading frame
PARP	poly-(ADP-ribose)-polymerase
PCI	proteasome, COP9, eIF3
PCR	polymerase chain reaction
Psi	precocious sexual inducer
REMI	restriction enzyme-mediated mutagenesis
RGS	regulator of G-signalling
RING	really interesting new gene
ROS	reactive oxygen species
rRNA	ribosomal RNA
S	Svedberg sedimentation rate
SCF	Skp1/Cdc53/F-box
SDS-PAGE	sodium dodecyl-sulphate polyacrylamide gel electrophporesis
SSC	sodium chloride / sodium citrate buffer
ST	sterigmatocystin
STRE	stress response element
SUMO	small ubiquitin-like modifier
Ubl	ubiquitin-like protein

CHAPTER 1: INTRODUCTION

1.1. Regulated protein turnover

Multicellular organisms accomplish a plethora of differentiation programmes in response to a variety of environmental inputs. Cellular differentiation relies on precise and controlled means of gene expression and protein turnover to ensure a flexible and defined spatiotemporal expression of gene products. To achieve this, several levels of regulation are employed, such as regulation of transcription initiation, mRNA localisation and stability, translational regulation, posttranslational modifications and variable turnover rates of proteins. The regulated protein degradation has been a focus of intensive research in recent years, resulting in identification of the ubiquitin-proteasome system as a general mechanism in eukaryotic cellular and developmental regulation (reviewed by Glickman and Ciechanover, 2002; Hochstrasser, 1996). The specific ubiquitin-proteasome system is exclusive to eukaryotes, although archaea possess a simplified structure homologous to the proteasome core (Puhler *et al.*, 1992) and a deubiquitinating enzyme has been described in pathogenic *E.coli*. with homologues in other prokaryotes (Catic *et al.*, 2007)

1.1.1. Ubiquitin-dependent protein degradation

Protein substrates are targeted for degradation by conjugation with ubiquitin, a highly evolutionarily conserved 76-residue polypeptide, added as polyubiquitin chains. Conjugation to the protein substrate proceeds via a three-step cascade mechanism. Initially, the ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-requiring reaction, generating a high-energy thiol-ester E1-S~ubiquitin intermediate. One of several ubiquitin-conjugating enzymes (E2) then transfers the activated ubiquitin from E1 via another high energy intermediate to the substrate protein. The E2 enzymes act in conjunction with an array of E3 ubiquitin ligases, which confer specificity by binding to specific degradation signals on protein substrates. There are two major classes of E3 enzymes, differing in their mode of ubiquitin transfer. HECT domain E3s bind ubiquitin via an active site Cys residue forming a third high energy intermediate before it is attached to the substrate, while RING finger-containing E3s mediate direct transfer of the activated ubiquitin from E2 to the substrate. In both cases, the ubiquitin ligase complex catalyzes a covalent attachment of a polyubiquitin chain to a ε-NH_2 group of a lysine residue of the target protein via an isopeptide bond. After multiple rounds of ubiquitin ligation, a multiubiquitin chain is synthesised, in which the C-terminal residue of each ubiquitin is linked to a lysine (K48) residue of the preceding ubiquitin molecule in the same way.

Polyubiquitinated target protein is recognized by a specific receptor on the proteasome and degraded, releasing free ubiquitin (Glickman and Ciechanover, 2002; Hochstrasser, 1996).

The 26S proteasome specifically degrades ubiquitinylated proteins to small peptides, forming the hub of protein degradation machinery. It consists of two subcomplexes, the 20S core particle conferring the catalytic activity and the 19S regulatory particle mediating substrate specificity and unfolding ubiquitinylated substrates for degradation. The S19 particle is composed of two eight-subunit subcomplexes: the base, which contains ATPase subunits and the lid, which can associate or disassociate from the proteasome. Numerous associated proteins and auxiliary factors can additionally interact with the regulatory particle. Both proteolysis itself and protein unfolding require energy provided by ATPase subunits in the base of the 19S particle. The structure of the 26S proteasome is reviewed in detail in (Bochtler *et al.*, 1999).

Ubiquitin-mediated proteolysis of endogenous proteins plays an important role in a variety of cellular processes and is one of the primary mechanisms governing passage through the cell cycle and division (Deshaies 1997). Other processes involving controlled protein degradation include differentiation and development, response to stress and extracellular effectors, regulation of ion channels and the secretory pathway, DNA repair, transcriptional regulation and circadian rhythms. A list of proteins targeted by the ubiquitin system is still growing and includes, among others, cyclins, cyclin-dependent kinase inhibitors, tumour suppressors and

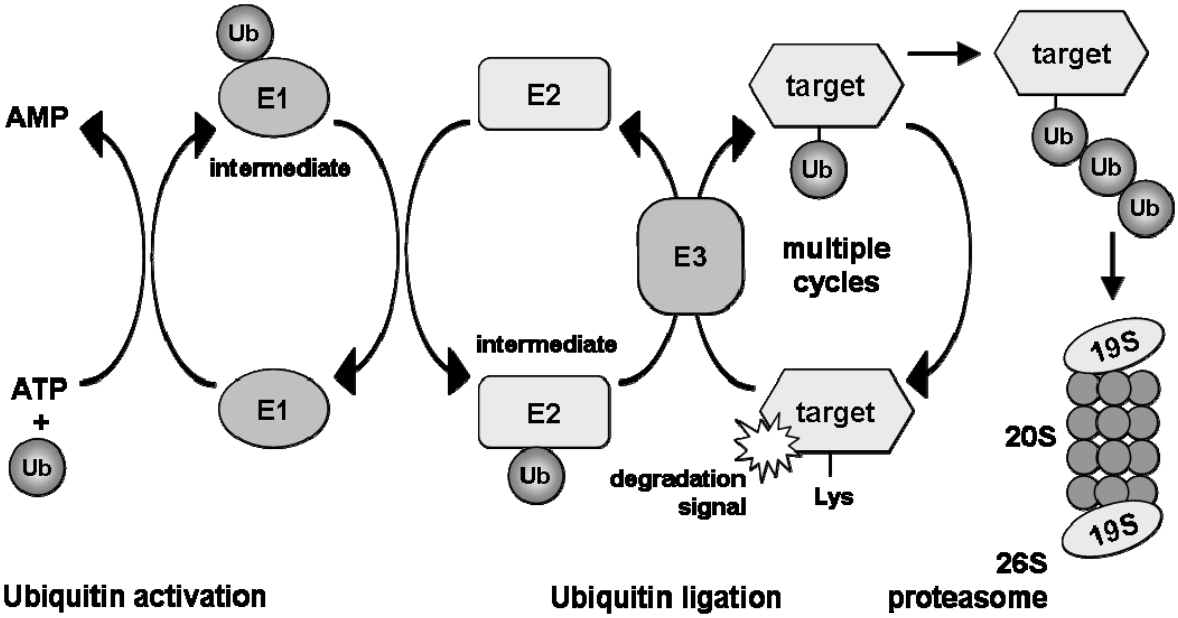


Figure 1. Ubiquitin-dependent protein degradation pathway. Ubiquitin is activated by the E1 enzyme in an ATP-dependent manner, transferred via a high-energy intermediate to one of E2 enzymes and conjugated to a lysine on a target protein by one of E3 ubiquitin ligase complexes. Multiple cycles of ubiquitin ligation result in polyubiquitin chain formation. Ubiquitination requires a specific degradation signal. The ubiquitin chain is recognized by a receptor on the 26 proteasome and the ubiquitinated protein is degraded.

transcriptional regulators. The question of how the ubiquitin system achieves its high specificity and selectivity is the focus of current investigation. Substrates must be specifically recognized by the appropriate E3 ligase as a prerequisite to their ubiquitination. To achieve recognition the substrate, the E3 enzyme or both must usually undergo a modification, such as substrate phosphorylation or oligomerization. The stability of some transcription factors can also depend on their dissociation from DNA.

1.1.2. Ubiquitin ligases and their regulation

The ubiquitin system has a hierarchic structure. A single E1 activates the whole pool of cellular ubiquitin and interacts with all of several E2 enzymes. Each E2 in turn interacts with several E3s, each of which targets several substrates for ubiquitination by means of one of many adaptor subunits. E3 ubiquitin ligases play a key role in the ubiquitin-mediated proteolytic cascade since they serve as the specific recognition factors of the system. They display the greatest variety among its components and are heterogeneous, but can be nevertheless divided into two major groups differing in structure and mode of action.

Of many E3 ligases known, the anaphase-promoting complex (APC) and cullin-RING-finger ligase (CRL) complexes are most prominent. A few of them are monomeric, such as Mdm2 (Schoenfeld *et al.*, 2000) which contains the RING finger and the substrate binding site in the same molecule. Most of them, however, have a modular structure composed of four subunits. The archetypal CRL complex, SCF (Skp1/Cdc53/F-box), involved in degradation of signal- and cell-cycle induced phosphorylated proteins, is the best described of four types of multimeric CRLs (Strack *et al.*, 2000). The cullin (Cul1) has a curved, yet rigid, N-terminal stalk, which binds Skp1 (S-phase kinase-associated protein), whereas the RING-H2 domain subunit (Roc1/Rbx1/Hrt1) interacts with the C-terminal globular domain. Skp1 recruits the F-box specific substrate receptors and the RING subunit recruits the ubiquitin-conjugating enzyme (E2) to form the active ligase complex (Figure 2). The F-box protein is responsible for substrate recognition and binding using one of many protein-protein interaction motifs and because it is the most variable component, different SCF complexes are designated by their accessory F-box subunit (e.g. SCF^{Cdc4}). The number of F-box and other adapter proteins, and therefore the potential number of CRLs, is staggering. Whereas *S. cerevisiae* has 18 characterised or putative adaptor subunits and at least 11 different known complexes, higher eukaryotes may contain hundreds, as evidenced by nearly 700 F-box proteins encoded by *A. thaliana* genome (Gagne *et al.*, 2002).

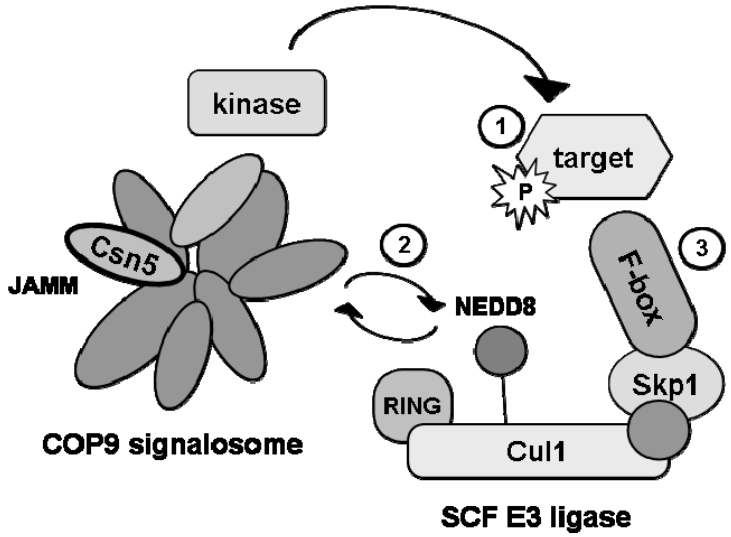


Figure 2. SCF ubiquitin ligase and regulation of its activity. The four subunits of a typical E3 ligase are shown, using the Cul1-containing SCF ligase as an example. The activity of SCF towards its substrate is regulated in three ways: 1. substrate phosphorylation, 2. cullin neddylation and deneddylation and 3. control of F-box protein stability.

Distinct ubiquitin ligases recognize different degradation signals, and therefore target distinct subsets of proteins for degradation. The activity of SCF towards its ubiquitinylated substrates is regulated mainly via timely phosphorylation of the substrates and posttranslational modification of cullins (see 1.1.4), but also on the level of F-box protein stability. Phosphorylation is a widespread regulatory mechanism and targets numerous substrates to SCF ligases, including the cyclin-dependent kinase (CDK) inhibitor Sic1 and the central transcriptional activator Gcn4 to SCF^{Cdc4} in *S. cerevisiae* (Meimoun *et al.*, 2000; Nash *et al.*, 2001), cyclin E to SCF^{Cdc4} and the CDK inhibitor p27 to SCF^{Skp2} in humans (Tsvetkov *et al.*, 1999). On the other hand, the substrate receptors of CRLs are often unstable, sometimes dependent on their subcellular localisation. This is most evident in *S. cerevisiae*, in which F-box proteins are degraded with a half-life of 5–30 minutes after assembly with Skp1 (Zhou and Howley, 1998). Although the destabilization and setting the steady-state level of receptors was proposed to occur primarily by autoubiquitinylation, sometimes they are turned over by pathways requiring other CRLs (reviewed by Deshaies, 1999).

1.1.3. Ubiquitin-like proteins

In recent years, a number of ubiquitin-like proteins (Ubls) have emerged in all eukaryotic organisms, often significantly diverged from ubiquitin itself but also covalently modifying other proteins via Ubl-actvating and -conjugating enzymes (reviewed by Kerscher *et al.*, 2006). Nedd8/Rub1 is a small ubiquitin-like protein, which was originally identified as a developmentally down-regulated in the mouse (Kumar *et al.*, 1993). It was later found to be conjugated to Cdc53, a cullin component of the SCF ligase complex in *S. cerevisiae* (Lammer *et al.*, 1998) and neddylation plays a role in regulating the activity of ubiquitin ligases (see below). SUMO, originally called Ubl1 or sentrin (Shen *et al.*, 1996), was first described as a conjugate of RanGAP1, a regulator of protein and ribonucleoprotein transport across the

nuclear pore complex (Matunis *et al.*, 1996). Since then SUMO was implicated in various other cellular processes, such as transcriptional regulation, apoptosis, protein stability, cell cycle and response to stress (reviewed by Kroetz, 2005). Other Ubls include Urm1, which functions during invasive growth and budding in yeast and shows similarity to prokaryotic proteins essential for molybdopterin and thiamine biosynthesis (Furukawa *et al.*, 2000; Goehring *et al.*, 2003) and two interferon-inducible modifiers. ISG15 plays a role in immune response and cancer and functions as an antiviral molecule (Dao and Zhang, 2005; Lenschow *et al.*, 2007) while Fat10, consisting of two ubiquitin-like domains, is involved in activation of apoptosis (Liu *et al.*, 1999; Raasi *et al.*, 2001). Finally Apg12, although not related to ubiquitin, is conjugated by a similar mechanism and important in autophagy in yeast and mammals (Mizushima *et al.*, 1998a; Mizushima *et al.*, 1998b). Most of Ubls are conjugated to lysine residues and competition between ubiquitination and other modifications of the same residue is emerging as an important regulatory mechanism.

1.1.4. Neddylation regulates ubiquitin ligase activity

Nedd8 exhibits more homology to ubiquitin than other ubiquitin-like modifiers, such as SUMO, and is covalently conjugated to other proteins by a mechanism similar of the ubiquitination pathway (Gong and Yeh, 1999; Liakopoulos *et al.*, 1998). Rub1 conjugation requires at least three proteins. Ula1 (APP-BP1 in mammals) and Uba3 are related to the N- and C-terminal domains of the E1 ubiquitin-activating enzyme, respectively, and together activate Rub1 by forming a thiol-ester bond. It is then linked to Ubc12, a protein related to E2 ubiquitin-conjugating enzymes and functioning analogously to E2 enzymes. Finally, neddylation is completed by formation of an isopeptide bond between Nedd8 and a conserved lysine residue. However, no specific Rub1-ligating E3 enzyme has been described up to date.

All members of the cullin family evaluated so far are modified by covalent attachment of Nedd8 to a conserved lysine residue in the cullin homology domain (Hori *et al.*, 1999). Neddylation enhances Cul1-dependent ubiquitin-ligase activity *in vitro* (Podust et al., 2000), possibly by facilitating the recruitment of ubiquitin-loaded E2s. Genetic studies indicate that neddylation is crucial for function of Cul1 *in vivo* and accordingly, Nedd8 is essential in numerous organisms from *S. pombe* to mice (Osaka *et al.*, 2000; Tateishi *et al.*, 2001), with the notable exception of *S. cerevisiae* (Lammer et al., 1998). Typically only a fraction of the total cullin pool is neddylated, which indicates that perhaps only a subset of cullin molecules is fully active. Nedd8 conjugated to cullins is detached by the COP9 signalosome (CSN) in a process known as deneddylation (see 1.2.2). Paradoxically, genetic studies indicate that CSN is a positive regulator of CRLs, in that loss-of function mutations in CSN subunits mimic or

enhance the detrimental effects of mutations in CRL subunits (Cope *et al.*, 2002; Pintard *et al.*, 2003). It led to a theory that dynamic cycles of Nedd8 attachment and removal might be required to sustain CRL function. This theory is supported by the role of Cand1 (cullin-associated and neddylation-dissociated protein-1), a protein that specifically associates with deneddylated cullins to sequester them in an unassembled and inactive state (Zheng *et al.*, 2002). Binding of Cand1 to Cul1, mutually exclusive with the COP9 signalosome interaction, enhances deneddylation of the cullin, thus potentially facilitating the neddylation cycle (Min *et al.*, 2005). Putative Cand1 homologues have been identified in most eukaryotic model organisms, except for *S. cerevisiae*.

Until recently, the only reported Nedd8 substrates were cullins, but Xirodimas *et al.* (2004) showed that p53 tumour suppressor can be neddylated by the Mdm2 ligase, which inhibits its transcriptional activity. Mdm2 might thus constitute a common component of the ubiquitin and NEDD8 conjugation pathway and suggests the existence of diverse mechanisms involving interplay between ubiquitination, phosphorylation and neddylation, by which E3 ligases can control the function of substrate proteins.

1.2. COP9 signalosome

1.2.1. Architecture and conservation

The COP9 (constitutive photomorphogenesis 9) signalosome, also called, CSN is a multiprotein complex highly conserved in eukaryotes and initially discovered in *A. thaliana* as a suppressor of light-dependent development (Wei *et al.*, 1994) and subsequently isolated from human cells (Seeger *et al.*, 1998). Together with the 19S proteasome lid (see 1.1.1) and the eukaryotic translation initiation factor 3 (eIF3), the CSN forms a conserved family of PCI multiprotein complexes sharing common architecture. Their subunits contain either a PCI (proteasome, COP9, eIF3) domain or an MPN (Mpr1p, Pad1 N-terminal) domain, which are thought to stabilise protein-protein interactions, although the mechanism of their actions remains unclear (Glickman *et al.*, 1998; Kim *et al.*, 2001). In higher eukaryotes, COP9 has a size of between 450 kDa in mammals and 500 kDa in plants, is localised mostly in the nucleus and is composed of eight subunits named CSN1 to CSN8 (Deng *et al.*, 2000). CSN5/Jab1 is, together with CSN2, the most conserved subunit and the only subunit conserved among all eukaryotes including budding yeast (Maytal-Kivity *et al.*, 2003). Six of the subunits contain PCI domains and two (CSN5 and CSN6) contain MPN domains. This subunit composition is similar to the architecture of the 19S proteasome lid, with Rpn7, 6, 3, 5, 11, 8, 9 and 12 corresponding to CSN1-8 (Kim *et al.*, 2001). Electron microscopy studies, however, revealed

that the overall architecture of CSN and LID are not identical, although they share an asymmetric shape enclosing a central groove (Kapelari *et al.*, 2000).

Apart from the sequence homologies, components of the mammalian proteasome lid and eIF3 sometimes co-purify with CSN (Seeger *et al.*, 1998). The CSN subunits form a complex interaction map with each other as well as with a multitude of other proteins (Figure 3) (reviewed by Wei and Deng, 2003). CSN subunits in plants and mammals are known to interact directly with the proteasome (Kwok *et al.*, 1999) and, in all investigated model organisms, with RING domain E3 ubiquitin ligases (Schwechheimer *et al.*, 2001; Suzuki *et al.*, 2002). Two of the subunits, CSN2 and CSN7, are phosphorylated, presumably by one of kinases associated with the complex, which include the inositol 1,3,4-triphosphate 5/6 kinase, as well as protein kinases C and D (Sun *et al.*, 2002; Uhle *et al.*, 2003). Besides the eight-subunit holocomplex, smaller subcomplexes, often localised in the cytoplasm and usually containing CSN5 have been reported in mammalian cells (Tomoda *et al.*, 2002; Tomoda *et al.*, 2005). They seem to play independent roles in the regulation of protein degradation and localisation of ubiquitinylated substrates.

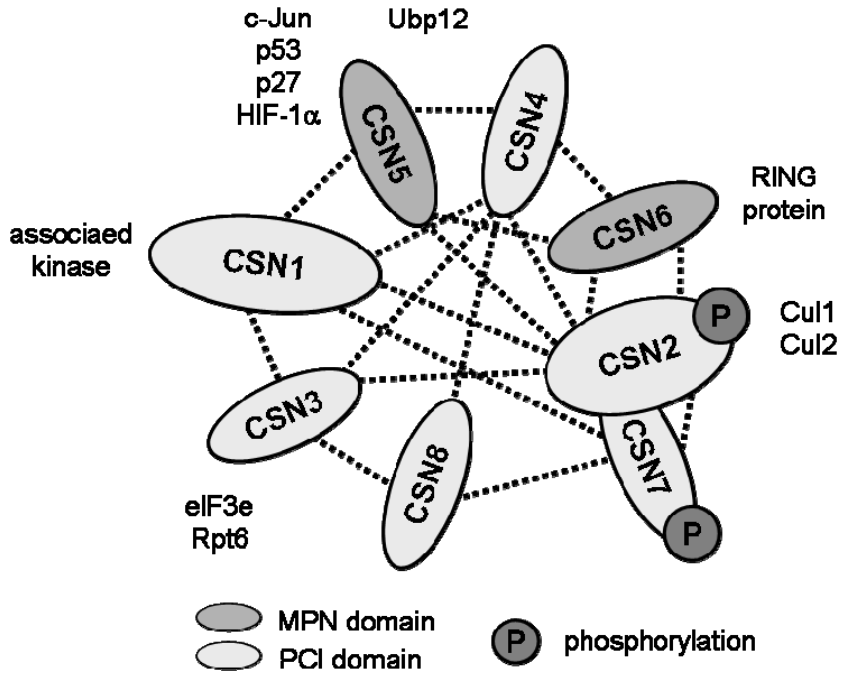


Figure 3. The COP9 signalosome architecture and subunit interactions. Experimentally demonstrated protein-protein interactions between the subunits are marked with dotted lines (Fu *et al.*, 2001). Several selected interactions with other proteins are shown. CSN1 associates with inositol 1,3,4-triphosphate 5/6 kinase, CSN2 with E3 ligase cullin subunits, CSN3 with subunits of the eiF3 (sIF3e) and 19S proteasome lid (Rpt6), CSN5 with c-Jun transcription factor, p27 CDK inhibitor, p53 tumour suppressor and HIF-1a hypoxia-inducible factor, CSN with RING-domain E3 ligase subunit. CSN complex associates with deubiquitinylating enzyme Ubp12. Modified from (Kapelari *et al.*, 2000).

1.2.2. Deneddylation, phosphorylation and JAMM

The CSN functions by regulating ubiquitin-dependent protein degradation on various levels. As mentioned above, the COP9 signalosome is involved in deneddylation of the cullin subunits of RING-H2 E3 ubiquitin ligases, as shown first for Cul1 (Lyapina *et al.*, 2001; Zhou *et al.*, 2001) and regulating their activity towards the substrate proteins. The deneddylase catalytic activity is intrinsic to the CSN and resides in CSN5 subunit, but requires the assembled complex to function (Lyapina *et al.*, 2001). Cleavage of Nedd8 is not achieved by a conventional thiol-protease but by a metalloisopeptidase activity located in the so-called Jab1/MPN or MPN+ domain, conserved between pro- and eukaryotes, including all Csn5 and proteasome lid Rpn11 orthologs. The domain contains a His-X-His-X_{10}-Asp motif, accompanied by an upstream conserved Glu (Figure 4), termed JAMM (Jab1/MPN domain metalloenzyme). The JAMM motif is essential for CSN function in Drosophila development (see 1.2.3), but studies in *S. pombe* showed that mutations in this motif do not impair complex formation (Cope *et al.*, 2002). Structural studies of the prokaryotic JAMM domain revealed that it possesses key features of a zinc metalloprotease, with active site His and Asp residues coordinating a zinc ion and Glu serving as an acid-base catalyst (Ambroggio *et al.*, 2004; Tran *et al.*, 2003). Nevertheless, the knowledge about the structural context of the domain within the CSN complex is still lacking.

In addition to deneddylation, CSN mediates phosphorylation and deubiquitination of substrates, which affects their fate toward the proteasome system and consequently stability and

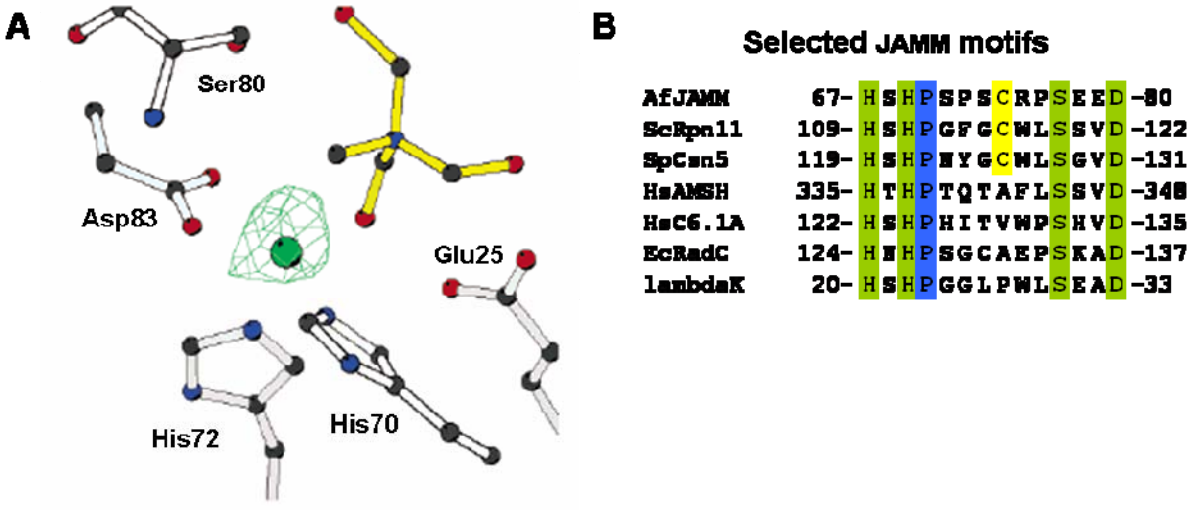


Figure 4. The JAMM motif structure and conservation. (A) A view of the Jab1/MPN active site of the *Archeoglobus fulgidus* AF2198 protein. The zinc ion coordinated by the histidine and aspartate residues is shown as a green sphere in the middle. **(B)** A selection of JAMM motifs from proteins of diverse functions. The canonical JAMM residues are highlighted in green. Af: *A. fulgidus*, Sc: *S. cerevisiae*, Sp: *S. pombe*, Hs: *Homo sapiens*, Ec: *E. coli*. Modified from (Ambroggio *et al.*, 2004; Tran *et al.*, 2003).

activity (Naumann *et al.*, 1999; Sun *et al.*, 2002). The protein kinases shown to associate with the COP9 signalosome (see 1.2.1) phosphorylate proteins such as p53 (Bech-Otschir *et al.*, 2001) and c-Jun (Naumann *et al.*, 1999), altering their susceptibility to ubiquitination and sometimes activity. The CSN inhibits cullin activity by recruiting the deubiquitinating enzyme Ubp12, which counteracts the intrinsic ubiquitin polymerizing activity of the catalytic core (Zhou *et al.*, 2003). On the other hand, CSN-catalysed deneddylation and Ubp12-mediated deubiquitination cooperate in counteracting the instability of Cullin-RING ligase specific adaptors (Wee *et al.*, 2005). The precise coordination of these diverse activities and their overall effect on protein stability are not yet understood. Two general hypotheses of the CSN and its associated activities have been suggested. One stresses the importance of the similarities and interactions between CSN and the 26S proteasome individual subunits, suggesting that it might act as an alternative or additional proteasome lid or alter the activity of the S19 lid complex (Wei and Deng, 1999). It was recently supported by showing that CSN directly interacts with the 26S proteasome and competes with the lid, which has consequences for the peptidase activity of the 26S proteasome *in vitro* (Huang *et al.*, 2005). The second hypothesis suggests that the COP9 signalosome acts as a platform with scaffolding function for many associated enzymes such as kinases, deubiquitinases and E3 ubiquitin ligases (Bech-Otschir *et al.*, 2002).

1.2.3. Function in cellular processes

Since its discovery, the COP9 signalosome has been identified and characterized in mammalian cells, insects, yeasts and fungi as a general regulator of protein degradation and signal transduction (Freilich *et al.*, 1999; He *et al.*, 2005; Mundt *et al.*, 1999; Seeger *et al.*, 1998). Its direct interaction partners and proteins influenced by the modulation of the ubiquitination pathway include transcription factors, cell cycle regulators, hormone receptors and tumour suppressors (reviewed by Wei and Deng, 2003). Not surprisingly, CSN is involved in a wide array of cellular and developmental processes, including cell cycle regulation, MAPK kinase signalling, hormone signalling and DNA repair (Doronkin *et al.*, 2003; Harari-Steinberg and Chamovitz, 2004; Liu *et al.*, 2003; Lykke-Andersen *et al.*, 2003; Naumann *et al.*, 1999; Oron *et al.*, 2002; Schwechheimer *et al.*, 2001). In plants and animals it is an essential regulator of development and its defects result in pleiotropic phenotypes leading to embryonic lethality. In contrast, CSN is not essential for viability in fungi, but is involved in circadian clock regulation, cell cycle and the pheromone response (He *et al.*, 2005; Maytal-Kivity *et al.*, 2002). Recently, a first complete fungal COP9 signalosome was identified and isolated in *Aspergillus nidulans* (Busch *et al.*, 2007).

1.3. *Aspergillus nidulans* physiology and life cycle

1.3.1. *A. nidulans* as a model of eukaryotic cell biology and gene regulation

The aspergilli are a ubiquitous group of filamentous ascomycetes comprising 185 species, many of which are of medical and industrial significance. They are ubiquitous saprophytes equipped with a metabolic versatility, which allows it to survive over a wide range of environmental conditions and nutrient sources. *A. fumigatus* is a major allergen and an opportunistic pathogen, leading to invasive aspergillosis with a high mortality rate in immunocompromised patients (reviewed by Brakhage, 2005). *A. oryzae* and *A. sojae* are widely employed for food refining in Asia, e.g. for the production of sake and soy sauce. *A. niger* is valued in biotechnology for its ability to secrete large amounts of metabolites and is the main industrial producer of citric acid. *A. flavus* is a plant pathogen producing aflatoxin and a subject of research on fungal secondary metabolites.

Within this genus, *Aspergillus nidulans* (previously known as *Emericella nidulans*) has a central role as a model organism, established since the 1950s (Pontecorvo *et al.*, 1953). Research in this fungus has contributed to the understanding of genetic and metabolic regulation (especially carbon and nitrogen regulation), development, cell polarity, cell cycle control, chromatin structure, cytoskeletal function, DNA repair, pH control and human genetic diseases. It grows as a haploid mycelium, allowing easy selection of mutants and Mendelian analysis in a single generation. In contrast to other aspergilli, *A. nidulans* possesses a well-characterized sexual cycle and thus a well-developed genetic system. The analysis of *A. fumigatus* and *A. oryzae* genomes, however, implies they might be capable of sexual reproduction and suggests descent from a sexually-reproducing ancestor (Galagan *et al.*, 2005).

The whole genome of *A. nidulans* strain FGSC A4 has been recently sequenced as a part of the Fungal Genome Initiative at the Broad Institute (Cambridge, MA, USA) in collaboration with Monsanto (St. Louis, MO, USA). The 13X assembly comprising 96,3% of the complete genome was released in March 2003 and was subject to three rounds of automated gene annotation. The size of the *A. nidulans* genome is relatively small; approximately 30,6 megabases (Mb) distributed over 8 well-marked chromosomes containing an estimated 10,000-11,000 genes. The latest round of automated annotation has predicted 10,701 protein-coding genes. As of 2003, about 900 genes have been identified in *A. nidulans* by conventional matings, 432 have been mapped to locus, and 254 are cloned and sequenced according to the Broad Institute. A comparative study of *A. nidulans*, *A. fumigatus* and *A. oryzae* revealed that *A. nidulans* shares only 67% amino acid identity with the two other species across all predicted

orthologous proteins, although the three genomes show extensive synteny (Galagan *et al.*, 2005). These differences can be attributed to intensive rearrangement in regions enriched for secondary metabolite genes thought to have a role in niche adaptation in virulence, facilitating species-specific evolution. Genome analysis also revealed a number of conserved putative regulatory sequences, as well as stressed the importance and wide scope of translational regulation by upstream open reading frames in fungi.

Availability of genome information enables analyses on genome level and high-throughput studies on a previously impossible scale. In recent years a few large-scale transcription experiments were conducted in *A. nidulans*, mostly using as probes polymerase chain reaction (PCR) products from expressed sequence tag (EST) collections and thus limiting the number of genes studied (Sims *et al.*, 2004). The advent of genome sequencing has brought on more comprehensive studies (Mogensen *et al.*, 2006) and resulted in the establishment of at least two genome-wide DNA microarray platforms for analysis of transcriptional changes (David *et al.*, 2006; PFGRC, 2005), one of which is used in the course of this work.

1.3.2. Germination and vegetative growth

A. nidulans is a fast growing saprophytic soil organism. A single colony is capable of hyphal growth as a haploid mycelium, as well as reproduction and can be regarded as a developmental unit. This filamentous fungus is able to reproduce both asexually and sexually and in both cases development includes steps of increasing morphological complexity (see Figure 5). These processes require coordinated spatiotemporal regulation of cellular processes in order to differentiate complex three-dimensional structures. An important switch for perception of extracellular signals and subsequent transduction of this signal are heterotrimeric G proteins composed of α, β and γ subunits, for example Ras proteins. Activated Ras proteins stimulate the production of regulatory cAMP, which activates protein kinase A that in turn modifies the activity of specific transcription factors by phosphorylation. A regulatory role in the integration of carbon source availability and the onset of germination in *A. nidulans* is proposed for RasA protein. High levels of RasA are initially essential for germination, but subsequent gradual decrease in its activity is required for further development (Som and Kolaparthi, 1994).

When the dormant asexual or sexual spore germinates, it re-enters the cell cycle and goes through a short period of isotropic swelling. A key feature of germination is the switch from isotropic swelling to polarized growth by apical extension and mitotic cell divisions leading to the emergence of the germ tube and the formation of the multinucleate vegetative mycelium. Polarised growth requires the gene products of the *pod*, *sep* and *hyp* genes (Harris *et al.*, 1999;

Kaminskyj and Hamer, 1998), a mitogen-activated protein kinase MpkA (Bussink and Osmani, 1999) and the protein phosphatase PphA (Kosmidou *et al.*, 2001).During growth, perforated septae are formed, isolating 3-4 nuclei per cell, which presumably requires a mitotic division (Momany *et al.*, 1999). The cytoskeletal transport system is crucial for delivery of vesicles containing cell wall material and exoenzymes during cell wall formation at the growing tip. Major components of this system are the structural tubulins and actins as well as motor proteins like dyneins and kinesins. Microtubule-associated transport is also essential in nuclear positioning in growing hyphae and has been extensively studied (reviewed by Xiang and Fischer, 2004). Nuclear distribution is affected in mutants of the *nud* (Ahn and Morris, 2001) and *tub* genes (Kirk and Morris, 1991) resulting in vegetative growth defects. The hyphae can establish new polarity axes and branch laterally after breaking down the cell wall material at the branching point (Momany *et al.*, 1999).

A. nidulans mycelium can grow as a homokaryon or heterokaryon (containing genetically different nuclei in one compartment after hyphal fusion), and diploids can be spontaneously generated at low frequency by fusion of nuclei. The combination of heterokaryon and diploid formation enables the fungus to undergo non-sexual genetic variation via a mitotic crossing-over in a so-called parasexual cycle, a phenomenon which has been extensively used for genetic mapping (Clutterbuck, 1992). Mitotic recombination was shown to accelerate adaptation in *A. nidulans* (Schoustra *et al.*, 2007).

1.3.3. Competence establishment and balance of developmental programs

Hyphae establish developmental competence for further differentiation about 16 to 20 hours after germination. Environmental factors, such as temperature, nutritional status and cell density are known to be involved in this time-dependent process, but little is known about the underlying molecular mechanisms and only a few *aco* genes contributing to the establishment of competence have been identified (Butnick *et al.*, 1984). Prerequisite for further development of the competent mycelium is an air/medium interface, though some extreme conditions allow differentiation in submerged culture. *A. nidulans* is able to reproduce asexually via uninuclear conidiospores and sexually, forming complex fruiting bodies called cleistothecia, which contain binucleate meiotic ascospores. An overview of the life cycle is presented in Figure 5. The two propagation cycles have the potential to proceed independently, though only extreme conditions lead to formation of exclusively conidiospores or cleistothecia.

A number of environmental and genetic factors govern the determination of developmental fate and morphogenesis of reproductive organs, with aeration and light being the main physical

stimuli. Aeration directs the developmental program towards conidiation, whereas high CO_2 content induces fruit body formation (Axelrod *et al.*, 1973). Light influences the asexual / sexual development ratio and *A. nidulans* produces predominantly conidiospores when exposed

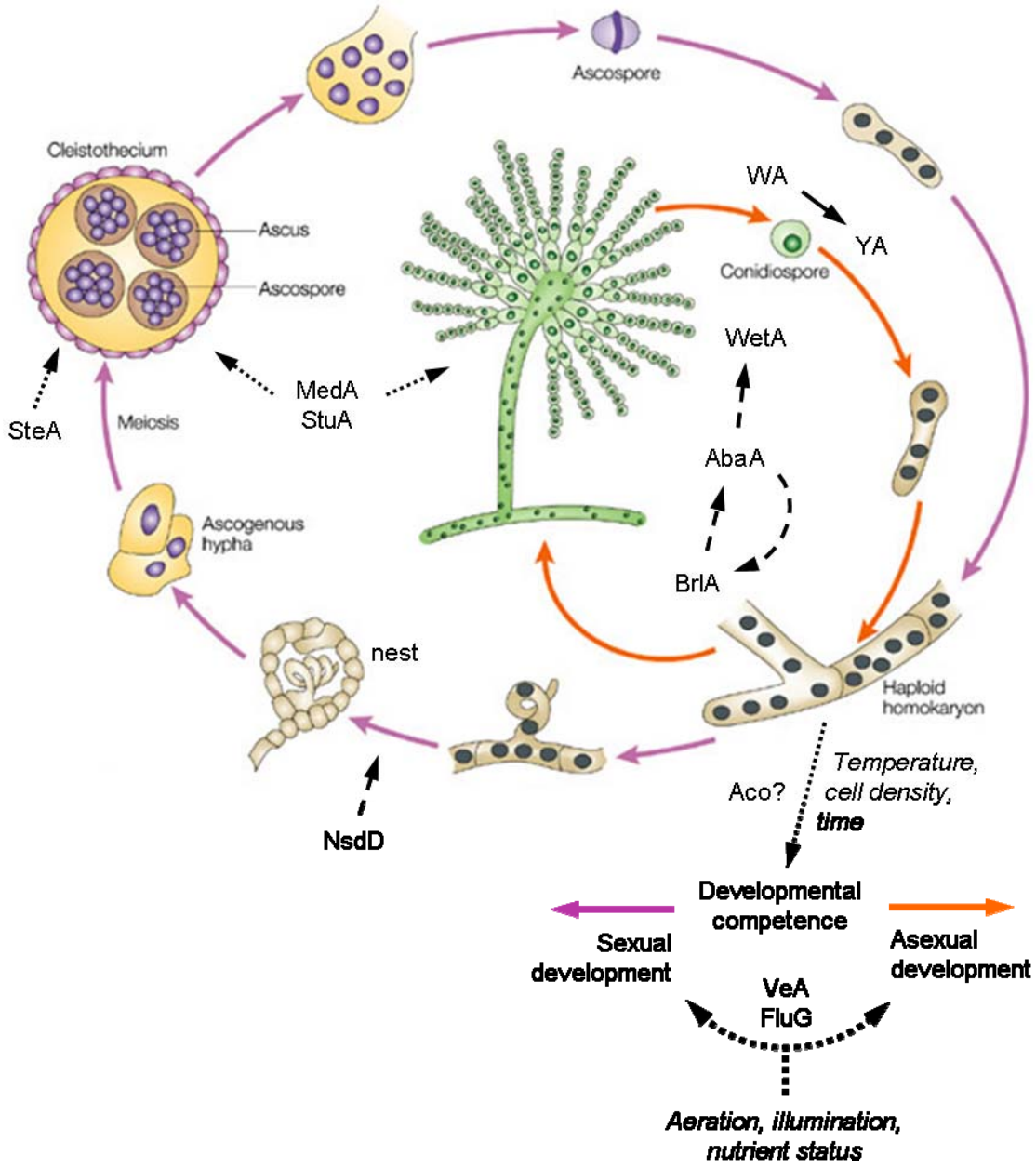


Figure 5. *Aspergillus nidulans* life cycle and some aspects of its regulation. After achieving developmental competence by the growing mycelium, asexual cycle (orange) or sexual cycle (purple) can be initiated, usually in parallel, leading to the production of mitotic conidiospores or meiotic ascospores, respectively. A number of environmental and genetic factors govern the determination of developmental fate and morphogenesis of reproductive organs. Arrows show biochemical or morphological processes, whereas dashed arrows show transcriptional dependency and dotted arrows show an uncharacterized regulation. Modified from (Casselton and Zolan, 2002). Aco proteins are important at the stage of competence establishment, VeA is involved in receiving the light signal (see 1.3.3) and FluG is required for synthesis of a signalling molecule playing a role in developmental activation (see 1.4.2). BrlA, Aba and WetA constitute the central asexual regulatory pathway while WA and YA play a role in conidiophore maturation (see 1.4.1). MedA and Stua are spatiotemporal developmental modulators (see 1.4.3) and both NsdD and SteA are transcription factors regulating exclusively the sexual cycle (see 1.4.4).

to light and denser cleistothecia but less conidia when incubated in the dark for the first 24 hours after inoculation (Zonneveld, 1977). Specifically, asexual sporulation is stimulated and sexual development repressed by red light, reminiscent of a phytochrome response. Indeed, a fungal bacterial-like phytochrome FphA was shown to acts as a red-light sensor and repress sexual development under red-light conditions (Blumenstein *et al.*, 2005). Depending on exposure to light, it seems to exist in two interconversible states, either promoting conidiation (red light) or fruit body formation (far-red light). After light is reception by a phytochrome, the downstream developmental response to the light signal is mediated by the *veA* gene product (Mooney *et al.*, 1990)(see Figure 5), whose expression is significantly increased during sexual development (Kim *et al.*, 2002). Many of the laboratory strains carry the *veA1* mutation, causing induction of asexual sporulation independently of light, indicating that VeA represses initiation of asexual development.

1.3.4. Asexual reproduction

During the asexual reproductive cycle, *A. nidulans* differentiates mitotically derived conidiospores about 48 hours after germination. Formation of the asexual developmental unit, the spore-bearing conidiophore follows a linear cascade of morphological events and has been intensively studied (as reviewed by Adams *et al.*, 1998). The development is initiated when the vegetative mycelium differentiates a foot cell, from which a stalk develops by vertical apical extension. A spherical vesicle is formed at the end of the stalk, from which a layer of cells grows by budding similar to yeast pseudohyphal growth. The budding is accompanied by mitotic divisions, resulting in two layers of uninucleate sterigmata, called metulae and phialides. Their budding at the tip results in isogenic haploid conidiospores enclosed by a thick hydrophobic cell wall. The distal spores arrest in G1 phase and become green coloured by a characteristic polyketide pigment. Each phialide mother cell produces long chains of

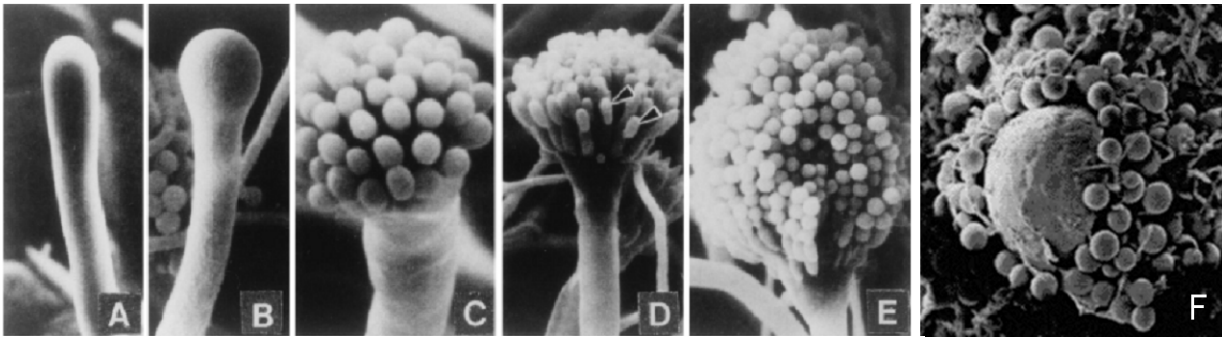


Figure 6. Morphological changes during A. nidulans conidiophore and fruit body formation.
(A) Early conidiophore stalk. **(B)** Vesicle formation from the tip of the stalk. **(C)** Developing metulae. **(D)** Developing phialides. **(E)** Mature conidiophores bearing chains of conidia. **(F)** Mature cleistothecium surrounded by Hülle cells. A-E reproduced from (Sewall *et al.*, 1990), F © Reinhard Fischer.

conidiospores, which can be easily dispersed. The steps are shown in Figure 6.

1.3.5. Sexual reproduction

A. nidulans is a homothallic (self-fertile) fungus, able to develop fruiting bodies in the absence of a mating partner and is therefore especially tractable for genetic analyses. Sexual propagation results in the differentiation of meiotic ascospores about 100 hours after germination. Development of the closed, spherical fruiting bodies (cleistothecia) comprises several morphological processes (Braus *et al.*, 2002; Poeggeler *et al.*, 2006). About 50 hours after germination, sexual development is initiated by fusion of ascogenous hyphae, which are surrounded by a mass specialised hyphae, termed nests, and globose Hülle cells. These cells presumably have a nursing function and express many tissue-specific proteins such as phenol oxidase (laccase II), catalase-peroxidase and mutanase (Hermann *et al.*, 1983; Scherer *et al.*, 2002; Wei *et al.*, 2001). Within the nest, a cleistothecial primordium develops, later differentiating into a micro-cleistothecium and partitioning into sterile hyphae which later form the fruit body wall (Champe *et al.*, 1994) and inner fertile cells. Asci arise from croziers, which are formed by simultaneous division of the two nuclei of the terminal cell of an ascogenous hypha. The crozier comprises a uninucleate tip cell, a binucleate penultimate cell and a uninucleate basal cell. As growth proceeds, the ascus is formed by enlargement of the penultimate cell, which is accompanied by fusion of its two nuclei to form a transient diploid zygote that undergoes meiosis immediately. The four nuclei produced by meiosis divide mitotically to generate eight nuclei, each of which is included in one of the eight ascospores in each ascus. The nucleus in each ascospore undergoes a single mitotic division resulting in mature binucleate ascospores. During ascosporogenesis, ascus wall accumulates a characteristic red pigment called asperthecin. Each cleistothecium can contain up to 80,000 ascospores that are the meiotic progeny of a single ascogenous hypha (Champe *et al.*, 1994). Crossing two *A. nidulans* strains requires a heterokaryon, which facilitates preferential crozier forming between unlike haploid nuclei. Recent analysis demonstrated that, contrary to what was previously thought, cleistothecia could stem from more than one fertilization event.

1.4. Genetic regulation of development

Sexual and asexual reproduction in *A. nidulans* requires the transformation of undifferentiated hyphae into complex structures consisting of specialized cell types. Genetic control of growth and development of *A. nidulans* is subject to intensive research. Many regulatory proteins and dependencies have been proposed (see Figure 7and Figure 8), but their precise contribution to developmental decisions in *A. nidulans* remains to be clarified. Recently,

nutrient sensing signal transduction pathways influencing developmental progression were reconstructed *in silico* by homology with *S. cerevisiae* and *S. pombe*, but the function of only a few predicted components has been experimentally validated up to date (Muthuvijayan and Marten, 2004).

1.4.1. Central asexual regulatory pathway

Specific regulation of asexual development is driven by a central regulatory cascade comprising two major transcription factors, BrlA and AbaA (Figure 7). Activation of *brlA* gene is necessary and sufficient for conidiophore development. *brlA* produces two overlapping transcripts, *brlAα* and *brlAβ*, both accumulating early in during development. Expression of *brlA* is subject to complex regulation, in that activation of the two transcripts is regulated at different levels. While *brlAα* is regulated at the transcriptional level, *brlAβ* is regulated at both the transcriptional and translational levels. *brlAα* expression requires both *abaA* and *brlA*, but lack of *abaA* can be overcome by overexpressing *brlAβ*. brlA β mRNA is constitutively transcribed, but its translation is repressed by a short microORF upstream of the *brlA* transcription start site, which prevents premature development (Han *et al.*, 1993; Prade and Timberlake, 1993). It was suggested that this complex locus has evolved to provide a mechanism to separate responses to the multiple regulatory inputs activating and maintaining

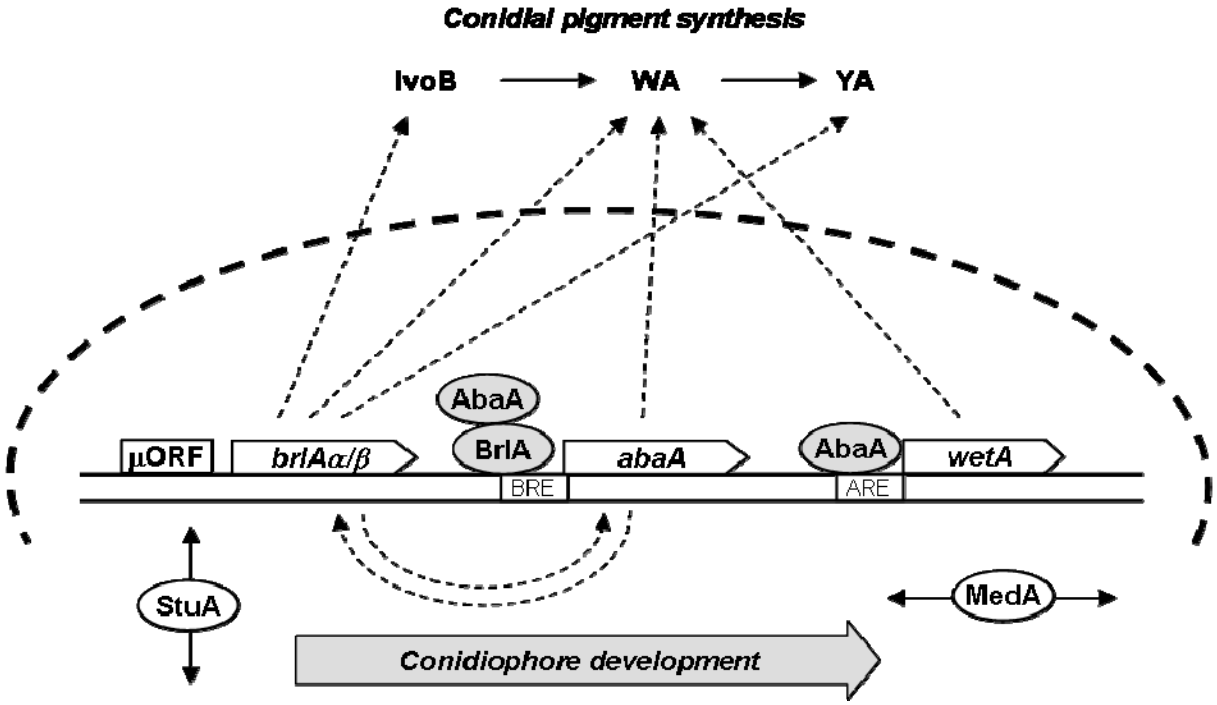


Figure 7. The central asexual regulatory pathway. BrlA, AbaA and WetA constitute the main pathway of interregulated transcription factors in the nucleus, influencing other genes involved in conidiophore and conidia maturation. StuA and MedA are developmental modifiers. The grey arrow indicates progress of conidiophore development in time.

brlA expression throughout development.

brlAβ, encoding a zinc-finger family transcription factor, is the main effector and is translated about 20 hours after germination and activates transcription of other developmental genes such as *wetA* and *rodA*, as well as *abaA* (Chang and Timberlake, 1993). It binds to the 5'-MRAGGGR-3' sequences, called BREs (BrlA response elements), present in the promoters of target genes (Chang and Timberlake, 1993). *abaA* encodes a transcription factor closely related to yeast Tec1 playing a role in pseudohyphal growth and to mammalian TEF-1 (transcription enhancer factor 1) involved in early muscle development (Gavrias *et al.*, 1996; Melin *et al.*, 1993; Xiao *et al.*, 1991). It is activated by *brlA* during the middle stages of conidiophore development after sterigmata differentiation and is required for the switch from sterigmata budding to the formation of conidiospores (Andrianopoulos and Timberlake, 1994). The *abaA* gene product activates transcription of several conidiation-specific genes including *wetA*, *yA* and *wA,* as well as its own expression, initiating a positive feedback loop. It does so by binding to the 5'-CATTCY-3' sequences called AREs (AbaA response elements)(Andrianopoulos and Timberlake, 1994). WetA is a regulatory gene activating expression of spore-specific genes, including *wA*, and is essential for conidia maturation (Marshall and Timberlake, 1991). The genes *ivoA*, *ivoB*, *wA* and *yA* are involved in the biosynthesis of the green polyketide-derived conidiospore pigment. The latter two genes are also subject to regulation by BrlA and AbaA (Andrianopoulos and Timberlake, 1994; Aramayo and Timberlake, 1993).

1.4.2. Activation of the central cascade

One group of mutant strains blocked early in development comprises the "fluffy" mutants with a characteristic phenotype, generating profuse aerial hyphae lacking development into conidiophores (Wieser and Adams, 1995). The analysis of recessive fluffy mutations led to the characterization of six genes, designated *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE* (Wieser *et al.*, 1994). Loss-of-function mutations in any one of these six genes result in fluffy colony morphology and greatly reduce *brlA* expression. *fluG* is required for synthesis of a low-molecular-weight factor that signals the activation of the major pathway for conidiophore development independent of the environmental conditions. The FluG factor probably initiates asexual development at a certain threshold (Lee and Adams, 1994). VeA was proposed to interact with a protein involved in the production of the FluG factor (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 is a RGS (regulator of G-signalling) domain protein, controlling the activity of an inhibitory heterotrimeric G protein pathway whose inactivation is essential for both asexual and sexual sporulation, as presented in Figure 7 and Figure 8 (Rosen *et al.*, 1999;

Yu *et al.*, 1996). It consists of *fadA*, encoding a G protein α-subunit, *sfaD*, encoding a β-subunit and *gpgA*, encoding the only known *A. nidulans* γ-subunit (Seo *et al.*, 2005). Interestingly, SfaD and GpgA are also necessary for proper self-fertilization and later cleistothecia formation. *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. The understanding of the mechanism by which FlbA and FadA regulate conidiation was recently extended by characterization of a cAMP-dependent protein kinase catalytic subunit PkaA. Deletion of *pkaA* leads to hyperconidiation, while *pkaA* overexpresssion inhibits *brlA* expression and conidiation (Shimizu and Keller, 2001). Deletion of *pkaA* restores conidiation in *flbA* and *fadA* mutants, suggesting that FlbA and FadA-dependent inhibition of conidiation is mediated in part through PkaA. *flbB*, *flbC*, and *flbD* are likely to function as DNA binding proteins and could control the transcriptional activation of other developmental regulators, like *brlA*, in response to sporulation signals (Wieser *et al.*, 1994).

1.4.3. Developmental modulators

Once developmental cycles are induced, several regulatory mechanisms seem to co-ordinate their molecular control. Two developmental regulatory genes, *stuA* and *medA*, are necessary for the precise spatial pattern seen in the multicellular conidiophore. Mutations in either gene give rise to spatially deranged conidiophores, but both *stuA* and *medA* mutants are able to produce some viable conidia. Transcript levels of *stuA* rise at competence and increasing developmental *stuA* thresholds are probably required for vegetative growth, asexual propagation and sexual development (Wu and Miller, 1997). Similarly to *brlA* locus, *stuA* gene produces two overlapping transcripts and its expression is in turn modulated by BrlA. *A. nidulans stuA* deletion strains develop disorganised asexual structures with shortened conidiospores budding directly from the vesicle (Miller *et al.*, 1991). On the molecular level, StuA is required for the correct cellular spatial distribution of BrlA and AbaA (Miller *et al.*, 1992). The parallel expression of StuA and BrlA during conidiophore development establishes antagonistic spatial transcription factor gradients and allows a controlled transition to the budding growth required for sterigmata formation. *stuA* encodes a transcription factor with significant similarity to the fungal transcriptional regulators that regulate development or cell progression, especially the yeast Phd1, belonging to a sub-family possessing the conserved APSES domain. StuA is also required for ascosporogenesis and multicellular development during sexual reproduction, including Hülle cell formation (Dutton *et al.*, 1997). On the other hand, MedA is responsible for proper temporal expression of *brlA* transcripts and also functions as co-activator of *abaA*

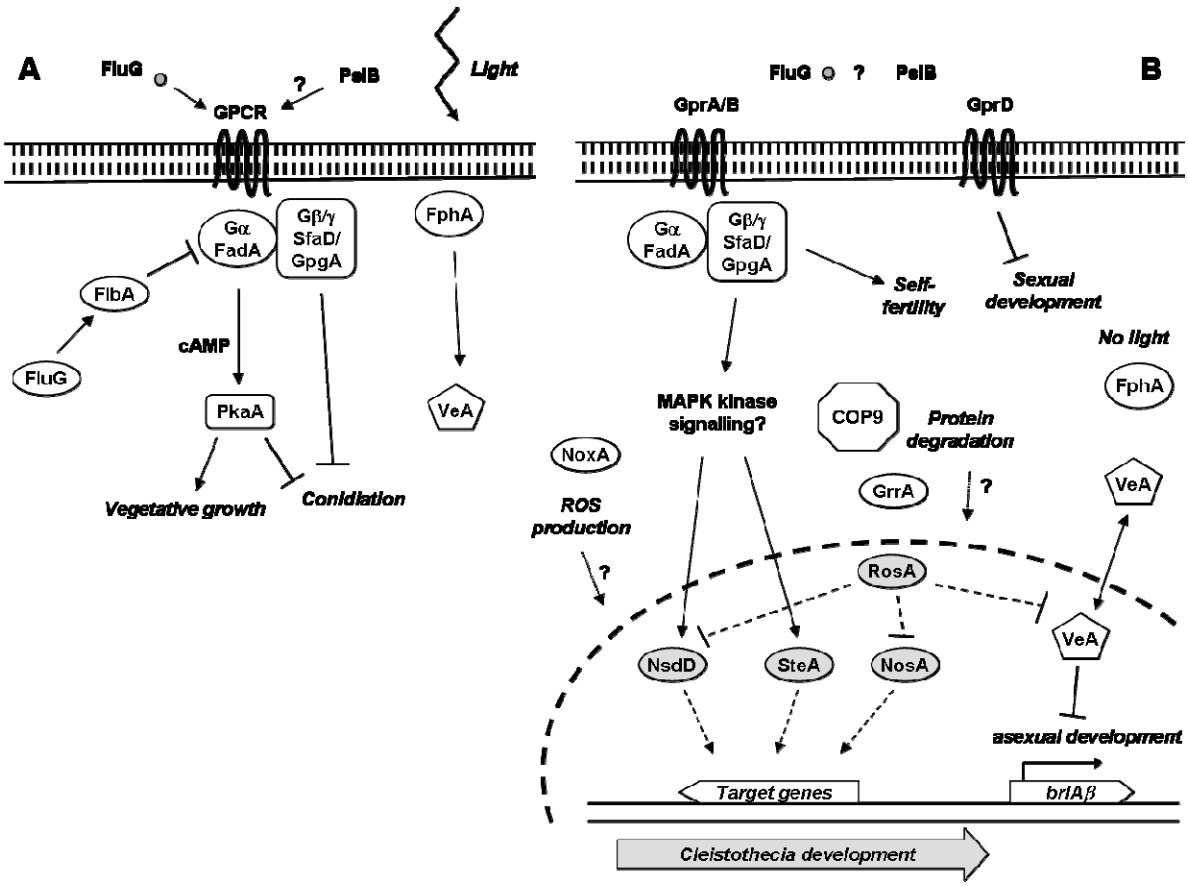


Figure 8. The proposed signalling pathways regulating *A. nidulans* growth and development.
(A) Activation of the central asexual regulatory cascade by G-protein signalling and protein kinase A. **(B)** Regulation of sexual development activated by G-protein signalling and MAPK kinase pathway and transcription factors (shown in grey) implicated in sexual development. The grey arrow indicates progress of cleistothecia development in time.

expression (Busby *et al.*, 1996). *medA* transcript levels are high during vegetative growth and decline following developmental induction. Strains with *medA* defects also show disorganised asexual structures, resulting in the production of multiple layers of sterigmata before conidia form. Occasionally, *medA* mutant conidiophores bear secondary conidiophores that sometimes produce conidia. medA also has impact on sexual development and the mutant strains are acleistothecial, but produce Hülle cells. Like *stuA* and *brlA*, the *medA* locus also has two transcription start sites that give rise to two mRNAs, both containing multiple mini ORFs, which suggests the possibility of translational regulation (Miller, 1993).

Both the asexual and the sexual signalling pathways share signal transduction compounds. Considering that conidiation starts earlier than sexual development, intrinsic signals play an important role in the decision to start cleistothecia formation. The developmental modifiers demonstrate that a sophisticated cross-talk must exist between the two pathways. A genetic interaction between FluG and VeA influencing the production of the extracellular signal supports this model (Mooney *et al.*, 1990).

1.4.4. Regulation of sexual reproduction

The precise timing of the events leading to cleistothecium formation is not known to date. The knowledge about the sexual regulatory circuits in *A. nidulans* is rather fragmentary and is deduced mainly from the physiological defects of mutant strains. As mentioned in 1.4.3, G-protein signalling plays a role in both developmental pathways. The signals in these signalling pathways are perceived by transmembrane G-protein coupled receptors (GPCRs). Three of 16 proposed GPCRs of *A. nidulans*, the hormone receptors GprA and GprB as well as GprD, are involved in regulation of sexual development (Yu, 2006). Additionally, the MAPK (mitogen-activated protein kinase) module containing Sak/HogA, and several transcription factors including SteA, NsdD, StuA, DopA, MedA and CpcA are involved in the regulation of fruit body formation (reviewed in Poeggeler *et al.*, 2006). Two of these transcription factors, NsdD and SteA, are known to play a role exclusively in cleistothecia formation. *A. nidulans nsdD* null mutants lack all sexual tissue types and its overexpresssion represses the asexual sporulation program (Han *et al.*, 2001). *nsdD* encodes a GATA-type transcription factor with the type a zinc finger DNA-binding domain. Based on genetic studies, it is predicted to act downstream of VeA. In contrast, *steA* deletion mutants are acleistothecial but development of Hülle cells is unaffected (Vallim *et al.* 2000). *steA* gene encodes a protein with a homeodomain similar to those of fungal Ste12 proteins, and possessing a C-terminal C_2/H_2-Zn^{+2} finger domain. Although SteA function is restricted to the sexual cycle, cross regulation between the two developmental pathways exists. NsdD and SteA are well characterized, however, most of their target genes are yet unknown.

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). One of the genes involved in ascospore formation is *grrA*, encoding a protein similar to yeast F-box protein Grr1p, which functions as a substrate receptor for ubiquitin ligase. *grrA* is induced and expressed during cleistothecial development of *A. nidulans*. *grrA* deletion strain resembles the wild-type hyphal growth, asexual sporulation, Hülle cell formation and development of cleistothecia, but is unable to produce mature ascospores due to a block in meiosis (Krappmann *et al.*, 2006). Considering the defects in fruit body development in mutants lacking the subunits of the COP9 signalosome (see 1.7), it is likely that sexual differentiation might require not only specific activation of developmental-specific genes, but also specific destruction of proteins specific for hyphal growth.

Recently, two novel putative zinc-finger transcription factors involved in sexual development, have been described in *A. nidulans*, called RosA and NosA (Vienken *et al.*, 2005; Vienken and Fischer, 2006), related to Pro1 of *Sordaria macrospora*. Both NosA and RosA regulate sexual development. RosA plays a role in early decisions and represses sexual development upon integration of several environmental signals Transcript levels of the sexual developmental regulators *nsdD*, *veA*, and *stuA* are increased in *ΔrosA* strains. In contrast to this, NosA activity is required for primordium maturation. The two factors are genetically linked, because RosA also represses NosA expression, but their exact place in the signalling pathway remains unclear.

The potential role of endogenous reactive oxygen species (ROS) in fungal sexual development has also come into focus in recent years (reviewed by Aguirre *et al.*, 2005). According to current hypothesis of Aguirre *et al*., a switch between growth and differentiation occurs when a transient increase in ROS levels, beyond the cellular capability to neutralize them, is produced, followed by expression of antioxidant detoxifying enzymes (such as superoxide dismutases (SODs), catalases, catalase-peroxidases and peroxiredoxins), shutting the signal off.

Hülle cells and cleistothecia initials produce superoxide and H_2O_2 in a process dependent on *noxA* gene encoding a member of a novel NADPH oxidase subfamily. Deletion of *noxA* specifically blocks differentiation of cleistothecia, without affecting hyphal growth or asexual development. Accordingly, *noxA* is induced during sexual development, peaking at the time of cleistothecia differentiation in parallel with the Hülle cell-associated catalase peroxidase gene *cpeA*. This expression pattern is not dependent on transcription factors SteA and StuA, but stress MAPK signalling is implicated in this process (Lara-Ortiz *et al.*, 2003). The signal transduction pathways used to detect ROS are conserved between yeast and *Aspergillus*, although their function has not been validated experimentally in this fungus yet.

1.5. Secondary metabolism and its regulation in development

Fungi are capable of producing a wide range of secondary metabolites. Many of them are of medical, industrial or agricultural importance and their biosynthesis is usually associated with cell differentiation or development, although the mechanisms underlying this connection are only beginning to be understood. Secondary metabolites associated with sporulation can be placed into three broad categories: metabolites that activate sporulation, pigments required for sporulation structures (such as melanins), and toxic metabolites secreted by growing colonies at the approximate time of sporulation (for example mycotoxins). Knowledge about biosynthesis

of hormones, pheromones and other signal molecules in *A. nidulans* is still 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. The tryptophan-related secondary metabolite auxin was identified as a probable contributor for differentiation processes (Eckert *et al.*, 1999). The diffusible extracellular FluG factor is presumably also derived from amino acids, since its production depends on *fluG* that encodes a proposed glutamine synthetase activity (Lee and Adams, 1994). Secondary metabolite production in fungi is connected both with asexual sporulation and sexual fruit body formation (reviewed by Calvo *et al.*, 2002). Melanins are associated with developmental structures, while synthesis of sterigmatocystin and other *Aspergillus* toxins is connected with the asexual development via a G-protein-mediated signalling pathway.

1.5.1. Psi factors

Oxylipins represent a vast and diverse family of secondary metabolites that originate from the oxidation or further conversion of polyunsaturated fatty acids. In *Aspergillus nidulans*, endogenous lipogenic signal molecules called psi factors (for precocious sexual inducer), derived from linoleic acid, play a role in regulation of cleistothecium and conidiophore development (Figure 9, panel A) (Champe *et al.*, 1987). Psi factors are a mixture of differently hydroxylated oleic (18:1) and linoleic (18:2) acid derivatives. They comprise three interconvertible compounds: 5,8-dihydroxylinoleic acid cyclic lactone PsiAα, 8-hydroxylinoleic acid PsiBα and 5,8-dihydroxylinoleic acid PsiCα, which are structural relatives to vertebrate eicosanoid hormones (Calvo *et al.*, 1999; Champe and el-Zayat, 1989). The proportion of these compounds controls the ratio of asexual to sexual spore development, with

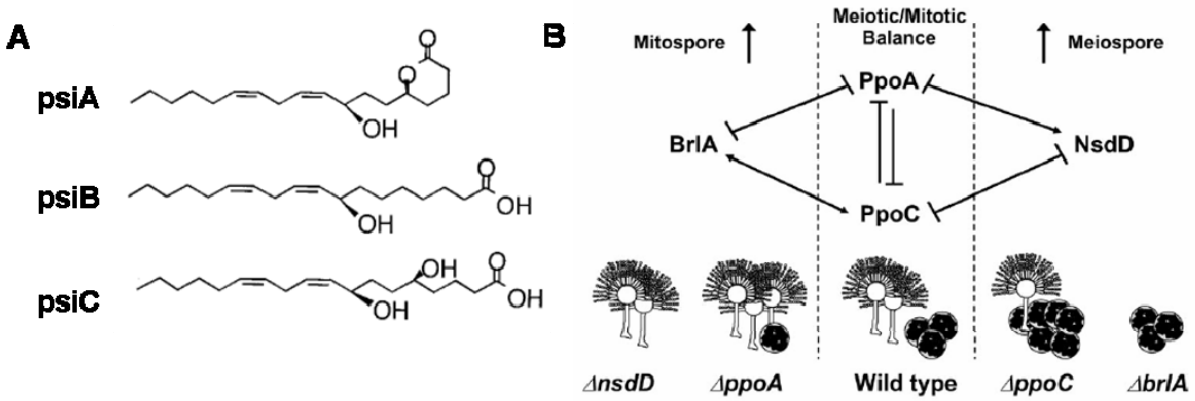


Figure 9. *A. nidulans* psi factors and oxygenases responsible for their synthesis. (A) Structures of the psi factors. **(B)** Model depicting the genetic relationship between PpoC, PpoA, BrlA, and NsdD to establish the meiotic/mitotic ratio in *A. nidulans*. PpoA and PpoC are involved in linoleic and oleic acid oxylipin production, respectively. The numbers of individual conidiophores and cleistothecia are indicative of the asexual/sexual ratio in each mutant and are shown not in scale. Modified from (Calvo *et al.*, 2002; Tsitsigiannis *et al.*, 2004a).

PsiBα and PsiCα stimulating, and PsiAα inhibiting sexual spore development (Champe *et al.*, 1987). Purified linoleic acid and hydroxyperoxy linoleic acids also exhibit sporogenic activity in several *Aspergillus* species. The psi factor signalling seems to be regulated in turn by the light-dependent developmental regulator *veA*, as the sexual inducing activity of the PsiC factor is abolished in *veA1* mutant strains (Calvo *et al.*, 1999). *veA* also seems to affect the overall fatty acid composition of the fungal cell (Calvo *et al.*, 2001).

Three fatty acid dioxygenases, PpoA, PpoB and PpoC are necessary for psi factor biosynthesis and the balance of asexual and sexual spore production. PpoA, involved in biosynthesis of psiBα, is known to be required for sexual development and its overexpresssion reduces asexual sporulation while deletion of *ppoC* gene significantly increases meiospore production and decreases mitospore development (Tsitsigiannis *et al.*, 2004a). Psi factors communicate with transcriptional factors regulating fungal differentiation. It was shown that *ppoC* and *ppoA* expression is dependent upon, and regulates the expression of, *nsdD* and *brlA*. It has been also suggested that Ppo oxylipin products antagonistically signal the generation of Ppo substrates. Deletion of *ppoC* leads to transcriptional activation of the of genes involved in fatty acid biosynthesis and a concomitant increase in the total amount of fatty acids in the fungal thallus (Tsitsigiannis *et al.*, 2004a) (Figure 9, panel B). On the other hand, *ΔppoA* reduces transcription of the lipogenic genes, indicating that PpoC and PpoA regulate signalling cascades that couple meiospore and mitospore production to a host of other developmental programmes in *A. nidulans*, including fatty acid anabolism (Tsitsigiannis *et al.*, 2004a). 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).

1.5.2. Pigments

A. nidulans produces pigments in various cell types and they are often derived from amino acids as precursor molecules. Melanins are dark brown pigments formed by oxidative polymerization of phenolic compounds or and are synthesized during spore formation for deposition in the cell wall. Fungal melanins can be formed from tyrosine by a tyrosinase (phenoloxidase) or from malonyl-CoA via the DHN pathway by a polyketide synthase (reviewed by Plonka and Grabacka, 2006). It is partly secreted and partly accumulates in the cell wall of elder fungal hyphae, where it can comprise up to 20% cell wall dry weight (Bull, 1970). Other pigments are restricted to the reproductive cycles, where they probably function to

protect nucleic acids from damage by ultraviolet light. The conidiospore pigment probably originates from tryptophan; 6-hydroxy-N-acetyl-tryptophan is converted to a yellow pigment precursor and subsequently to the green pigment by 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. Spatiotemporal expression of these conidia-specific genes is regulated mainly by the central asexual regulatory pathway (see 1.4.1). The biosynthesis pathway of the red ascospore pigment, asperthecin, is yet unknown. A phenol oxidase (laccase II) is specifically expressed during early sexual development in primordia and Hülle cells, but its contribution to pigment synthesis is unclear (Scherer and Fischer, 1998).

1.5.3. Toxins and antibiotics

The aspergilli are capable of producing polyketide-derived toxins as well as β-lactam antibiotics. *A. flavus* is a known producer of aflatoxin, while *A. nidulans* synthesizes sterigmatocystin, which is the penultimate precursor in the aflatoxin biosynthesis pathway. Genes required for the synthesis of aflatoxin and sterigmatocystin are well conserved between aspergilli and are located in large gene clusters. *A. nidulans* contains a cluster of 25 genes that encode enzymes required to synthesise sterigmatocystin (ST) (Brown *et al.*, 1996). One ST cluster gene, *aflR*, functions as a pathway specific transcriptional activator of other genes in the ST pathway, and *aflR* expression is regulated during the life cycle. The general mechanisms controlling the activation of *aflR* and synthesis of sterigmatocystin in conjunction with sporulation have not been understood yet. It is known, however, that the cross-talk between asexual development and ST synthesis is mediated via the G-protein signalling pathway. The proposed FluG factor stimulates both development-specific events and activation of FlbA, which in turn inactivates FadA-dependent proliferation signalling. FadA signalling antagonizes both sporulation and ST production and FadA inactivation by FlbA is a prerequisite for both processes (Hicks *et al.*, 1997).

Much effort was directed into the study of mechanisms that regulate biosynthesis of β-lactam antibiotic penicillin (reviewed by Brakhage, 1997). The biosynthesis of this secondary metabolite is catalyzed by three enzymes encoded by *acvA*, *ipnA* and *aat* genes, organized into a gene cluster. To date, no penicillin-specific regulators have been thoroughly characterised, but several regulators affect the synthesis of the antibiotic. Regulation of penicillin biosynthesis seems to be connected to production of sterigmatocystin. Whereas FadA inhibits the ST pathway, it activates penicillin biosynthesis (Tag *et al.*, 2000). These two metabolites are also

oppositely regulated by pH (penicillin is favoured in alkali environments, and sterigmatocystin is favoured in acidic environments). Furthermore, the expression of both sterigmatocystin and penicillin genes is adversely regulated by VeA (Kato *et al.*, 2003), suggesting that the differential regulation is correlated not only with sporulation, but also with early stages of reproductive cycle determination. On the other hand, a putative protein methyltransferase LaeA has been identified as a global regulator of secondary metabolism in *Aspergillus* species, controlling ST and penicillin synthesis (Bok and Keller, 2004). *laeA* is negatively regulated by AflR in a unique feedback loop, as well as by two signal transduction elements, protein kinase A and RasA (see 1.4.2), but it does not affect sporulation, indicating that its primary targets seem to be metabolic gene clusters allowing for uncoupling the control of secondary metabolism and development.

1.6. Structure and plasticity of *A. nidulans* cell wall

1.6.1. Cell wall structure

The fungal wall is a unique structure, differing in its composition and properties both from plant and bacterial cell walls. It provides the cell with a rigid structural framework, protects against mechanical injury, prevents osmotic lysis, and provides passive protection against the entry of potentially harmful macromolecules while maintaining permeability. The main components of *A. nidulans* cell wall are chitin, 1,3-β- and 1,6-β-glucan, galactomannan and proteins (Figure 10). The cell wall polysaccharides can be divided in two fractions: the alkali soluble fraction is composed mainly of α-1,3-glucans with some galactomannan. The main components of the alkali insoluble fraction, believed to be responsible for rigidity, are galactomannan, chitin and β-1,3-glucan. Galactomannan is composed of a core chain of β-1,5-mannose with short side chains of β-1,5-galactofuranose residues. A considerable amount of linear β-1,3/1,4-glucan was also found. The cell wall contains considerable amounts of melanin, which can comprise up to 20% cell wall dry weight and is important for protection against reactive oxygen species (Bernard and Latge, 2001; Bull, 1970; Zonneveld, 1971). Cell wall-associated proteins are commonly anchored to the extracellular side of the cellular membrane by a covalent glycosyl-phosphatidylinositol (GPI) linkage. Based on the studies in *A. fumigatus*, the cell wall does not seem to harbour proteins covalently attached to structural polysaccharides. The cell wall structure is rigid, but not inert and it also serves as a dynamic interface of the cell with the environment. The processes associated with fungal cell wall biogenesis are still not completely understood, considering that an estimated 20 % of *S. cerevisiae* genes control functions that are in some way related to this process. Much less is

known about conidial cell wall organisation, except for the outer layer, composed of small hydrophobic proteins known as hydrophobins (such as RodA and DewA) organized in a so-called rodlet structure, which seem to be necessary for efficient spore dispersal (Stringer *et al.*, 1991; Stringer and Timberlake, 1995).

1.6.2. Cell wall biosynthesis and remodelling

The wall is a dynamic structure, subject to change during spore formation and germination, polarized growth, hyphal branching and septum formation in filamentous fungi. The current understanding of cell wall polysaccharide biosynthesis suggests that initial formation of β-1,3-glucan and chitin is followed by branching and formation of covalent linkages between β-1,3/1,6-glucan core and chitin, galactomannan and β-1,3/1,4-glucan which constitute the three-dimensional structural core (Bernard and Latge, 2001). β-(1,3)-glucan synthase complex of filamentous fungi is membrane-bound. *A. nidulans fksA* gene encodes a protein very similar to yeast Fks β-glucan synthases (Kelly *et al.*, 1996), but no α-1,3-glucan synthases have been experimentally characterized yet.

Cell wall polymer branching and cross-linking, as well as plasticity during morphogenesis depend upon the activities of a range of hydrolytic enzymes, including glucanases, chitinases and proteases, often associated with the cell wall. Early studies have shown that most of the

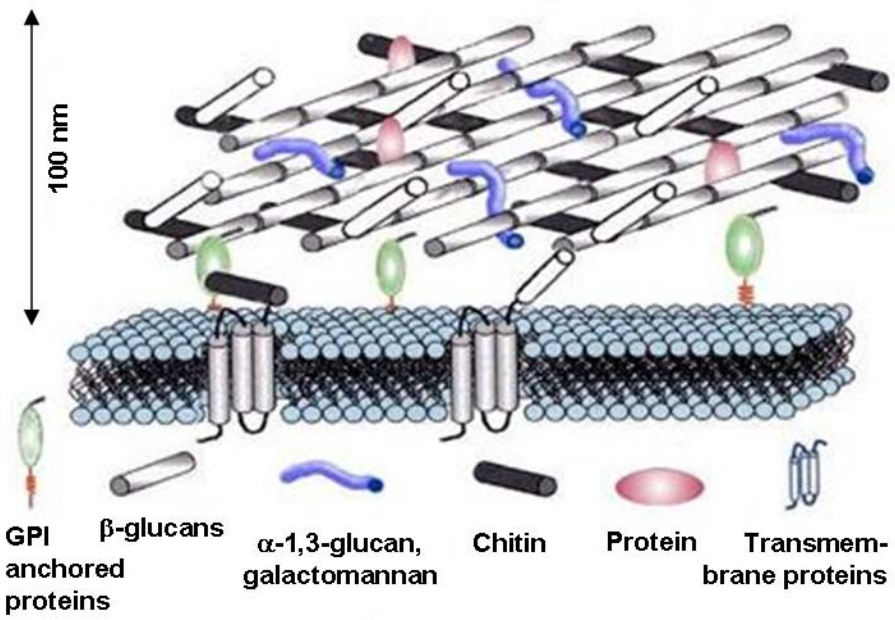


Figure 10. Structure of *Aspergillus* cell wall. Chitin and β-glucans form a rigid, alkali-insoluble network intertwined by amorphous, alkali-soluble α-1,3-glucan and galactomannan. Many cell wall synthesis and remodeling enzymes are located in the vicinity of the wall, either attached by a GPI anchor or integral to cell membrane, such as glucan and chitin synthases. Modified from (Latge, 2003).

wall-associated hydrolytic enzymes are soluble, but their wall-bound fraction has a higher specific activity, with laminarinase (β-1,3-glucanase) showing least difference in specific activity. Interestingly, apical cell walls seem to be better substrates for soluble autolysins and lateral walls for wall-bound enzymes (Polacheck and Rosenberger, 1978). Little is known about α-glucanase encoding genes, with the exception of *mutA*, which is developmentally regulated (see 4.3.2) (Wei *et al.*, 2001). Three endo-β-glucanase genes have been described in *A. nidulans* so far; *eglA, eglB* and *eglC*. *eglA* encodes an endo-1,4-β glucanase and *eglC* an endo-1,3-β glucanase dependent on the presence of the *nsdD*, but with no significant deletion phenotype (Chikamatsu *et al.*, 1999; Choi *et al.*, 2005). Cell wall chitinases of filamentous fungi are important during autolysis and might also have a role in sporulation. The *A. nidulans* genome contains least 12 conserved active-site domains for chitinases of glycosyl hydrolase family 18. Disruption of *chiA* chitinase leads to decrease in the frequency of spore germination and reduced hyphal growth rate (Takaya *et al.*, 1998). Another chitinase *chiB* is dispensable for cell growth, but involved in autolytic processes under starvation conditions (Yamazaki *et al.*, 2007).

1.7. Scope and aim of this work

Since the advent of genome sequencing, more and more high-throughput analysis methods allowing for monitoring mRNA and protein changes in a cell on a global scale have emerged. The DNA microarrays (Schena *et al.*, 1995), previously based on incomplete cDNA or expressed sequence tag (EST) libraries, especially benefited from the post-genomic information, becoming not only a method for studying global regulatory networks affecting gene expression, but also a powerful tool for validating the genome annotation efforts. In combination with traditional methods of molecular biology on single gene and protein level, it is now possible to study the structural features of proteins concomitantly with their general role in the cellular processes.

The COP9 signalosome was identified as a non-essential but key regulator of sexual development in *Aspergillus nidulans* (Busch *et al.*, 2003) and a regulator of the stability of proteins involved in the circadian clock of *Neurospora crassa* (He *et al.*, 2005). Four characterized *A. nidulans csn* mutants (*ΔcsnA, ΔcsnB, ΔcsnD* and *ΔcsnE*) share an identical developmental phenotype. They are blocked in fruit body formation at the level of primordia, while initiating preferentially the sexual cycle when induced in pregrown competenent mycelia, indicating partial blindness to light-induced repressing signals. Genetic data suggest that CSN acts upstream of VeA in the regulatory pathway (Busch *et al.*, 2003). The mutants also produce

a yet unidentified red pigment, which suggests disruption of secondary metabolite synthesis pathways.

In this work, we addressed the question of the molecular basis of the *csn* phenotype and the place that the CSN takes in *A. nidulans* light-dependent, developmental and metabolic signalling. These functions of the complex could depend either on the specific deneddylation activity of the *csnE* subunit or on other activities requiring the complex as a whole, such as regulating associated kinase activities. We wanted to distinguish between these two roles and to see which activities underlie the developmental and metabolic functions of CSN, respectively. On the other hand, we were interested in placing the COP9 signalosome in the broader context of the signalling pathways controlling *A. nidulans* light-dependent development and secondary metabolism. Accordingly, this study focuses on two aspects of the CSN function in *Aspergillus* development: the deneddylating activity and the Nedd8/rub1 cycle in this fungus identifying downstream targets of the signalosome as a developmental regulator of gene transcription.

Taking advantage of the non-lethal *ΔcsnE* phenotype and apparent conservation of the ubiquitin system and its regulatory mechanisms, in the first part of this work we have performed mutational analysis of the CsnE subunit JAMM motif and confirmed the role of deneddylation in sexual fruit body formation. Additionally, we identified *rubA*, a homologue of Nedd8, as a gene essential for *A. nidulans* growth.

The pleiotropic phenotype of the *csn* mutants suggested changes at multiple levels of developmental regulation. This fact, combined with the availability of genome-wide *Aspergillus* microarrays prompted us to investigate the COP9 function during development using transcriptome profiling. Such an approach allows identifying global changes in gene expression before the morphological changes become apparent. Although many microarray experiments were reported in recent years in *A. nidulans*, only one study up to date used chips with complete genome coverage (David *et al.*, 2006). In the second part of this work, we present the results of a genome-wide transcriptome analysis study of the *ΔcsnE* mutant, elucidating the effect of CSN on many levels of cellular signalling in regulation of developmental balance and in secondary metabolite biosynthesis, as well as in cell wall recycling during development.

CHAPTER 2: MATERIALS AND METHODS

2.1. Strains and growth conditions

2.1.1. Strains

E. coli strain DH5α (Woodcock *et al.*, 1989) was used for preparation of plasmid DNA and was cultivated at 37°C on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with antibiotics: 100 μg/ml ampicilin, 25 μg/ml chloramphenicol or 100 μg/ml zeocine (Cayla, Toulouse, France) when appropriate or LBLS medium (1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) when selection for zeocine resistance was applied. *E. coli* strain KS272 (F⁻ *lacX74 galE galK thi rpsL phoA* (*Pvu*II)) was used for propagation of the recombination vector pKOBEG (Chaveroche *et al.*, 2000) and of *A. nidulans* recombinant plasmids. Expression of pKOBEG genes under the control of pBAD promoter was induced with 0.2% arabinose. Fungal strains used and constructed in this study are summarized in Table 1. The *A. nidulans* strains FGSC A4 (Glasgow wild-type) and TNO2A3 (*pyrG89; pyroA4; ΔnkuA::argB*) were provided by the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, USA).

Table 1. *A. nidulans* strains used in this study.

Strain	Description	Reference
A4	Glasgow wild type	FGSC[a]
AGB209	*pyroA4; pyrG98/pyr-4+ ΔcsnE*	Busch *et al*., 2003
TNO2A3	*pyrG89; pyroA4;Δ nkuA::argB*	Nayak *et al*., 2006
AGB152	*pyroA4; pyrG98*	Busch *et al*., 2003
AGB244	*pyroA4; pyrG98/pyr-4+ ΔcsnE/csnE1::bleo*	This work
AGB245	*pyroA4; pyrG98/pyr-4+ ΔcsnE/csnE2::bleo*	This work

a. Fungal Genetics Stock Center (University of Kansas Medical Center, KS, USA);

2.1.2. *Aspergillus* media and growth conditions

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) was used for cultivation of *A. nidulans* strains at 30°C or 37°C (Barratt *et al.*, 1965) supplemented as needed with 4.8 μM pyridoxine-HCl and 5 mM uridine/uracil. 10 μg/ml phleomycine (Cayla, Toulouse, France) was used to select for the presence of the *ble* gene of *Streptoalloteichus hindustanus*. For plates, 2% agar was added. Vegetative mycelia were obtained from submerged liquid culture grown at 10^6 spores/ml and

development was allowed by an air-medium interface. Asexual sporulation was induced by incubation on non-sealed plates in continuous white light and cleistothecia formation by oxygen-limiting conditions on parafilm-sealed plates grown in the dark (Clutterbuck, 1974). To obtain homogenized cell material, *A. nidulans* mycelia were filtered through sterile Miracloth (Merck Chemicals, Nottingham, UK), shock-frozen, ground in liquid nitrogen with a mortar and pestle and stored at -80°C.

Radial growth tests were performed with about 500 conidiospores dropped on a minimal medium plate, with growth rates recorded as colony diameter within time. Stress resistance tests were performed by growing colonies from around 500 conidiospores on minimal medium plates containing 0.02% 4-NQO, 0.03% H_2O_2, 4% ethanol, 0.02% benomyl or 0.008% nocodazol.

For the transcriptome analysis experiments, FGSC A4 and AGB209 strains were grown from freshly harvested spores at 37°C in minimal medium (Barratt *et al.*, 1965) supplemented with 4.8 μM pyridoxine-HCl. For plates, 2% agar was added. To obtain vegetative mycelia, strains were grown at $1.5x10^6$ spores/ml in shaking flasks at for 14 or 20 hours. To achieve developmental induction, $4x10^6$ spores per plate were incubated on plates covered with cellophane film (Merck Chemicals, Nottingham, UK) for 48 hours. Asexual sporulation was induced by incubation on non-sealed plates in continuous white light and cleistothecia formation by oxygen-limiting conditions on parafilm-sealed plates grown in the dark (Clutterbuck, 1974). For each time point, two RNA extractions were carried out from biological material from 2 flasks (vegetative growth) or 4 plates (developmental induction) and the RNA samples were pooled before further processing (see Figure 12). Pooled RNAs were obtained from three subsequent cultivations, referred to as “experiment 1”, “experiment 2” and “experiment 3”.

2.1.3. Microscopic analysis

A. nidulans colonies were observed under an Olympus SZX12 binocular (Olympus, Hamburg, Germany) and cleistothecial development was investigated using a Zeiss Axiolab (Zeiss AG, Oberkochen, Germany) microscope. Colony surface and cleistothecia were photographed with a Kappa PS30 camera (Kappa Opto-electronics, Gleichen, Germany)

2.2. Genetic manipulation

2.2.1. Transformation procedures

E.coli cells were routinely transformed using the chemical competence method (Inoue *et al.*, 1990). When transforming plasmids into KS272 for recombination, calcium shock method was

used (Hanahan *et al.*, 1991) and subsequently electroporation. Electro-competent *E.coli* cells were obtained by washing exponentially growing cells three times with ice-cold distilled H_2O and concentrating 300-fold with 10% glycerol. Electroporation was carried out in 0.2-cm electroporation chambers and using a BioRad GenePulser II set to the following parameters: 200 Ω, 25 µF and 2.5 kV. Shocked cells were diluted 3-fold in LB, incubated for 1 h at 30°C without aeration and subsequently plated on appropriate media. *S. cerevisiae* strains were transformed according to Elble (1992). *A. nidulans* transformations were performed using the polyethylene glycol-mediated fusion of protoplasts (Punt and van den Hondel, 1992) with the following modification: transformed protoplasts were embedded in 5 ml warm minimal medium containing 0,7% agar and supplements poured on solid medium instead of mechanical spreading. 5 mg/ml Glucanex (Novozymes, Bagsvaerd, Denmark) and lysosyme (Sigma-Aldrich, Munich, Germany) were used as protoplasting enzymes.

2.2.2. Recombinant DNA methods

Standard protocols of recombinant DNA technology were carried out according to Sambrook *et al*. (1989). *Taq* (Fermentas GmbH, St. Leon-Rot, Germany), *Pfu* (Fermentas) and *Kod* (Novagen, Nottingham, UK) polymerases were used in PCR reactions (Saiki *et al.*, 1985) depending on requirements. Annealing temperatures for oligonucleotide primers were calculated using OligoCalc tool (http://www.basic.northwestern.edu/biotools/oligocalc.html) with the basic temperature calculation method. Essential cloning and mutagenesis steps were verified by sequencing at the Göttingen Genomics Laboratory. All restriction enzymes used were from Fermentas and custom oligonucleotides were ordered from Operon Europe (Cologne, Germany). pBSKII+ vector (Stratagene) was used routinely for cloning procedures. Plasmid DNA isolation from overnight *E. coli* cultures was performed by alkaline lysis. High quality plasmid preparation was performed with Qiagen (Hilden, Germany) columns according to the manufacturer's instructions.

A. nidulans genomic DNA was isolated form a volume of 0.5 ml of ground mycelia according to Kolar *et al.* (Kolar *et al.*, 1988). For Southern hybridisation, genomic DNA was digested overgnight with appropriate restriction enzymes and separated on agarose gel. RNA was isolated from a volume of 200 µl ground mycelia with the TRIzol reagent (Invitrogen, Karlsruhe, Germany) as recommended by the manufacturer.

2.2.3. Hybridisation techniques

Non-radioactive Southern hybridisation experiments were performed using the GeneImages™ CDP-Star Detection Kit (GE Healthcare Life Sciences, Munich, Germany) with

fluorescein-labelled probes synthesised using the Gene Images™ Random-Prime DNA Labelling Kit (GE Healthcare Life Sciences) according to the manufacturer's instructions. Before hybridisation, separated DNA was denatured and depurinated according to a standard protocol (Sambrook *et al.*, 1989) and transferred to HybondN membranes (Amersham plc, HP7 9NA UK) by dry capillary blot and crosslinked for 1 minute with UV or by baking at 80°C for 30 min.

Table 2. Plasmids used and generated in this study.

Plasmid	Description	Reference
pME2237	*csnE* genomic, 6 kb fragment *Eco*RI in pBSK+	Busch *et al*., 2003
pME1510	REMI vector *PgpdA::ble::TtrpC*::MCS of PBSK+ in pAN8-1	S. Busch, pers. communic.
pBSKII+	pBluescript II: cloning vector (*amp*^{R}; MCS)	Stratagene
pEG202	2-hybrid bait / BD (ampR; PADH:lexA:TADH, HIS3, 2µm)	Golemis and Brent, 1996
pJG4-5	2-hybrid prey / AD (ampR; PGAL1:B42:TADH, TRP1, 2µm)	Gyuris *et al*., 1993
pME2881	*csnE1* (D143N) in pME2237	This work
pME2882	*csnE2* (H134A,H136A,D143N) in pME2237	This work
pME2883	*csnE1* complementation *Bam*HI/*Eco*RI in pME1510	This work
pME2884	*csnE2* complementation *Bam*HI/*Eco*RI in pME1510	This work
pME2370	*csnE* bait (*csnE* cDNA *Eco*RI in pEG202)	Busch *et al*., 2007
pME2371	*csnE* prey (*csnE* cDNA *Eco*RI in pJG4-5)	Busch *et al*., 2007
pME2975	*csnF* bait (*csnF* cDNA *Eco*RI in pEG202)	Busch *et al*., 2007
pME2981	*csnF* prey (*csnF* cDNA *Eco*RI in pJG4-5)	Busch *et al*., 2007
pME3129	*csnE1* (D143N) bait in pEG202	This work
pME3130	*csnE2* (H134A,H136A,D143N) bait in pEG202	This work
pME3131	*csnE1* (D143N) prey in pJG4-5	This work
pME3132	*csnE2* (H134A,H136A,D143N) prey in pJG4-5	This work
pME2409	*zeo::AfPyrG::zeo* blaster cassette	S. Krappmann, pers. comm..
pKOBEG	*repA101ts; pBAD::Redαβγ; Cm*^{R}	Chaveroche *et al.,* 2000
pME2888	*rubA* genomic, 5.5 kb fragment *Cla*I/*Apa*I in pBSK+	This work
pME3005	*rubA* cDNA, *Kpn*I/*BamH*I in pBSK+	This work
pME3128	*ΔrubA*::*zeo*::*AfpyrG*::*zeo* deletion construct	This work

Northern hybridisation experiments were performed according to the protocol of Brown and Mackey (Brown and Mackey, 1997). RNA was transferred to HybondN membranes (Amersham plc) by capillary 20×SSC blot or semidry electroblot in 0.5×TBE buffer using a BioRad blotting apparatus. Hybridisation probes were labelled with [α-^{32}P]-dATP employing

the Prime-It®-II kit (Stratagene Europe, Amsterdam, Netherlands) or Hexa-Label kit (Fermentas) according to the manufacturer's instructions. Autoradiograms were exposed on BioMaxMS film (Kodak). DNA for *csnE* hybridisation probes was amplified from the plasmid pME2237 by PCR using the primers SB102 (5'-TAA TCT AGA GAG CTA GAG AAT GCT GTT ACC CTG-3') and SB103 (5'-TAA TCT GAC AAA GTC CTC GGC TTT GTT AAG CG-3').

For isolation of the genomic *rubA* clone, a BAC genomic library of *A. nidulans* was screened by colony hybridisation. A Hybond C membrane (Amersham plc) was placed on the colonies for 1 min, incubated on Whatman paper subsequently in denaturing solution (1.5 M NaCl, 500 mM NaOH), neutralizing solution (1.5 M NaCl, 500 mM Tris pH 7.2) and 2×SSC for 5 minutes each, dried, crosslinked with UV and treated with 100 μg/ml proteinase K in 2×SSC, 0.1% SDS for 1 hour at 65°C. Southern hybridisation was then performed following a standard protocol (Sambrook *et al.*, 1989). DNA for *rubA* hybridisation probe was amplified from genomic DNA using primers KN 34 (5'-ATG TTG ATC AAG GTC CGT ACA CTT ACC-3') and KN 35 (5'-CTA CTG AAG GGC GGC GCA GCC-3') and labelled with [α-^{32}P]-dATP employing the Prime-It®-II kit (Stratagene).

2.2.4. Mutagenesis of *csnE*, plasmid and strain construction

Site-directed mutagenesis of *csnE* in the plasmid pME2237 was performed by using the QuikChange Multi kit (Stratagene) following the manufacturer's instructions. The codons for His-134, His-136 and Asp-147 were substituted for Ala, Ala and Asn, respectively. The resulting mutant alleles *csnE1* (D147N) and *csnE2* (H134A. H136A, D147N) were cloned *BamH*I/*EcoR*I into pME1510 complementation plasmid containing the *ble* gene and the resulting pME2883 and pME2884 plasmids were ectopically integrated in one copy into the *A. nidulans csnE* deletion strain AGB209, resulting in strains AGB 244 and AGB 245, respectively. The correct substitutions in the mutant alleles were confirmed for by sequencing.

2.2.5. Isolation of *rubA*, plasmid and strain construction

BAC DNA was isolated using the modified alkaline lysis method (Sambrook *et al.*, 1989) followed by double phenol/chlorophorm extraction and the genomic fragment was subcloned into pBSK+ via *Cla*I/*Apa*I, yielding plasmid pME2888. *rubA* cDNA clone was isolated from an *A. nidulans* vegetative cDNA library by amplification with primers KN 32 (5'-CGG GTA CCA AGA TGT TGA TCA AGG TCC G-3') and KN 33 (5'- ATG GAT CCC TAC TGA AGG GCG GCG CA-3') containing *Bam*HI and *Kpn*I restriction sites and subcloned into pBSK via *Kpn*I/*Bam*HI, yielding plasmid pME3005. For construction of the deletion cassette, 300 bp

flanking arms of *rubA* were inserted into *EcoR*V and *Hpa*I sites of pME2409 plasmid via blunt-end ligation. This plasmid was co-transformed into *E.coli* strain KS272(pKOBEG) with pME2888 and homologous recombination was induced according to the protocol of Chaveroche et al. (2000), resulting in the deletion construct pME3128. The deletion plasmid was transformed into AGB152 and TNO2A3 strains and purified transformants were screened by Southern hybridisation.

2.3. Heterokaryon rescue of *rubA*

Essentiality of *rubA* was confirmed using the heterokaryon rescue technique (Osmani *et al.*, 2006b), modified to use the minimal growth media and *A. nidulans* polyethylene glycol-mediated transformation method described above. In this technique, gene deletion is carried out using a selection marker to replace the coding region of a target gene via homologous recombination in an *nkuA*-defective *A. nidulans* strain. If the deleted gene is essential, the transformant nuclei are able to propagate only in a forced heterokaryon with parental nuclei. The essentiality and heterokaryon formation can be confirmed by simple replica plating of spores derived from primary transformant colonies combined with diagnostic PCR or Southern blot analysis of material from primary transformants. The procedure is outlined in Figure 11, using *pyrG* nutritional marker as an example. If the spores can propagate only on non-selective medium and heterokaryon formation is confirmed, it means that the gene is essential. The terminal phenotype can be examined microscopically in germinating spores on both selective and non-selective medium. Two deletion constructs were used for heterokaryon rescue analysis of *rubA*. One was pME3128 carrying a *pyrG* marker and the other was constructed using a fusion PCR method (Szewczyk *et al.*, 2006) with the primers listed in Table 3 and was carrying a *ptrA* dominant marker. The fusion PCR reactions were kindly performed by Marcia Kress.

Table 3. Primers used for making the fusion PCR-based *rubA* deletion construct.

Primer	region	bp	Sequence 5'-3'
MK03Fw	*rubA* 5'	20	AGA CTG ATA TTC TCG AAA CG
MK25Rev	*rubA* 5'	39	ACC AAT GGG ATC CCG TAA TCT TGA GAA TGC GAT GCT CTA
MK26Fw	*ptrA*	39	TAG AGT ATC GCA TTC TCA AGA TTA CGG GAT CCC ATT GGT
MK27Rev	*ptrA*	42	GTC CTC AAA TCA AAT TCC ACA GCA TCT TTG TTT GTA TTA TAC
MK28Fw	*rubA* 3'	42	GTA TAA TAC AAA CAA AGA TGC TGT GGA ATT TGA TTT GAG GAC
MK07Rev	*rubA* 3'	20	TCA TGC CTT CAT GAC TTT CG

2.4. Protein methods

2.4.1. Yeast two hybrid analysis

The transcriptional activation of a LexA/LacZ-based reporter system with plasmids pEG202 and pJG4-5 was used to identify interacting proteins (Gyuris *et al.*, 1993). For the bait

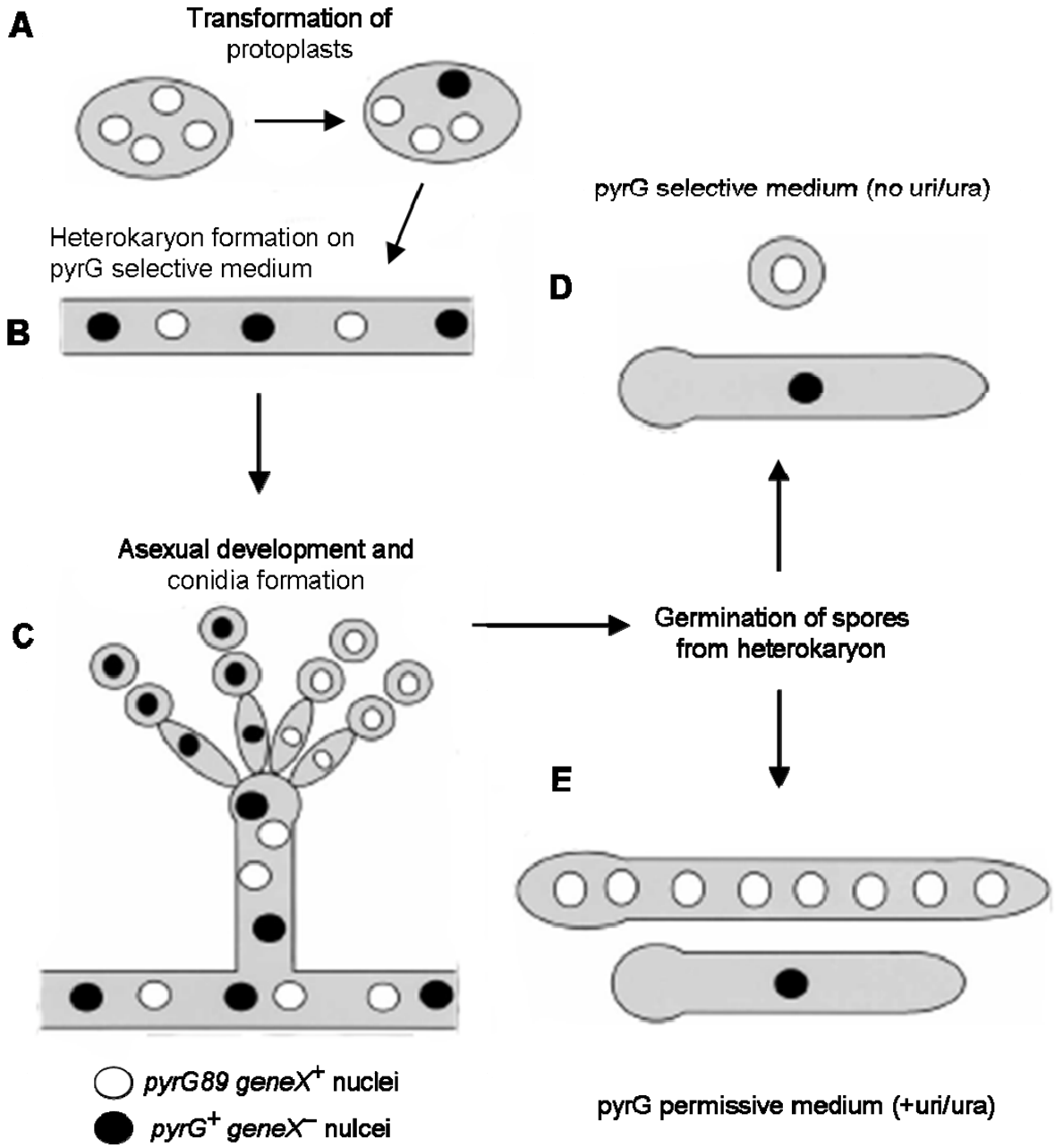


Figure 11 Overview of the heterokaryon rescue method. (A) *pyrG89* protoplasts containing multiple nuclei are transformed with a deletion cassette containing the *pyrG*$^{+}$ marker. **(B)** Protoplasts are regenerated on selective plates, forcing heterokaryon formation containing *pyrG89 geneX*$^{+}$ nuclei and *pyrG*$^{+}$ *geneX*$^{-}$ nuclei in a common cytoplasm. **(C)** Colonies undergo asexual spore formation producing spores with either parental or transformant nuclei. **(D,E)** Spores from heterokaryon colonies are replica streaked onto pyrG selective medium **(D)** or pyrG non-selective medium **(E)**. On selective plates, *pyrG89 geneX*$^{+}$ spores cannot germinate due to lack of pyrG and *pyrG*$^{+}$ *geneX*$^{-}$ spores germinate but arrest or due to lack of geneX function at a stage dependent on the gene function. On permissive plates *pyrG89 geneX*$^{+}$ spores can germinate and form colonies and *pyrG*$^{+}$ *geneX*$^{-}$ spores germinate but arrest with the same terminal phenotype as on selective media. Modified from (Osmani *et al.*, 2006b).

constructs, *csnE1* and *csnE2* mutagenised regions were amplified by PCR using primers containing *Nco*I sites (KN52: 5'-GTC CAT GGA CCA AAG ACC CTC ACT-3' and KN53: 5'-ATC CAT GGC TAA GTA GAC TCT ACC GTC TGA-3') and the digested PCR fragments were cloned into the *Nco*I site of pME2370, resulting in bait plasmids pME3129 and pME3130. For the prey constructs, *csnE1* and *csnE2* mutagenised regions were cloned into pME2371 with *Nco*I/*Mlu*I, resulting in prey plasmids pME3131 and pME3132. The constructs were transformed into the yeast two-hybrid reporter strain EGY48-p1840 (MATα, *his3*, *trp1*, *ura3-52*, *leu2*::*pLEU2-LexAop6*, *ura3*::*lacZ-LexAop2*)(Golemis and Brent, 1996). For interaction tests, overnight yeast cultures were resuspended in saline and 10 µl of an appropriate dilution was dropped on SC-4 (-his, -leu, -trp, -ura) plates containing 2% galactose as a carbon source (growth tests) or SC-plates containing 2% galactose, supplemented with 0.2 g/l leucine and covered with filter paper (β-galactosidase tests). Cells were grown for 72 h at 30°C and for β-galactosidase tests the filter was lifted, shock frozen in liquid nitrogen and soaked in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) on a Whatman paper supplemented with 40 µl 1% XGal in DMF. The filters were incubated at 30°C and inspected after 1 to 6 h.

2.4.2. Yeast protein isolation and analysis

Yeast whole cell extracts of were prepared from cultures grown to exponential phase. Cells were washed in ice-cold buffer B (50 mM Tris-HCl pH 7,5, 1 mM EDTA, 50 mM dithiothreitol), lysed with glass beads in 200 µl of buffer B + PIM (1 mM each phenylmethylsulfonyl fluoride, tosyl-L-Lys-chloromethylketone, tosyl-L-Phe-chloromethylketone, *p*-aminobenzamidine-HCl and *o*-phenanthroline) + 3% Triton X-100 + 0,8% SDS at 4°C and centrifuged at 3500 rpm for 15 min. Total protein concentration was determined by the procedure of Bradford (Bradford, 1976). 30 µg of protein from each strain was denatured in SDS loading dye by heating at 65°C for 15 min and were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane.

Fusion proteins were analysed by Western hybridisation using a polyclonal goat anti-LexA (D-19) antibody (Santa Cruz Biotechnology, Heidelberg, Germany) and a secondary donkey anti-goat antibody coupled with horseradish peroxidase (Santa Cruz Biotechnology). Detection was carried out using the ECL method (Tesfaigzi *et al.*, 1994)

2.4.3. *A. nidulans* protein isolation and enzyme assays

Crude extracts were prepared by grinding mycelia to a fine powder and extracting soluble proteins with B-buffer (100 mM Tris-HCl, 200 mM NaCl, 20% glycerol, 5 mM EDTA, pH 8)

at 4°C in the presence of cOmplete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) diluted as suggested by the manufacturer**.** Crude protein extract was dialysed overnight at 4°C against 100 mM sodium acetate buffer, pH 5.0 and protein content was determined by the procedure of Bradford (Bradford, 1976). Laminarin hydrolysing activity was measured in 100 mM acetate buffer, pH 5.0 as follows: 25 µg of dialysed crude extract was incubated with 3% laminarin azure (Sigma-Aldrich) in a reaction volume of 200 µl for 40 minutes at 37°C. The reaction was terminated by addition of 1.2 ml ethanol. The precipitated substrate was removed by centrifugation (10 min, 2,000×g) and the absorbance of the supernatant was measured at 595 nm.

2.5. RNA and transcriptome profiling

2.5.1. RNA extraction and labelling

A. nidulans mycelium was filtered through Miracloth, ground in liquid nitrogen and stored at -80°C. RNA was extracted from a volume of 2 ml ground mycelia with Trizol (Invitrogen), followed by a double phenol/chlorophorm extraction. The RNA was then treated with DNAse I (Roche) for 20 min at 37°C in the presence of RNAseOUT™ (Invitrogen), extracted with phenol/chlorophorm/isoamyl alcohol and additionally purified using RNeasy columns (Qiagen). Quality and quantity of RNA was determined using a 2100 Bioanalyser (Agilent Technologies, Böblingen, Germany). For each array hybridisation, 1 µg RNA was amplified using the Message Amp^{TM} II kit (Ambion, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, the RNA was reverse transcribed with an oligo(dT) primer bearing a T7 promoter using ArrayScript^{TM} reverse transcriptase (Ambion), producing first strand cDNA, followed by a second strand synthesis and purification. Resulting full-length cDNAs were transcribed *in vitro* with T7 RNA polymerase incorporating a modified nucleotide, 5-(3-aminoallyl)-UTP (aaUTP) to synthesize amplified antisense aminoallyl-RNA (aRNA) and purified. 2 µg resulting aRNA was labelled with NHS ester-activated Cy-3 or Cy-5 dyes (Amersham plc) via coupling to the reactive amino group of aaUTP. The dye incorporation rate of labelled purified aRNA was measured using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and was found to be approximately at 80-100 pmol/µg in all labelling reactions.

2.5.2. Array preparation and hybridisation

The *Aspergillus nidulans* microarrays were provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA funded Pathogen Functional Genomics Resource Center at the J. Craig Venter Institute (Rockville, ML, USA). The polymer-coated

glass arrays contained 11,481 70-mer oligonucleotide probes spotted in duplicate, providing complete genome coverage. The slides were prehybridised with backing slides in 500 µl 5×SSC, 0.1% SDS, 1% BSA for 1 hour at 65°C. After washing in distilled water and isopropanol the slides were dried in nitrogen stream.

Table 4. Primers used to generate Northern blot hybridization probes for validation of selected genes.

Name	bp	Gene / locus	Sequence 5' – 3'
KN 89	21	AN3239.3 cds 5'	ATG GCG GCG GCG AGC AGC CGT
KN 90	30	AN3239.3 cds 3'	CAT TCC ATA TAC AAT AAT TCT ACC CAC CTC
KN 91	26	AN8532.3 cds 5'	ATG CAC AGA CCC CCA CCT TGG TCT CT
KN 92	27	AN8532.3 cds 3'	CTA CAA AAG CAG GTA TCC CAC CAC TCC
KN 93	26	AN1832.3 cds 5'	ATG CAG CTC CTC GCT CTC ACT TTG GC
KN 94	26	AN1832.3 cds 3'	TTA GGT GCA GTG CAG CTC TCC ACC TT
KN 95	25	AN10782.3 cds 5'	ATG GCG ACG CAA GAG AGG AAG TAC C
KN 96	24	AN10782.3 cds 3'	TTA GTG GCC TGT CGT CGG AAG GCC
KN 97	35	AN5997.3 cds 5'	ATG AAT GTA TAT TTC CTG AAA GAG TTA GCG TGG AA
KN 98	30	AN5997.3 cds 3'	TTA CTT GAG GGG AAT GAA ACG GGA AGA GTG
KN 112	24	*mutA* cds 5'	ATG AAG ATC TTC CAC CGC TGC TGC
KN 113	30	*mutA* cds 3'	CTA GGC GCT AAA AGA GCC AAC ATA AAC ATT
KN 116	24	AN7893.3 cds 5'	ATG GGC AGT GTC GAG ACG ATC CCA
KN 117	31	AN7893.3 cds 3'	TTA TTT GAG CTT TTC ACC AGT CTT CCC CAT G
KN 118	23	AN5311.3 cds 5'	ATG CGC TTC TCC CTC GCT GCC GT
KN 119	29	AN5311.3 cds 3'	TTA AAG GTA GAT GTA GCA GAA CGG CCC AC
KN 120	21	*ppoC* cds 5'	GAT GCG GTT GCG CTC GTG CGC
KN 121	32	*ppoC* cds 3'	TCA AGC AGA ATC AAT CTG CTT CTT AGG AGT GA
KN 122	24	AN5046.3 cds 5'	ATG CAA TTC TCC GCC ATC GTC CTC
KN 123	23	AN5046.3 cds 3'	CTA GTG GGT GCA GAT GCA GAC CT
KN 124	25	AN2505.3 cds 5'	ATG ACC AGG AAC CTG GAC TCC ATC C
KN 125	23	AN2505.3 cds 3'	CTA ACG GAG CCA GAA ACC ACG GG
KN 126	27	*catD* cds 5'	ATG GTG ACA ACA GCA CAG AGC CAA TGC
KN 127	24	*catD* cds 3'	TCA GGG CCC ATA AAC CGC CAT AGC A

1.5 µg (ca. 130 pmol) of each labelled aRNA was hybridised to the slides using the Agilent "60-mer Oligo Microarray Processing" protocol and buffers. Hybridisation was carried overnight at 65°C in Agilent hybridisation chambers in 500 µl buffer volume. The slides were

washed subsequentially in 2×SSC, 0.2% SDS; 2×SSC and 0.2×SSC for 5 minutes each at 30°C and dried in a nitrogen stream. All steps were performed in an ozone-free environment. Following hybridisation, spot intensities were determined using an Agilent G2505B DNA microarray scanner.

2.5.3. Validation of microarray data

Transcriptome profiling results of selected genes were validated using Northern hybridisation. Experiments were performed according to the protocol of Brown and Mackey (Brown and Mackey, 1997) and hybridisation probes were labelled with [α-^{32}P]-dATP employing the Prime-It®-II kit (Stratagene). Autoradiograms were exposed on BioMaxMS film (Kodak Molecular Imaging, New Haven, CT, USA) or using a Bio-Imaging Analyzer BAS-1500 (Fujifilm Life Science, Stamford, CT, USA). DNA for hybridisation probes was amplified from a sexually induced cDNA library or genomic DNA by PCR using the primers listed in Table 4. Primers used to generate Northern blot hybridization probes for validation of selected genes.

2.6. Computational methods

2.6.1. Sequence analysis

DNA sequence analysis was performed with the Lasergene software from DNASTAR (Madison, WI, USA). Pairwise alignments and the multiple sequence alignment with hierarchical clustering was performed using the CLUSTALW algorithm (Corpet, 1988). Deduced and confirmed protein sequences for performing alignments were retrieved from the Entrez protein database at NCBI. Phylogenetic average distance trees of cullins and Rub1/Nedd8 based on percent sequence identity were constructed using the Jalview application (http://www.ebi.ac.uk/jalview/).

2.6.2. Microarray experimental design and statistical analysis

For transcriptional profiling of wt and the *ΔcsnE* strain during different developmental stages we chose to use a B-swap design using 8 microarrays for comparisons (see Figure 12). It does not allow for identifying absolute changes in gene expression between developmental stages in one strain, but provides statistically robust information about changes in transcription at every developmental stage (Kerr and Churchill, 2001).

Microarray intensity data were extracted using the "Automatic Image Processing for Microarrays" software (Katzer, 2004, PhD thesis). Normalization of the raw intensity data was performed with a non-linear loess regression method (Yang *et al.*, 2002). Differentially

expressed genes were identified by an ANOVA fixed effects model (Landgrebe *et al.*, 2004) where adjusted p-values were obtained by the Benjamini-Hochberg method to control the False Discovery Rate (Bretz *et al.*, 2005). Normalization and statistical computation was done for two independent datasets derived from a high gain and a low gain scan, allowing replacement of saturated features in the high gain scan with data from the low gain measurement. "Contrasts" refer to $\log_2$ normalized intensity ratios between wild-type and *ΔcsnE* samples. Genes with $\log_2$ ratios ≥ 3.2 and adjusted p values ≤ 0.01 were regarded as significantly regulated and genes with $\log_2$ ratios ≥ 2 and adjusted p values ≤ 0.01 were regarded as moderately regulated. Only the significantly regulated genes were included in the functional category analysis. Microarray scanning, data extraction and analysis were performed by dr Reinhard Hitt and Lennart Opitz at the Transkriptom Analyse Labor (TAL) of the Medical Faculty, University of Göttingen.

2.6.3. Data mining and cis-element discovery

To allow functional data sorting, gene probes were annotated manually by homology searches using a translated nucleotide BLAST algorithm (blastx)(Altschul *et al.*, 1997) tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) with the default algorithm parameters. A functional category was assigned to the gene probe only if the sequence with a known or predicted function showing the highest similarity to the query had an alignment similarity score

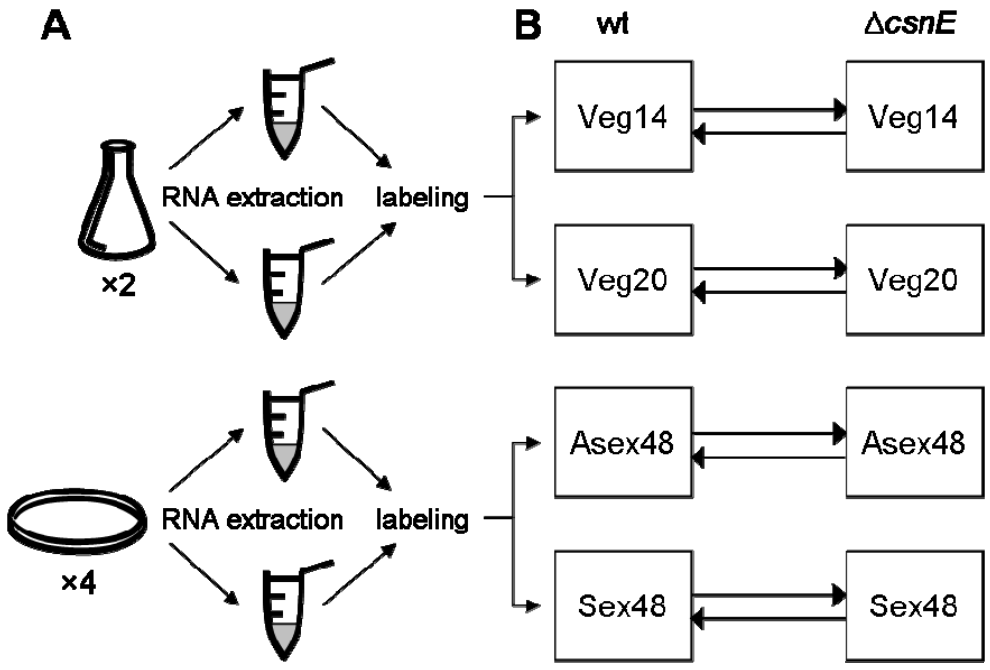


Figure 12. Microarray hybridization experimental setup. (A) Sample collection and preparation. For each of four time points RNA was extracted and labelled as indicated. **(B)** A repeated swap design was used, where wt and *ΔcsnE* aRNAs isolated from cultures at the same developmental stage were always hybridized on one array, but with reversing the assignment of dye labelling in two of four hybridizations. Each arrow in **(B)** indicates duplicate hybridization experiment between the aRNAs from indicated time points and the arrowhead points to the Cy5-labelled aRNA.

of 80 or higher and Expect (E) value of e^{-10} or lower. The exception was the "fungal" category, where gene probes were assigned if no function could be deduced from homology searches, but the probe had significant homology (Expect value of e^{-10} or lower) to other fungal proteins or was unique to fungi (no hits with E value of e^{-10} or lower to sequences from other kingdoms). Each gene probe was assigned to one functional category only.

A complete set of putative promoter sequences up to 1,000 bp upstream of predicted *A. nidulans* ORFs was downloaded from the *Aspergillus nidulans* Database at the Broad Institute (http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/). To identify putative common regulatory elements in the promoters of genes co-regulated in the microarray experiment, Promoter analysis of statistically overrepresented transcription factor binding sites (TFBS) was carried out. Sequences 1,000 bp upstream of different groups of genes co-regulated in the microarray experiment were analysed for enrichment of binding sites using F-Match software (http://www.gene-regulation.com/cgi-bin/pub/programs/fmatch/ffa2.cgi) with p-value threshold = 0.001, using a fungal matrix profile. A set of 150 random upstream sequences, manually filtered out not to contain any sequences upstream of genes which were significantly differentially expressed in the microarray experiment, was used as a control. Enriched transcription factor binding sites were independently mapped to the promoter regions of single genes using MatInspector program from the Genomatix suite (Quandt *et al.*, 1995) (http://www.genomatix.de/products/MatInspector/index.html) and P-match (Chekmenev *et al.*, 2005) (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi) software. The results of these two mapping methods were inspected and compared manually and binding sites with core matrix similarity score 0.95 or higher were visualised. Motif logos illustrating the consensus sequences of binding sites were obtained using WebLogo software (http://weblogo.berkeley.edu).

CHAPTER 3: RESULTS

3.1. The role of neddylation and deneddylation in *A. nidulans*

3.1.1. The JAMM deneddylase motif is conserved in filamentous fungi

To investigate the conservation of the *csnE* Jab1/MPN+ motif, we have compared the *A. nidulans* CsnE protein sequence with its homologues from yeasts, filamentous fungi and higher eukaryotes. Other deduced fungal proteins included a predicted CsnE protein from *A. fumigatus* and the Csn5 protein from *N. crassa* which was shown to form an incomplete CSN complex *in vivo* (He *et al.*, 2005). Figure 13 shows that the JAMM motif is conserved in all analyzed groups of organisms and therefore is most probably conferring the deneddylase activity in *Aspergillus*. There are two other proteins with a conserved Jab/MPN+ motif in the *A nidulans* genome. One (AN4492.3) is a homologue of the 19S proteasome lid subunit Rpn11 (Verma *et al.*, 2002) known to possess deubiquitinating activity and the other (AN3003.3) is similar to AMSH, an endosome-related ubiquitin isopeptidase (McCullough *et al.*, 2004). There is also one protein (AN4523.3) containing a putative partial MPN+ domain. Therefore the observed viability of *csn* mutants is not likely to stem from a redundant deneddylase. Therefore mutagenesis of the active metalloprotease site should be sufficient to show directly if deneddylation activity is necessary for the morphogenetic role of CSN.

Figure 13. The JAMM deneddylase motif is conserved in filamentous fungi. JAMM domain CSN5 proteins of ascomycetes and higher eukaryotes were aligned by ClustalW with the archaea JAMM protein AF2198 (Ambroggio *et al.*, 2004). Identical residues are marked in red, strongly similar in green and slightly similar in blue. an - *Aspergillus nidulans*, af - *Aspergillus fumigatus*, nc - *Neurospora crassa*, hs - *Homo sapiens,* at – *Arabidopsis thaliana,* sp - *Schizosaccharomyces pombe*, sc – *Saccharomyces cerevisiae.* Sequences obtained from GenBank. Conserved JAMM motif residues His143, His145 and Asp156 indicated with stars were mutagenised creating *csnE1* (D156N) and *csnE2* (H143A, H145A, D156N) alleles.

3.1.2. Construction of deneddylase-defective *csnE* alleles and *A. nidulans* strains

To examine the role of the JAMM deneddylase motif in sexual development, we constructed two different mutant alleles. The first one, *csnE1*, carries a mutation only in the codon for the active site aspartic acid (D156N) and the second one, *csnE2*, is also mutated in codons for two active site histidines responsible for coordinating the zinc ion (H143A, H145A, D156N, see Figure 13). The substitutions were introduced by PCR-based mutagenesis to the pME2237 plasmid carrying a 6kb *Eco*RI genomic region of the *csnE* locus. Mutagenized alleles including the native promoter and terminator regions were cloned into a complementation vector containing a phleomycine resistance cassette, resulting in pME2883 and pME2884 plasmids. We have tested the complementation of the mutant phenotype by integrating these plasmids ectopically into the *A. nidulans csnE* deletion strain AGB209 (*pyroA4; pyrG98/pyr-4+ ΔcsnE*). The transformation yielded 6 *csnE1* transformants and 8 *csnE2* transformants, which were verified by Southern hybridisation (Figure 14, panels A-B) and resulted in strains AGB244 and AGB245, respectively. In Northern experiments using the PCR-amplified *csnE* fragment as a probe, *csnE* transcript could be detected in both strains in the amount similar to the wild-type (Figure 14, panel C), confirming that ectopic location does not hinder their transcription.

3.1.3. JAMM-defective *csnE* alleles are not able to complement the *csnE* deletion

The AGB244 and AGB245 strains were grown at asexually and sexually inducing conditions to examine the fruit body formation. After 96 hours of growth the wild-type (A4) strain developed dark coloured cleistothecia surrounded by nests and Hülle cells and the mature cleistothecia contained red-pigmented asci (Figure 15). Both *csnE1* (AGB244) and *csnE2* (AGB245) strains were not able to form cleistothecia or micro-cleistothecia, even after 120 hours of growth (not shown). Microscopic examination revealed that sexual development is blocked at the level of primordia, identical with the *ΔcsnE* strain (Figure 15, panel B). The generation of nests and Hülle cells is not affected. Similarly to the *csnE* deletion strain, the JAMM mutants produce red pigment present in some of the hyphae and released to the growth medium (Figure 15, panel C). Additionally, even in the absence of cleistothecia, both mutants are predominantly induced in the direction of sexual development, which can be seen by production of a large amount of nests in the presence of light. This phenotype is also consistent with the *csnE* deletion, which demonstrates that an intact JAMM motif is necessary for COP9 function in the light-signalling pathway, in subsequent fruit body formation and possibly also in the regulation of secondary metabolism. Both mutant strains exhibit identical

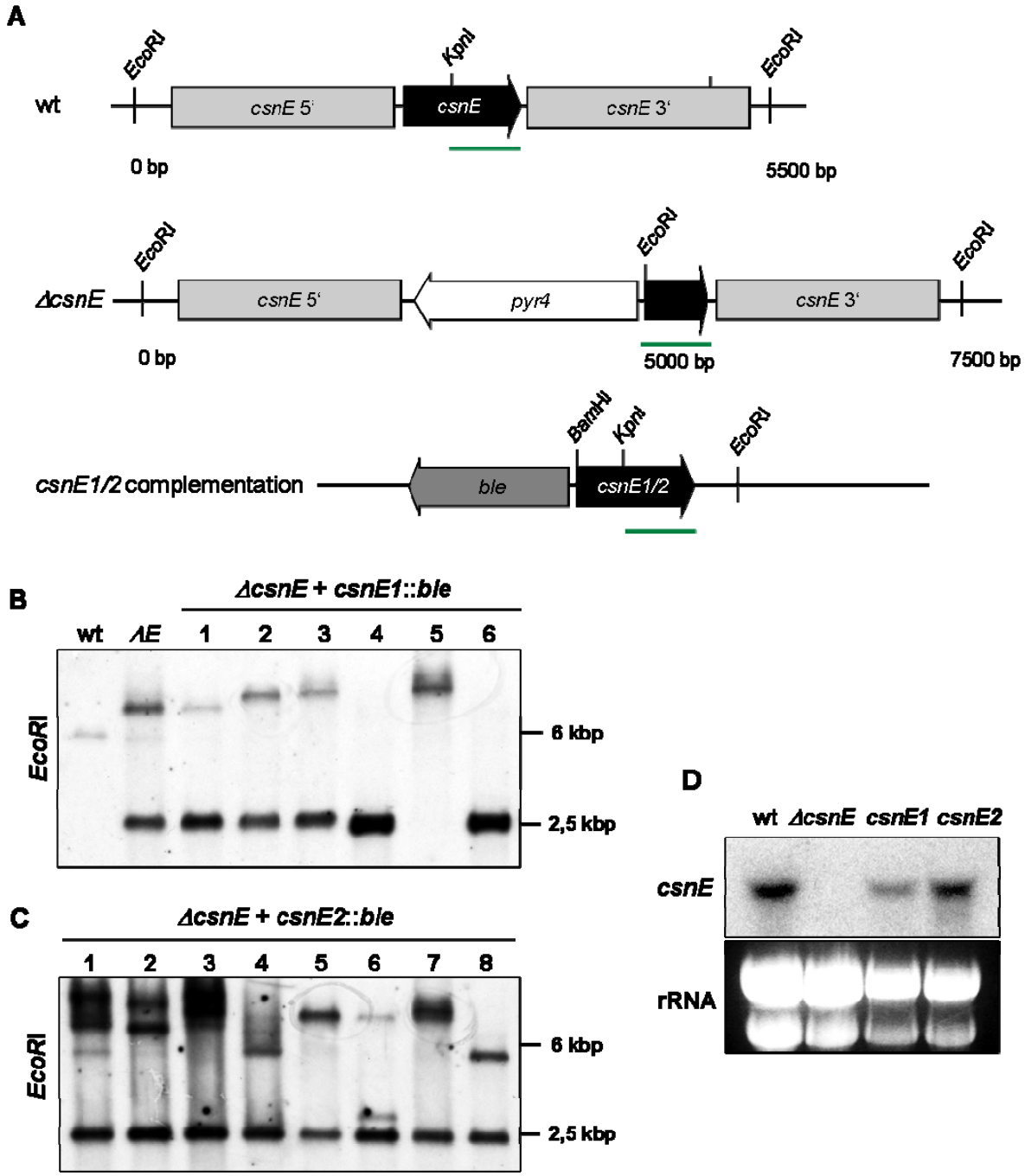


Figure 14. Construction of *A. nidulans* mutant strains AGB244 and AGB245. The plasmids containing mutant alleles *csnE1* and *csnE2* under the control of the native *csnE* promoter and a phleomycin resistance cassette were transformed into the *csnE* deletion strain AGB209. Integration of the *csnE::ble* resistance cassette into the genome was verified by Southern analysis with the 5' region of *csnE* as a probe. **(A)** A scheme of wild-type *csnE* locus, *csnE* deletion locus and ectopic *csnE1*$^{/B}$*::ble* complementation. Southern probes are marked as dark green lines. **(B)** Transformant nr 5 containing one ectopic integration replacing the deletion cassette was designated AGB244. **(C)** Transformant nr 7 containing one ectopic integration in addition to the deletion cassette was designated AGB245. **(D)** Ectopically integrated JAMM *csnE* mutant alleles are efficiently transcribed. **3**0 µg RNA extracted from wt (A4), *csnE* deletion (AGB209), AGB244 and AGB245 strains growing vegetatively was analyzed by Northern hybridization using a *csnE* fragment as a probe. rRNA is shown as a loading control.

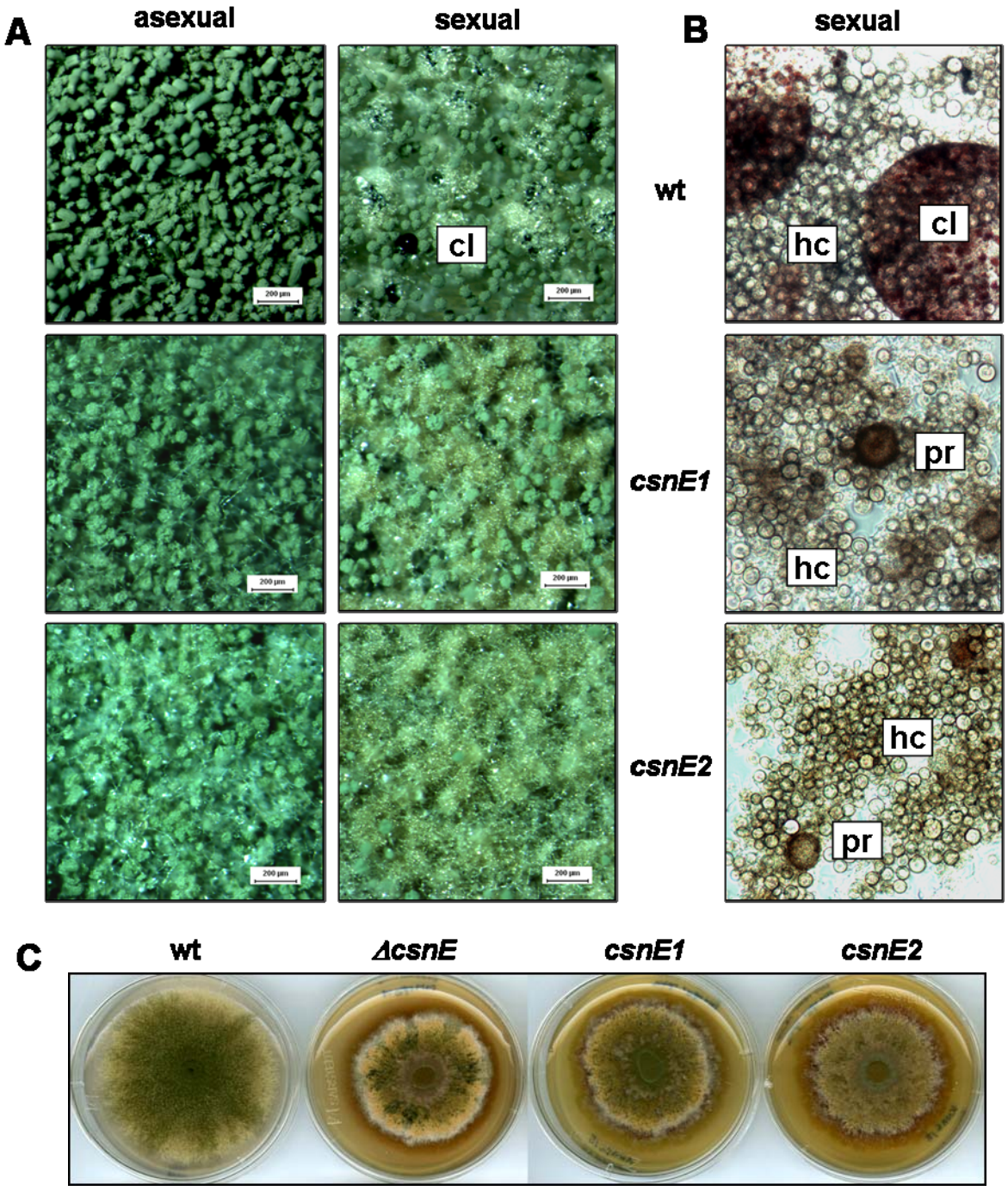


Figure 15. *A. nidulans* CsnE JAMM mutant phenotype mimics the deletion phenotype.
(A) View of the growing colony surface after 96 hours at 37°C in conditions inducing asexual or sexual development. Mature cleistothecia (cl) can be observed in the wt strain, but not in the mutant strains, which form only nests (yellow). **(B)** Microscopic view of the sexual structures. Cleistothecium development is blocked at the level of primordia (pr) in the mutant strains, but auxiliary Hülle cells (hc) are produced normally. **(C)** View of the growing colony after 72 hours in asexually inducing conditions. *ΔcsnE* strain, as well as *csnE1* and *csnE2* strains show an increased amount of nests (visible in yellow) forming on the colony surface. Additionally, a red colouring of the colony can be observed.

phenotypes, indicating that the inactivation of active site Asp156 is sufficient to disrupt the isopeptidase activity.

3.1.4. All *csnE* mutants are temperature- and stress sensitive

To further characterize both the *ΔcsnE* strain and the newly constructed *csnE1* and *csnE2* strains, we have tested their growth at different temperatures. As shown in Figure 16, panel A,

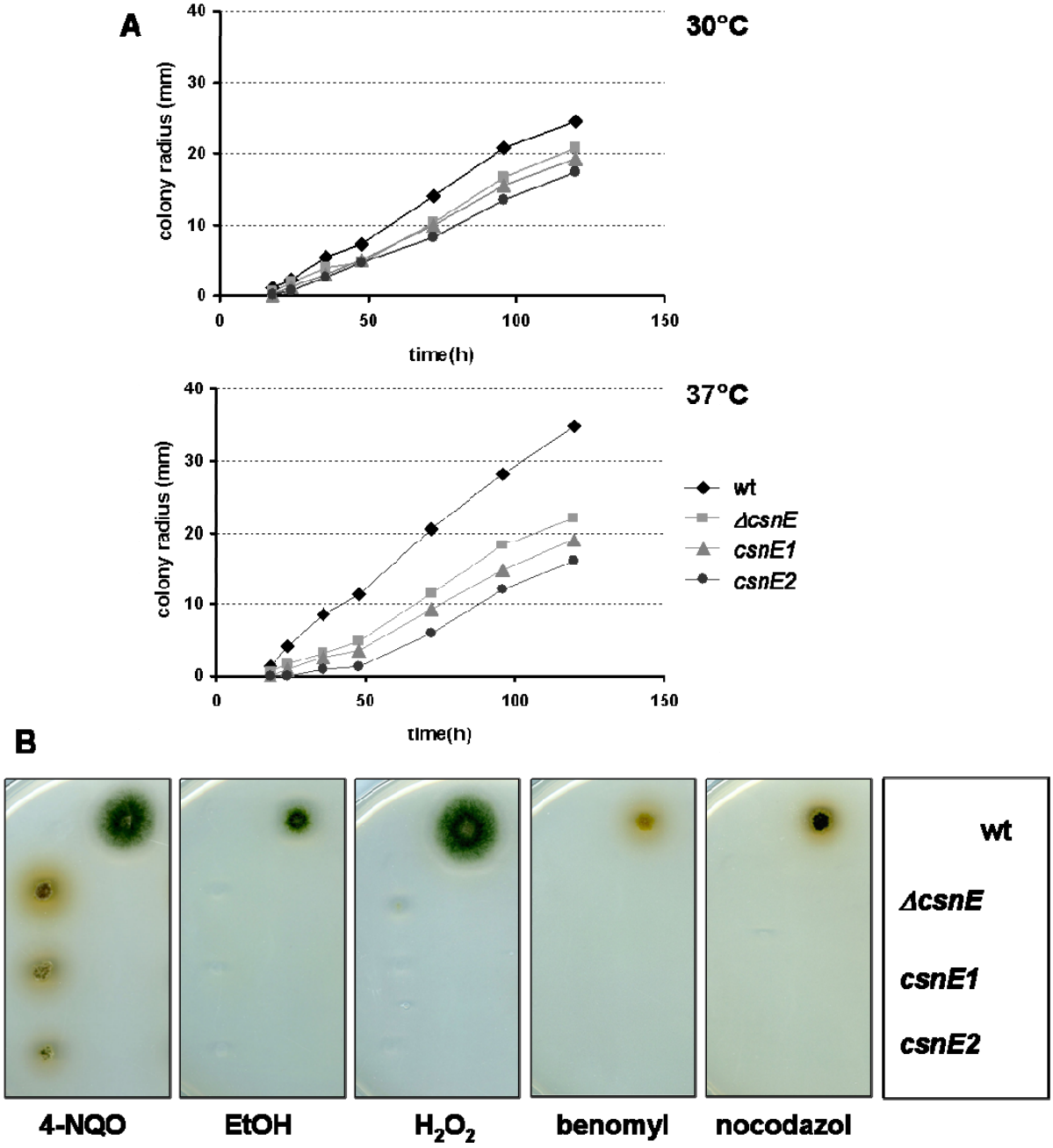


Figure 16. *csnE* mutant strains are sensitive to temperature and different stress-inducing agents. (A) *csnE* JAMM mutant strains, as well as the deletion strain grow at wild-type rate at 30°C, but are delayed in growth at 37°C. The results shown are an average of three independent cultivations, error bars not shown for clarity, standard deviation not exceeding 20% at time points > 36h. **(B)** *csnE* JAMM mutant strains are hypersensitive to DNA damage (4-NQO), ethanol stress, oxidative stress (H_2O_2) and microtubule stress (benomyl and nocodazol), consistent with the phenotype of *ΔcsnE* strain.

while all mutant strains show the same radial growth rate as the wild-type at 30°C, they are considerably delayed in growth at elevated temperature. The developmental block is not relieved when strains are grown at the lower temperature for up to 120 hours (not shown), suggesting that the temperature sensitivity is an independent phenotype, stemming rather from a general lowering of stress resistance.

A proteomic study of the *ΔcsnE* strain (Busch, personal communication) identified several proteins with differing expression levels between wild-type and the mutant during vegetative growth. Most of them appear to be associated with redox balance of the cell and response to oxidative stress (Busch, unpublished results). In order to investigate if this apparent regulation is reflected by heightened stress sensitivity, we have grown the *csnE* mutants in the presence of a number of factors known to induce oxidative as well as other types of stress. The strains appear to be hypersensitive to five stress-inducing agents (Figure 16, panel B). 4-NQO (4-nitroquinoline 1-oxide) is a DNA-damaging carcinogen (reviewed by Bailleul *et al.*, 1989); H_2O_2 increases intracellular peroxide (O_2^{2-}) levels, which leads to the direct oxidation of the sulphur-containing amino acids and generation of OH• radicals (reviewed by Singh, 1982). Products of oxidative ethanol metabolism damage membranes and functional proteins (reviewed by Fernandez-Checa *et al.*, 1997). Finally, benomyl and nocodazol bind to microtubules, interfering with their depolymerisation and polymerisation, respectively (Thyberg and Moskalewski, 1985; Willhite, 1983). At tested concentrations, *csnE* mutants grow weakly in the presence of 4-NQO and their growth is completely blocked in the presence of other agents. While ethanol-induced stress effects are very general and affect many cellular processes, sensitivity other agents suggest an additional role of *A. nidulans* CSN in oxidative balance regulation and oxidative stress response as well as in DNA repair and microtubule assembly and disassembly.

3.1.5. Mutant CsnE still interacts with CsnF *in vitro*

All subunits of the COP9 signalosome contain either a PCI or MPN domain, probably conferring protein-protein interactions (Kapelari *et al.*, 2000). A binary protein interaction map between *A. nidulans* COP9 signalosome subunits has been recently established using two-hybrid analysis (Busch *et al.*, 2007). It identified six direct protein-protein interactions between CSN subunits *in vitro*, one of them between CsnE and CsnF, both containing an MPN domain. Studies in *S. pombe* showed that mutations in the JAMM motif do not impair complex formation (Cope *et al.*, 2002), but there are major differences between the architecture of yeast and *Aspergillus* signalosomes (see Figure 29). We have therefore tested the interaction of CsnE1 and CsnE2 with CsnF using the two-hybrid method in which interaction of two partners

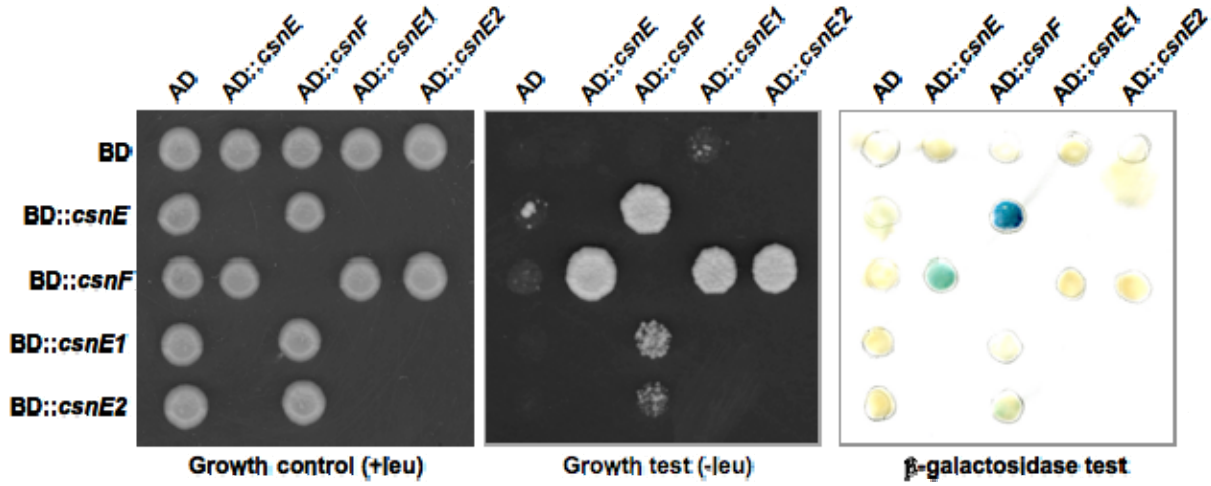


Figure 18. JAMM-defective CsnE interacts weakly with CsnF in a yeast two-hybrid assay. *csnE1* and *csnE2* alleles were fused to the sequences coding for the LexA-binding domain (BD) and the yeast GAL1 transcriptional activation domain (AD) and tested in combination with *csnF* fused to AD and BD, respectively, with the intact *csnE* as a positive control. Protein-protein interaction allows for prototrophic growth of the yeast host strain without leucine and for the activation of *lacZ* gene.

activates transcription of the *LEU* and l*acZ* genes in the two-hybrid yeast reporter strain EGY48-p1840 (MATα, *his3*, *trp1*, *ura3-52*, *leu2*::*pLEU2-LexAop6*, *ura3*::*lacZ-LexAop2*). The growth and expression of the β-galactosidase reporter were analysed on yeast SC medium with galactose and raffinose as carbon source and lacking leucine. *csnE1*, *csnE2* and *csnF* cDNA sequences were fused to the LexA DNA binding domain and the transcriptional activation domain. CsnE and CsnF fusion proteins were combined with the empty two hybrid vectors and with each other in the *S. cerevisiae* reporter strain. None of the fusion proteins shows significant background transcriptional activity. A strong interaction between CsnE and CsnF can be detected in both reporter systems, but an interaction between JAMM-defective CsnE versions and CsnF can be seen almost exclusively in the growth test, exhibiting stronger interaction when CsnE is fused to the activation domain (Figure 18). Considering that the growth test is more sensitive than the β-galactosidase assay, this result indicates that the binary interaction *in vitro* between mutant CsnE and CsnF is weakened, but not abolished. To test whether the weaker interaction of *csnE1* and *csnE2* alleles fused to the DNA-binding domain results from an impaired

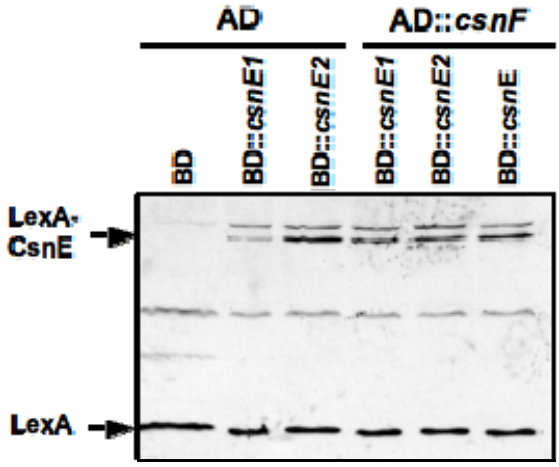


Figure 17. CsnE mutant alleles are efficiently expressed in yeast. The expression of *csnE* alleles fused to BD was confirmed by Western analysis using an anti-LexA antibody.

translation in yeast, we have analysed the expression of both wild-type and mutant alleles expressed in combination with either an empty activation domain or CsnF fused to the activation domain. Figure 17 shows that JAMM-defective alleles are expressed efficiently independently of the presence of the binding partner, which confirms the weakening of the CsnE1/Csn^{B}-CsnF interaction. CsnE seems to be present in two forms in yeast cells, which look identical to the two forms observed *in vivo* in *A. nidulans* in the absence of either CsnA or CsnD and suggesting a limited proteolysis of the subunits in the absence of a full complex (Busch *et al.*, 2007).

3.1.6. The *rubA* gene of *A. nidulans* encodes a highly conserved homologue of the ubiquitin-like modifier Nedd8

The effects of *csnE* mutagenesis indicate that the deneddylation activity of the COP9 underlies its role in sexual development, but is not crucial for growth and survival of *A. nidulans*. As the next step in investigating the role of cullin modification we have questioned the significance of neddylation in this organism. Mammalian Nedd8 is essential in cell cycle

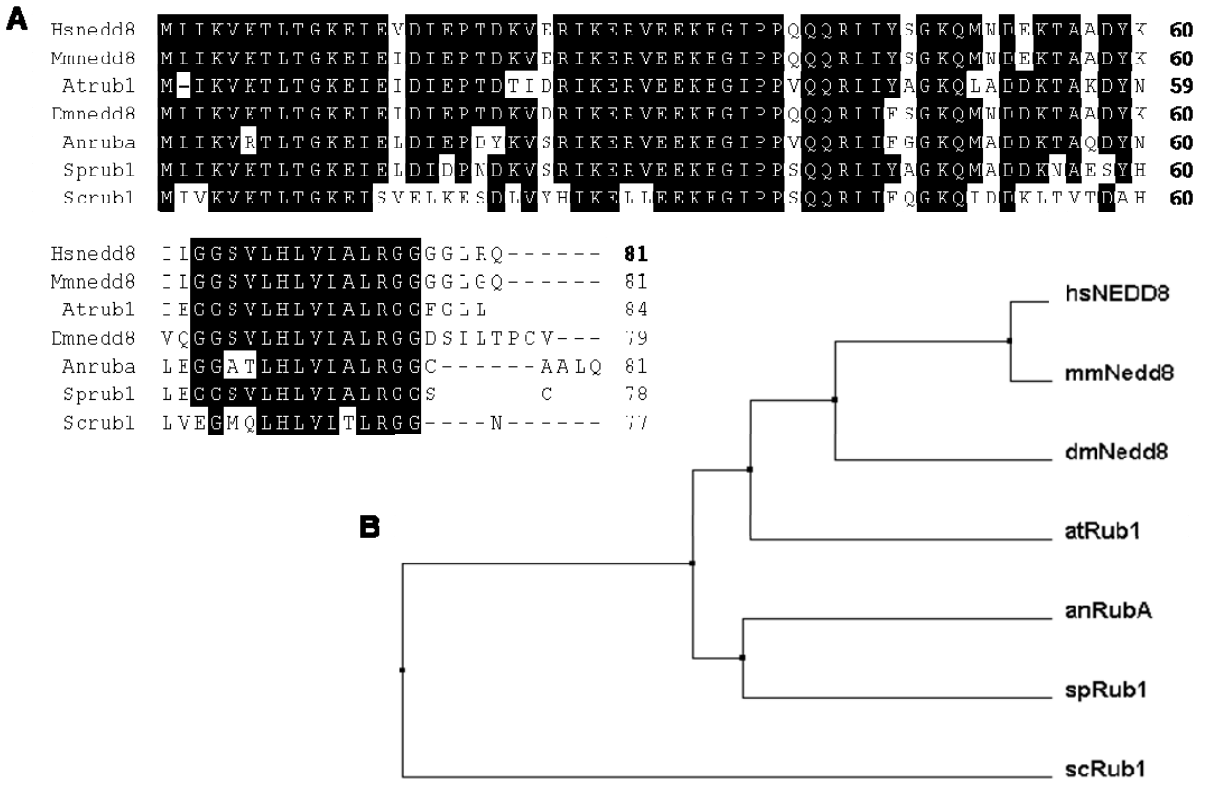


Figure 19. *A. nidulans* RubA is highly similar to *S. pombe* Rub1 and to mammalian Nedd8.
(A) Multiple alignment of NEDD8/Rub1 protein sequences of *Homo sapiens*, *Mus musculus*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Aspergillus nidulans* (AN6179.3), *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. The deduced peptide sequence of the *A. nidulans rubA* gene shows 80% amino acid identity with *S. pombe* Rub1, 75% with mammalian NEDD8 and 60% with *S. cerevisiae* Rub1. **(B)** Phylogenetic tree of the aligned sequences shows that *A. nidulans* RubA is most similar to fission yeast protein, but also to Nedd8 of higher eukaryotes.

control and embryogenesis (Tateishi *et al.*, 2001) and its importance is similar in other eukaryotes with the exception of *S. cerevisiae*, where Rub1 plays a role in growth and cell cycle progression, but is dispensable for survival (Lammer *et al.*, 1998). It is however essential in *S. pombe* (Osaka *et al.*, 2000). Similarity searches revealed one locus in the *A. nidulans* genome, AN6179.3, encoding a homologue of Rub1/NEDD8. The coding region spans 437 bp and includes three putative introns. It encodes an 81 amino acid predicted protein, with molecular weight of ca. 9 kDa calculated from the deduced sequence, showing very high similarity to the *S. pombe* (Osaka *et al.*, 2000) Rub1, Nedd8 of mammals (Kamitani *et al.*, 1997), plants (Pozo *et al.*, 1998) and insects, as well as a weaker similarity to *S. cerevisiae* Rub1 (Hochstrasser, 1996) (Figure 19). The final 5 C-terminal residues located after the conserved Gly75/Gly76 pair are presumably removed during the proteolytic activation, producing a predicted mature protein of 76 amino acids and calculated molecular weight of 8.5 kDa. The gene was named *rubA* in consistency with the *A. nidulans* nomenclature. We have isolated a 5.5 kb genomic *Cla*I/*Apa*I fragment containing the *rubA* locus from an *A. nidulans* BAC library via colony hybridisation with a *rubA*-specific probe. Amplification and sequencing of *rubA* cDNA from a vegetative library by PCR confirmed the predicted introns at 12-76 bp, 131-200 bp and 283-344 bp and established the length of the transcript at 246 bp (Figure 20, panel A).

3.1.7. *rubA* is essential for *A. nidulans* growth

We attempted to construct a *rubA* deletion strain to analyse the role of neddylation in the filamentous fungus. The complete *rubA* open reading frame was replaced by the *A. fumigatus pyrG* marker conferring uridine/uracil prototrophy flanked by *zeo* repeats as described in 2.2.5 and the deletion construct was transformed into AGB152 (*pyroA4; pyrG98*) strain. Three consecutive transformations yielded only a few transformants, shown by Southern blot analysis to contain only ectopic integration of the deletion cassette (not shown). To avoid ectopic integrations, TNO2a3 strain with an *nkuA* deletion, which is defective in non-homologous recombination (Nayak *et al.*, 2006), was used for further transformations. The analysis of resulting 6 transformants revealed the presence of both wild-type *rubA* locus and the homologous integration of the deletion cassette, indicating a formation of diploid cells (Figure 20, panel A). The scarce number of transformants in combination with diploid formation under conditions forcing homologous recombination suggested that *rubA* might be an essential gene, as some of the essential nuclear pore components showed similar symptoms in a previous study (Osmani *et al.*, 2006a). Therefore we analysed *rubA* using the heterokaryon rescue method

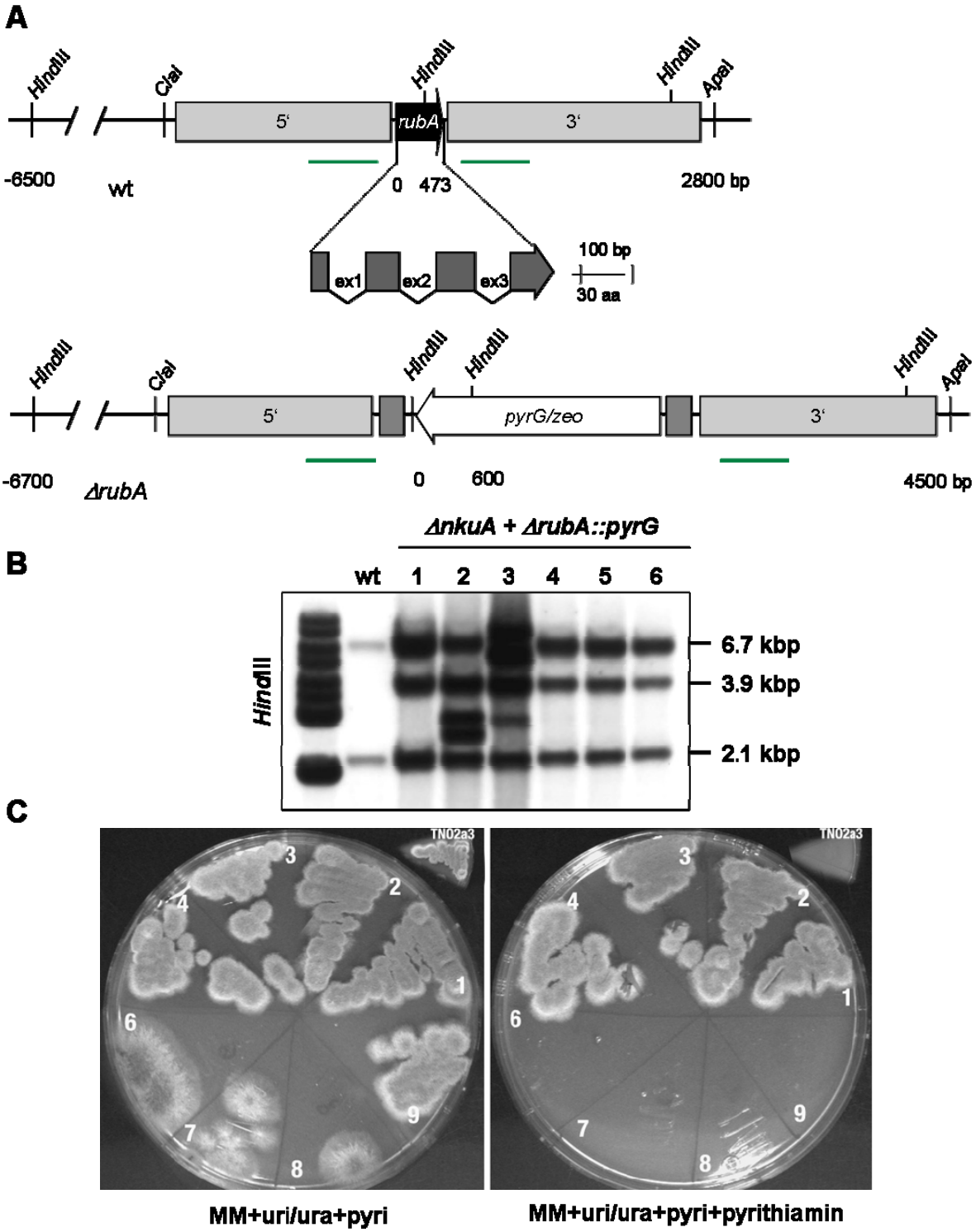


Figure 20. *rubA* is essential for *A. nidulans* growth. (A) The *rubA* genomic locus showing the exon-intron structure and construction of *A. nidulans rubA* deletion strains. The *rubA* deletion plasmid pME3128 was transformed into the *nkuA*-defective TNO2a3 strain. Southern probes are marked by dark grey lines. **(B)** Integration of the cassette was examined by Southern analysis with both the 5' and 3' flanking regions of *rubA* as probes and revealed the presence of wild-type bands as well as bands indicating a homologous integration of the deletion cassette into the genome, indicative of diploid formation. **(B)** Heterokaryon rescue of *rubA* deletion. Spores derived from 8 primary transformants were tested. Transformants 1-4 grew on selective medium, while transformants 6-9 showed heterokaryon formation. Photo in (B) was made by Marcia Kress.

(Osmani *et al.*, 2006b) utilizing an alternative deletion cassette where the *rubA* open reading frame was replaced by a dominant *ptrA* marker conferring pyrithiamine resistance. The method is based on an observation that if an essential gene is deleted, the null allele is maintained in spontaneously generated heterokaryons that consist of two genetically distinct types of nuclei. One nuclear type has the essential gene deleted but carries the *ptrA* gene (*ptrA*$^{+}$). The other has the wild-type allele of the essential gene but lacks the *ptrA* gene (*ptrA*$^{-}$). An essential gene can be distinguished by a simple growth test of conidia formed from primary transformants as it can only be propagated by heterokaryon rescue. The results of heterokaryon rescue analysis are shown in Figure, panel C. Spores derived from 4 out of 8 primary transformant colonies (6-9), resulting from integration of the deletion cassette in the TNO2a3 strain, could grow only on non-selective medium, indicating heterokaryon formation. Homologous integration of the *rubA* deletion cassette in these transformants was confirmed by PCR (Marcia Kress, not shown). The exact phenotype of *rubA* deletion during germination remains to be investigated, but preliminary observations show that *ΔrubA* spores fail to germinate and undergo cell division, confirming that *rubA* is essential for *A. nidulans* survival.

The mutagenesis experiments presented here show that all observable functions of the *A. nidulans* CSN depend on its JAMM motif and presumably on its deneddylase activity. While the deneddylation by the COP9 signalosome is dispensable for vegetative growth, neddylation of substrates is essential for the filamentous fungus.

3.2. Developmental transcriptome profiling analysis of an *A. nidulans* *ΔcsnE* strain

After establishing the role of neddylation in *A. nidulans* and the importance of the deneddylase motif in CSN function, the next step was to investigate the cellular mechanisms by which the fungal COP9 signalosome influences the developmental processes. To identify signalling pathways requiring CSN to complete sexual development even before the phenotype becomes apparent, we have used a DNA microarray approach. The viability of the CSN mutants allowed us to perform transcriptional profiling experiments across many developmental time points to address this question. Wild-type and *ΔcsnE* strains were compared at four time points, after 14 and 20 h of vegetative growth in liquid culture (referred to as V14 and V20, respectively) and after 48 h growth on solid medium, induced to develop either asexually or sexually (referred to as A48 and S48, respectively). Labelled amino allyl-RNAs from wt and *ΔcsnE* strains from the same developmental stage were always hybridised on the same array and a dye swap (see Materials and Methods) was employed to limit the labelling bias, according to the setup presented in Figure 12. Including the duplication of oligo spotting on the array, a total of 8 data points were obtained for each sample in each experiment.

3.2.1. Reproducibility of microarray hybridisation

The genome-wide transcriptome profiling experiment described above was performed twice, using a pooled RNA isolation from different cultivation series each time. Considering the unlimited growth of *A. nidulans* as a microbial mycelium and the pooling of different mycelium samples grown at the same time, additional biological replicates were not essential to achieve statistical significance. Nevertheless, we wanted to examine the variability reproducibility of the transcriptome profiling experiments with regards to the growth conditions. As shown on Figure 21, there is a much stronger correlation between the results of two experiments for the developmentally-induced samples after 48 h of asexual growth (correlation coefficient R=0.79) and sexual growth (R=0.67), than for the vegetative samples (R=0.55 after 14 h and R=0.56 after 20 h hours of growth), indicating that the effect developmental induction signals overcomes the inherent variability in cultivation conditions. We have decided to continue the analysis using the results of one experiment to avoid losing information about developmentally induced genes, which could be masked by differences in growth conditions. However, confirming the relative reproducibility of these growth conditions was necessary to select the best dataset for further analysis. We have analyzed the expression of two gene probes showing greatest differences in $\log_2$ contrasts between the two experiments after 14 and 20 h of

vegetative growth, respectively, by Northern hybridization, using RNA isolated from a third independent growth experiment (Figure 22). The Northern analysis confirms that expression pattern of AN8532.3 differs very strongly between the two experiments (panel A) and shows that expression of AN8532.3 at V14 (vegetative 14 hours) stage and of AN1832.3 at V20 (vegetative 20 hours) stage from experiment 2, but not experiment 1, can be reproduced in a third independent experiment. Based on these results, transcriptome profiling data from experiment 2 was used for further analysis, although the majority of genes showing significant changes in gene expression which are discussed in detail later were regulated in the same fashion in both experiments. The validation results of additional gene probes using RNA isolated from an independent experiment, shown in 3.2.3, further confirm the high reproducibility of experiment 2 dataset.

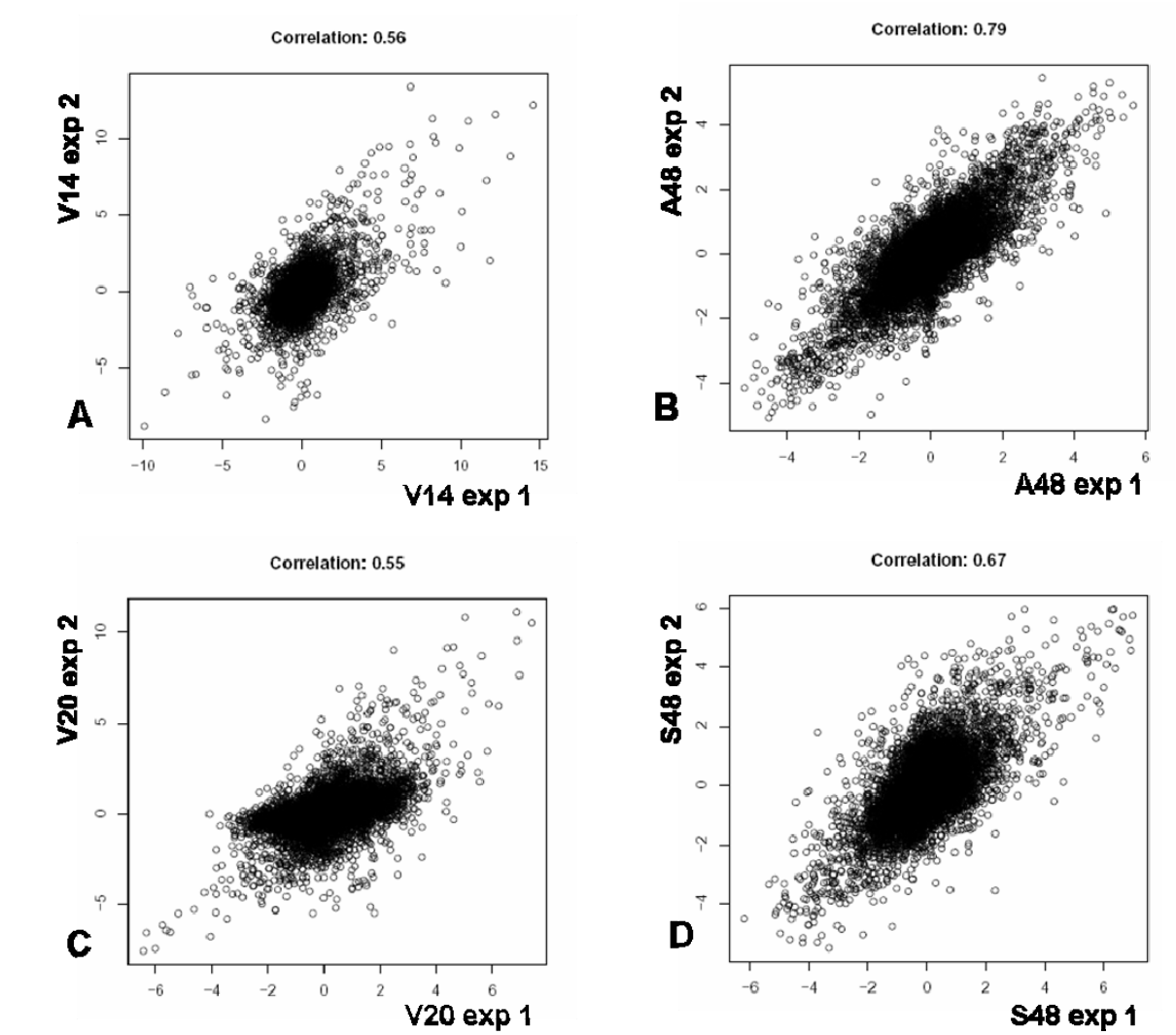
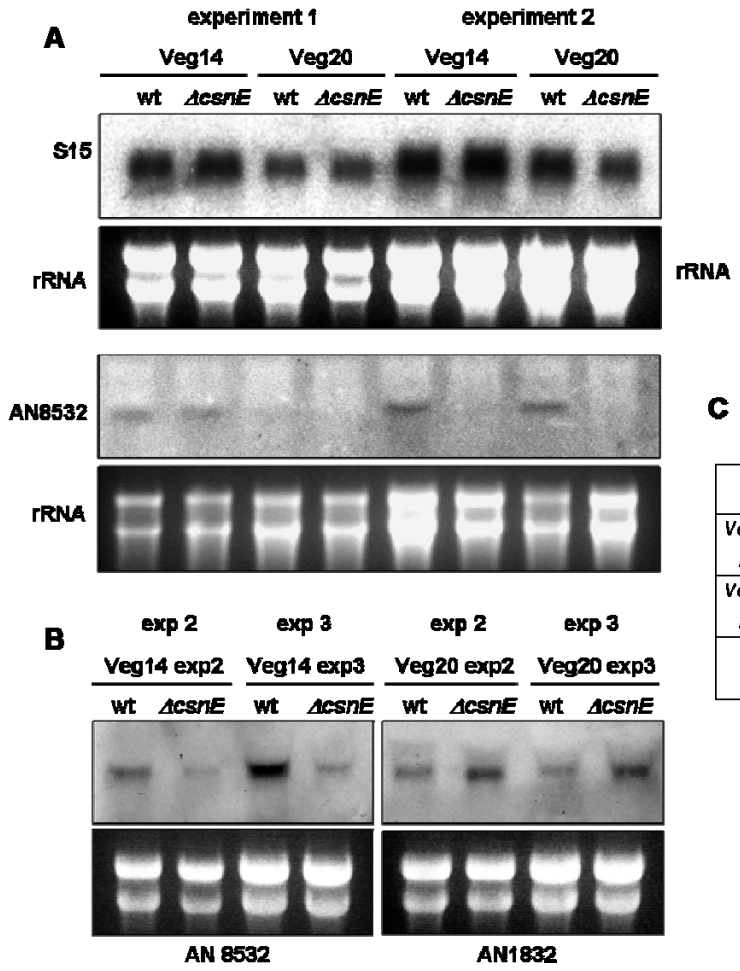


Figure 21. Reproducibility of microarray data. The correlation between two hybridization experiments is shown as correlation between the contrasts (normalized $\log_2$ ratios) of all data points representing single gene probes. **(A)** wt-*ΔcsnE* contrast at vegetative 14h, **(B)** wt-*ΔcsnE* contrast at vegetative 20h, **(C)** wt-*ΔcsnE* contrast at asexual 48h, (D) wt-*ΔcsnE* contrast at sexual 48h. Correlation analysis and visualisation was performed by Lennart Opitz, Transkriptom Analyse Labor.



C

	Log_2 contrast wt-ΔcsnE exp 1	Log_2 contrast wt-ΔcsnE exp 2
Vegetative 14h AN8532.3	0,49	4,90
Vegetative 20h AN1832.3	4,52	-0,32
int standard AN5997.3	0 (14h) 0,1 (20h)	-0,1 (14h) 0,1 (20h)

Figure 22. Validation of reproducibility of the two microarray hybridization experiments. Approximately 30 µg RNA from the two microarray experiments (exp1 and exp 2) or from an independent repetition at the same conditions (exp 3) was loaded on gel, blotted and hybridized with appropriate DNA probes. Non-regulated ribosomal protein S15 (AN5997.3) was used as an internal control and rRNA is shown as a loading control. **(A)** AN5997.3 is expressed on steady levels both at Veg14 and Veg20 stages in both experiments **(B)** Expression patterns of AN8532.3 and AN1832.3 can be reproduced in an independent experiment. **(C)** Variation of expression changes of the selected genes between the two experiments.

3.2.2. Deletion of *csnE* results in broad changes of gene expression during *A. nidulans* growth and development

In the light of almost identical *csn* mutant phenotypes, the transcriptomes of different *csn* deletions in *A. nidulans* might be similar, also considering the previous studies indicating that there are probably no stable CSN subcomplexes in filamentous fungi (Busch *et al.*, 2007). We selected the deletion mutant of *csnE*, encoding for the CSN5 subunit, to represent the CSN influence on transcription during development. The pleiotropic phenotype of *csn* deletion mutants, including a block in sexual development, disturbance in light-dependent development initiation and secondary metabolism suggested that *A. nidulans* CSN regulates more than one pathway and that the transcriptional effects on these pathways should be seen before the morphological phenotype could be observed.

To address this question, we performed a genome-wide transcriptome comparison of wild-type and *ΔcsnE* strains at four time points, at V14, V20, A48 and S48. *A. nidulans* mycelium achieves developmental competence about 16-18 hours after germination (Champe *et al.*, 1994), so the two selected vegetative time points reflect the transcriptomes before and after achieving competence, when the impact of CSN may differ. After 48 hours of sexually-induced growth, first morphological changes indicating the development of a fruit body are visible (Braus *et al.*, 2002). At this point, the sexual block phenotype is not obvious in the mutant strain yet, but the misregulation of light-dependency of development resulting in increased nest formation is already visible.

Table 5. Summary of transcriptional changes in *ΔcsnE* strain relative to the wild-type during development with log_2 contrast >3.2 (bold) or >2 (regular).

	Number of genes regulated in *ΔcsnE*					
Time point	**Up**			**Down**		
Vegetative 14h	**>3.2**	**74**	**33** *14 and 20h*	**>3.2**	**134**	**81** *14 and 20h*
	>2	226		>2	286	
Vegetative 20h	**>3.2**	**86**		**>3.2**	**130**	
	>2	264		>2	322	
Asexual 48h	**>3.2**	**115**	**58** *asexual and sexual*	**>3.2**	**115**	**62** *asexual and sexual*
	>2	395		>2	396	
Sexual 48h	**>3.2**	**104**		**>3.2**	**130**	
	>2	335		>2	355	

The microarray hybridisation experiments reveal a large number of genes that are differentially expressed in the *ΔcsnE* strain throughout development. In total, the expression levels of 584 unique genes, representing 5% of the genes on the array, are significantly altered (log_2 contrast value ≥ 3.2, $p \leq 0.01$) in the mutant strain as compared with the wild-type at least on one developmental stage (Table 5). The expression levels of 1625 genes, representing 14% of the genes on the array, are moderately altered (log_2 contrast value ≥ 2, $p \leq 0.01$). During vegetative growth, almost twice as many genes are significantly downregulated in the *csnE* mutant than upregulated, although the number of up- and downregulated genes is similar in both asexually and sexually developing cultures. Only around half of the genes differentially expressed either during early or late vegetative growth show parallel regulation at V14 and V20, indicating that the transcriptome regulation by CSN indeed changes after achieving of developmental competence. Around half of the genes differentially expressed after developmental induction exhibit parallel regulation in asexually- and sexually- inducing

conditions. Nine genes are significantly regulated in the mutant strain at all stages of growth and development, five of them being always upregulated, two always downregulated and two showing a mixed regulation (Table 6).

Table 6. Summary of the genes differentially expressed in the *ΔcsnE* mutant at all growth and developmental stages.

Gene ID	Function	Log_2 contrast wt-*ΔcsnE*				up/ down
		V14	V20	A48	S48	
AN7893.3	Putative citrinin biosynthesis oxygenase	-7,28	-7,44	-4,73	-4,24	↑
AN7902.3	Putative salicylate 1-monooxygenase	-6,95	-7,57	-4,55	-5,30	↑
cipB	Enoyl transferase / alcohol dehydrogenase	-6,81	-5,28	-4,14	-4,33	↑
AN11584.3	Hypothetical protein	-6,78	-6,42	-3,59	-4,38	↑
AN7894.3	Hypothetical protein, YCII-related domain	-6,13	-6,56	-3,93	-4,11	↑
AN5945.3	Conserved hypothetical *Aspergillus* protein	8,36	7,99	-3,55	-4,03	↓↑
AN0759.3	Conserved hypothetical protein, PLAC8 family	11,54	9,55	-3,81	-3,30	↓↑
AN5353.3	Conserved hypothetical fungal protein	6,36	7,70	3,54	4,07	↓
AN5046.3	Conserved hypothetical protein similar to defensin	12,17	10,52	3,67	4,03	↓

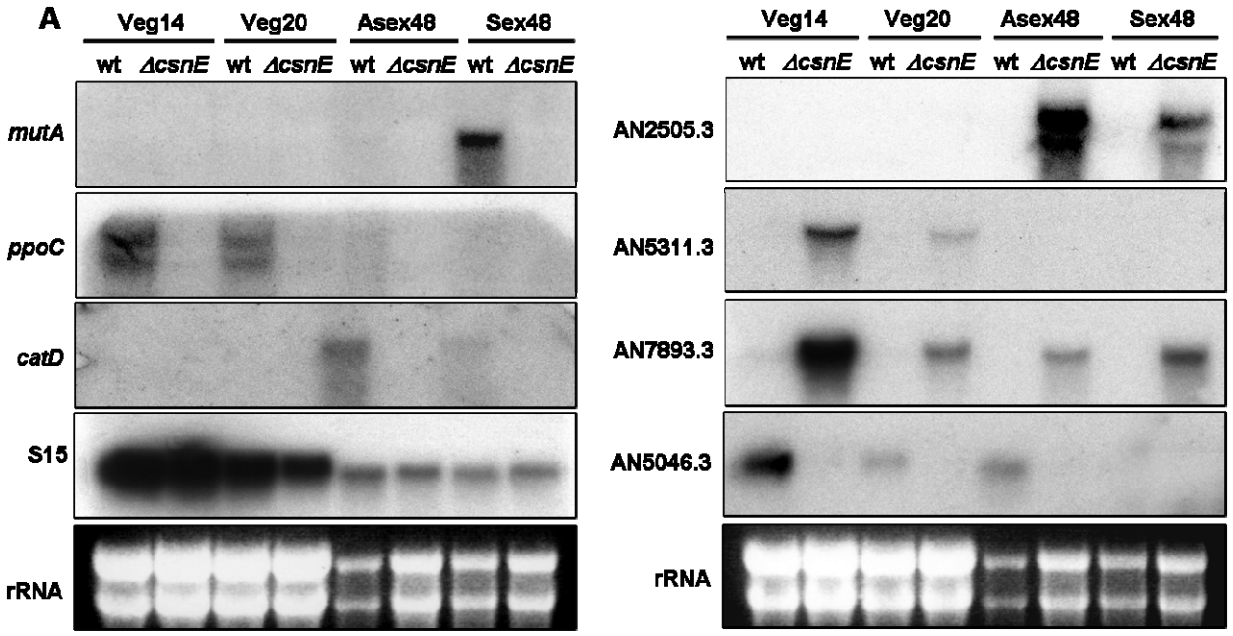
Arrows indicate change in *ΔcsnE* relative to wild-type.

As an initial validation of the microarray data, we examined the expression of mRNAs encoding the other CSN subunits in the transcriptome profiling experiment. As expected, their expression in the *csnE* deletion strain is not affected (data not shown), which is in agreement with the previous observation that the absence of CsnA or CsnD does not influence CsnE protein levels (Busch *et al.*, 2007). Similar to the previous observation in a *ΔcsnD* mutant (Busch *et al.*, 2003), the mRNA level of the developmental regulator *veA* (Kim *et al.*, 2002) do not change in the experiment, and neither does the transcription factor *nsdD* involved in fruit body development (Han *et al.*, 2001).

3.2.3. Northern analysis of selected gene mRNA levels validates the transcriptome profiling results

To further validate the transcriptome profiling results, we have selected 8 genes belonging to different functional categories and with different expression patterns in the transcriptome profiling experiment and analysed their expression by Northern hybridisation across the same time points as tested in the microarray analysis. Figure 23 shows that expression changes of all

tested genes analysed by Northern method correlate well with the microarray data. The transcriptional activation of dioxygenase *ppoC* (AN5028.3), putative catalase *catD* (AN8553.3) and α-glucanase *mutA* (AN7349.3) is suppressed in the *ΔcsnE* strain during sexual development, vegetative growth and asexual development, respectively, while AN2505.3 (encoding a putative F-box protein) is misactivated after induction of development and AN5311.3 (encoding a putative tyrosinase) during vegetative growth. Expression patterns of AN7893.3 (encoding a putative dehydrogenase) and AN5046.3 (encoding a protein similar to a defense-related peptide) are reversed at all times in the mutant.



B

Gene	Log₂ contrast wt-*ΔcsnE* V14	V20	A48	S48	Gene	Log₂ contrast wt-*ΔcsnE* V14	V20	A48	S48
mutA	0	0	4	4,4	AN2505	-0,3	-1,8	-3,6	-3,3
ppoC	6,4	3,2	3,7	2,5	AN5311	-5,4	-5,4	1,5	1,4
catD	-0,3	-0,4	4,7	4	AN7893	-7,3	-7,4	-4,7	-4,2
S15	0	-0,1	0,3	0,2	AN5046	12,2	10,5	3,7	4

Figure 23. Expression of selected genes from different functional groups. (A) Approximately 30 µg RNA from the same growth conditions as in the microarray experiment was loaded on gel, blotted and hybridized with appropriate DNA probes. Ribosomal protein S15 (AN5997.3) was used as an internal control. AN2505.3: putative F-box protein, AN5311.3: putative tyrosinase involved in melanin synthesis, AN7893.3: citrinin biosynthesis dehydrogenase homologue, AN5046.3: defensin homologue. *mutA*, *ppoC* and *catD* transcription require *csnE* for transcriptional activation during sexual development, vegetative growth and asexual development, respectively. AN2505.3 and AN5311.3 are misactivated in the *ΔcsnE* strain during development and vegetative growth, respectively. Patterns of AN7893.3 and AN5046.3 are reversed at all times in *ΔcsnE* strain. **(B)** normalized $\log_2$ ratios of expression of analyzed genes in microarray experiments, with negative values indicating upregulation and positive values indicating downregulation.

3.2.4. Transcriptional profile of the *ΔcsnE* strain reveals groups of genes especially affected by absence of CSN function

The genes differentially expressed in the mutant strain were assigned to 11 functional categories, based on existing automatic annotation available or similarity to characterized proteins from other organisms as examined by blastx searches (see Methods). The majority of genes were categorized by homology, because up to date less than 10% of the 10,701 putative *A. nidulans* genes have been assigned a function based on automated gene prediction tools. Figure 24 and Table 7 show an overview of functional gene categories up-and downregulated throughout development. In cases when the gene was conserved among fungi, but there was no indication of the gene product function, the gene was assigned to the "fungal" category. Interestingly, two genes always downregulated in the *ΔcsnE* belong to this category (Table 6), suggesting that the CSN might affect conserved fungal pathways of yet uncharacterized function. The largest group of genes misregulated throughout development encodes enzymes with oxidoreductase activity, some of them involved in both primary and secondary metabolism and assigned to these two functional categories accordingly, but predominantly of yet undetermined function and assigned to the "redox enzymes" category. In total, between 15% and 17% of all genes differentially expressed at each stage are associated with redox balance. The second largest group of genes expressed differentially at all developmental stages falls into "transport" category, encoding proteins facilitating membrane transport of different metabolites, predominantly carbohydrates, amino acids and toxins. Between 6% and 11% and of genes affected in the mutant at each stage fall to this category, although the difference in expression is notably higher during vegetative growth than during development.

The main metabolic networks involved in carbon metabolism, as defined by a recent transcriptional analysis study (David *et al.*, 2006) do not appear to be affected in the *ΔcsnE* mutant. Only a limited number of genes encoding for proteins predicted to be involved in carbohydrate, amino acid and fatty acid metabolism, as well as electron transport, is differentially expressed. On the other hand, consistent with the red-pigmented phenotype of the mutant (Busch *et al.*, 2003), many genes potentially involved in various secondary metabolism biosynthesis pathways are misregulated, especially during vegetative growth (Table 7), as discussed later in detail. Considering the developmental phenotype and the implication of Arabidopsis CSN in plant defense responses (Azevedo *et al.*, 2002), we have expected the genes associated with stress and defense and the regulation of transcription and development to be regulated. It appears to be the case at all time points, but only a small number of genes are affected. Finally, there are three groups of genes whose expression is affected only after the

initiation of development. Two are associated with protein degradation and include both downregulated proteases and upregulated components of the ubiquitin system (Table 7). The third and strikingly repressed group consists of genes encoding cell-wall degrading enzymes, associated both with autolysis and the degradation of plant cell wall components. This is by far the most prominent category of downregulated genes, especially at the S48 stage, where it amounts to 29 genes. Because of the apparent transcriptional control of cell wall degradation loci by *csnE*, below we focus largely on these genes and the integration of their regulation with developmental and secondary metabolism regulatory pathways.

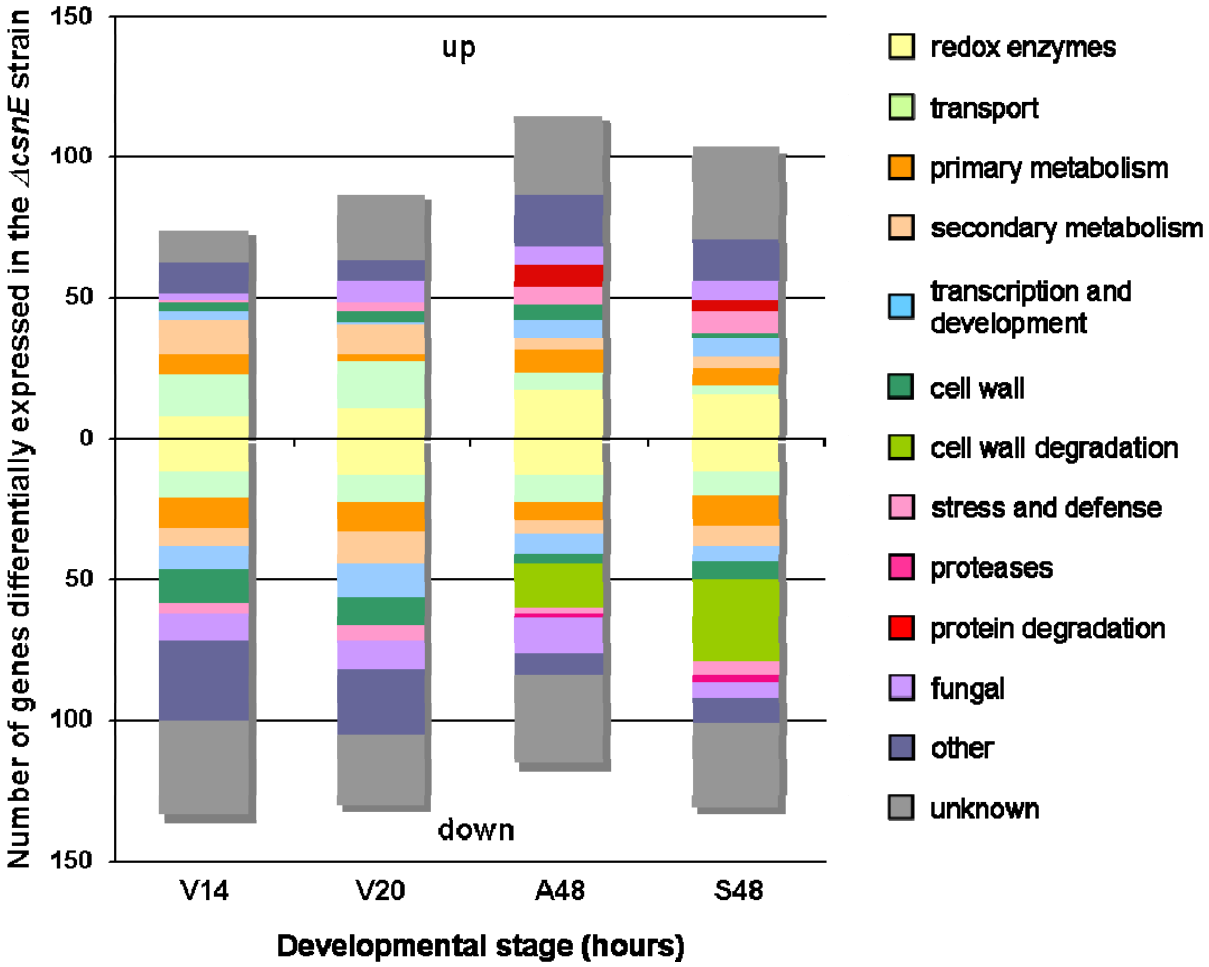


Figure 24. Functional category analysis of genes differentially expressed in the *ΔcsnE* mutant at different developmental stages. The genes were assigned to functional categories manually based on similarities to genes with known functions. V14, V20 – vegetative growth at 14 and 20 hours, A48 – asexual development at 48 hours, S48, sexual development at 48 hours. Positive values indicate numbers of genes upregulated in *ΔcsnE* strain at a given time point in comparison with the wt strain, negative values – downregulated in *ΔcsnE* strain. Absolute numbers of genes assigned to functional groups are shown, as listed in Table 7 below. "other" denotes genes with assigned function not falling into any of the main categories, while "fungal" denotes genes with no assigned function but significantly conserved or unique to fungi only.

Table 7. Absolute numbers of genes belonging to main functional categories up- and downregulated in the *ΔcsnE* mutant at different growth and development stages.

	V14		V20		A48		S48	
	up	down	up	down	up	down	up	down
redox enzymes	8	12	11	13	17	13	15	12
transport	15	9	16	10	7	10	4	8
primary metabolism	7	11	3	10	8	6	6	11
secondary metabolism	12	6	10	12	3	5	4	7
transcr. and development regulation	3	8	1	11	7	7	6	6
cell wall	3	12	4	10	5	4	2	6
cell wall degradation	0	0	0	0	0	15	0	29
stress and defence	1	4	3	6	7	2	8	5
Ub-dependent protein degradation	0	0	0	0	8	0	4	0
proteases	0	0	0	0	0	2	0	2
fungal	3	10	8	10	6	12	7	6
other	11	28	8	23	18	8	15	9
Unknown (hypothetical)	11	34	22	25	29	31	33	30

3.2.5. Expression of redox enzymes closely related to secondary metabolism is affected in *ΔcsnE* mutant at all growth and development stages

The largest group of genes differentially expressed in the absence of CSN at all time points are oxidoreductases. Redox balance and reactive oxygen species have been shown to play a role in regulating sexual development (Lara-Ortiz *et al.*, 2003) and many oxidoreductases are involved in biosynthesis of secondary metabolites in fungi (reviewed by Yu and Keller, 2005). A notable group among the affected oxidoreductases are cytochromes P450, belonging to a superfamily of heme-containing monooxygenases involved in various cellular activities, including xenobiotic metabolism and detoxification, biogenesis of cholesterol and, in fungi, secondary metabolism (Aninat *et al.*, 2005). Members of this oxygenase family are enriched in the CSN-regulated transcriptome, with 27% (11 out of 40) of cytochromes P450 predicted by hmmer/Pfam (Hidden Markov model protein domain search) in the *A. nidulans* genome regulated at least on one developmental stage. Although it is hard to conclude from available data what role CSN may play in regulating oxidative balance, it is supported by the fact that a gene encoding catalase D, existence of which was predicted by Kawasaki and Aguirre (2001), seems to require CSN for developmental transcriptional activation (Figure 23).

A relationship between fungal development, especially sexual sporulation, and secondary metabolite production has been long recognized (reviewed by Calvo *et al.*, 2002). It is therefore not surprising that genes associated with secondary metabolism account for 10% of total

misregulated genes in the *ΔcsnE* mutant during vegetative growth and 4% during development. It suggests that CSN influences these processes at early stages of signalling, before the onset of development itself, more likely at the stage of determining balance of development (analogous to *ppoC*). Three major groups of genes associated with biosynthesis of polyketide secondary metabolites are affected: 1) homologues of citrinin biosynthesis genes of *Monascus purpureus*, 2) genes involved in biosynthesis of pigments and 3) genes similar to sterigmatocystin synthesis genes. For a full list of genes and their regulation, see Table 8.

Citrinin is a polyketide-derived mycotoxin, produced by several *Monascus* ands *Aspergillus* species, but not described in *A. nidulans*. The homologues of citrinin biosynthesis genes are consistently upregulated in the mutant, especially during vegetative growth and AN7893.3, an oxygenase similar to *M. purpureus* CtnA (40% identity), is among the five genes upregulated all at the time points. Interestingly, all constantly upregulated genes, together with *cipB* oxidoreductase (Melin *et al.*, 2002) and homologues of *M. purpureus* proteins CtnB (45% identity), CtnR (34% identity) and citrinin polyketide synthase (Shimizu *et al.*, 2005)(45% identity), form a cluster on chromosome II (Figure 25). The cluster spans 13 genes, 12 out of which are upregulated in *ΔcsnE* strain on at least one stage of growth or development. *cipB* is homologous to an *Aspergillus terreus* enoyl transferase LovC involved in the synthesis of secondary metabolite lovastatin (Kennedy *et al.*, 1999), while other genes have a predicted biochemical function only, encoding for putative three oxygenases, a transporter and a transcription factor. All of these activities could have roles in secondary metabolite synthesis. The non-regulated locus AN11583.3 might not be a functional gene, as it encodes for a very short polypeptide with no similarities to other sequences. All other genes in the cluster are upregulated, suggesting that the genomic cluster is normally under repression mediated by the CSN and might be functional in *A. nidulans*, although it is not known to produce citrinin. A homologue of *M. purpureus* citrinin transporter CtnC, not localized on the cluster is moderately upregulated at V14. It should be noted that the automatic annotation of AN7903.3 is probably incorrect (the annotated locus spans only two polyketide synthase domains) and a longer, previously annotated sequence AN0523.2 was used for alignment with the polyketide synthase.

Differentially expressed pigment biosynthesis genes comprise four putative tyrosinases or tyrosinase domain containing proteins and *wA*, encoding the conidial yellow pigment biosynthesis polyketide synthase. The homologues of sterigmatocystin biosynthesis genes include putative o-methyltransferases and oxidoreductases, but none of actual genes known to be involved in ST biosynthesis are affected, suggesting that they are involved in other, not characterized secondary metabolism pathways. Genes belonging to these two groups, as well as

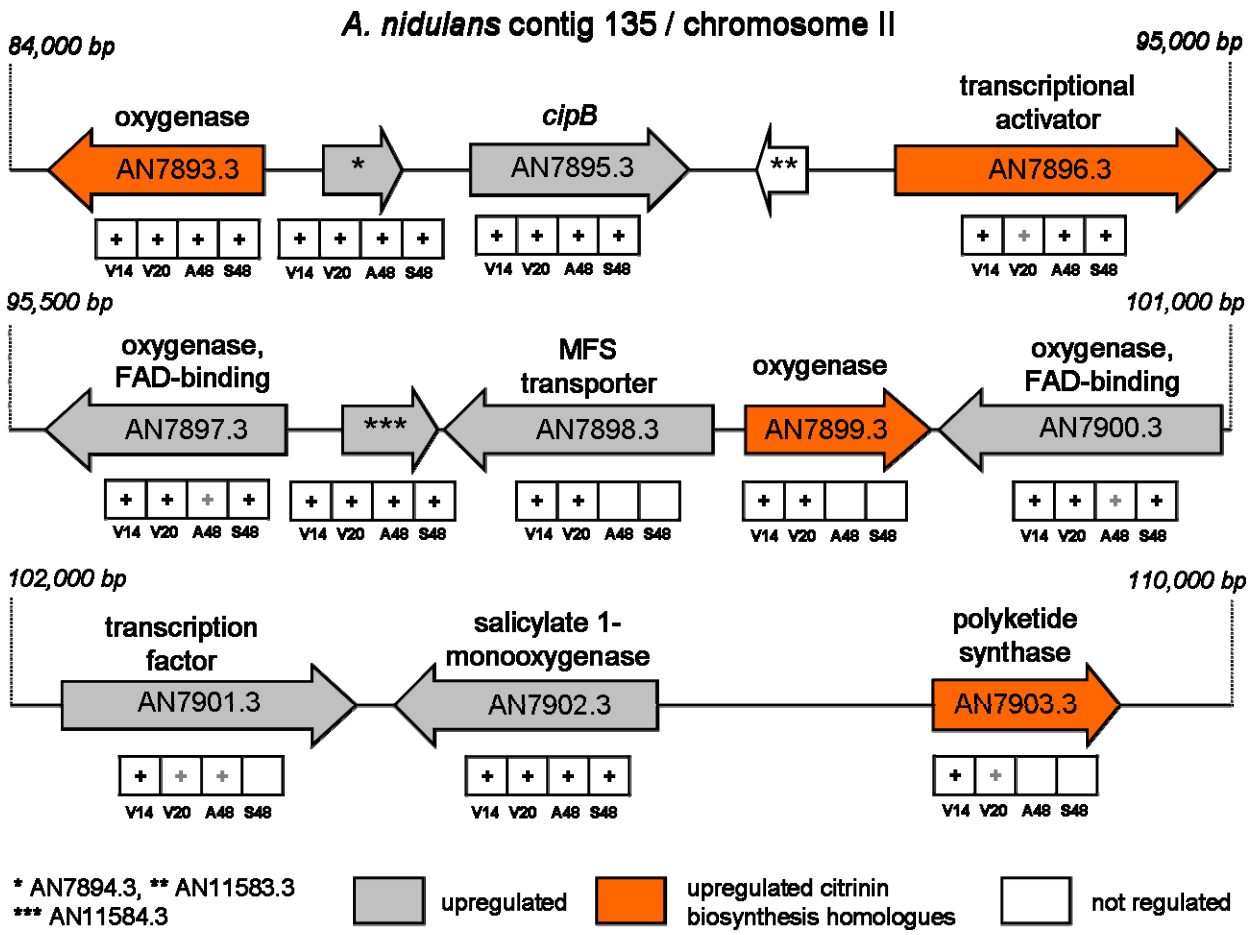
other putative secondary metabolism genes, are both up- and downregulated at different time points and do not provide a clear picture of possible regulation, although they seem to be more affected during vegetative growth.

Table 8. Selected genes associated with secondary metabolism differentially expressed in csnE strain.

Locus / Gene	Predicted function	Regulation in *ΔcsnE*			
Citrinin biosynthesis		**V14**	**V20**	**A48**	**S48**
AN7893.3	citrinin biosynthesis oxygenase	+	+	+	+
AN7899.3	citrinin biosynthesis oxidoreductase	+	+		
AN7903.3	citrinin polyketide synthase	+			
AN7896.3	citrinin biosynthesis transcriptional activator	+	-	+	+
Pigment biosynthesis					
AN5311.3	tyrosinase, GPI anchor motif	+	+		
AN1318.3	tyrosinase	+			
AN0230.3	putative tyrosinase			-	
AN8435.3	tyrosinase central domain protein				-
wA	conidial yellow pigment biosynthesis*			-	-
Similar to sterigmatocystin biosynthesis					
AN6945.3	o-methyltransferase	+	+		
AN3394.3	o-methylsterigmatocystin oxidoreductase	+	+		
AN6952.3	o-methyltransferase	+			
AN3236.3	putative o-methyltransferase	-	-		
AN2578.3	similar to sterigmatocystin biosynthesis peroxidase	-	-		
AN4008.3	o-methyltransferase B	-			
AN7881.3	similar to sterigmatocystin biosynthesis monooxygenase			+	
AN1616.3	norsolorinic acid reductase			-	
AN10023.3	similar to HypA protein				-
AN10044.3	similar to putative toxin biosynthesis protein				-
AN0146.3	similar to versicolorin reductase				-
Others					
AN9513.3	trichothecene 3-O-acetyltransferase	+	+		+
AN2859.3	dihydrodipicolinate synthase	+	+		
AN8433.3	nonribosomal peptide synthase	-			-
AN2548.3	trichothecene biosynthesis acetyltransferase	-			
AN6075.3	phenylalanine ammonia-lyase	-	-		
AN1242.3	nonribosomal peptide synthase		+		
AN6274.3	3-oxoacyl-acyl-carrier-protein reductase		+		
AN9499.3	isopenicillin N synthase family protein		-		
No number	cyclohexanone monooxygenase			-	-
AN2611.3	geranylgeranyl pyrophosphate synthase			-	-

* function validated experimentally, + upregulated, - downregulated

Figure 25 (next page). The genes upregulated in *ΔcsnE* mutant at all stages of growth and development are a part of a cluster on chromosome II potentially involved in secondary metabolite biosynthesis. The cluster consists of 13 predicted genes, 12 of which are upregulated at east on one growth or development stage. Predicted gene function based on sequence homology is shown, with the exception of already characterized *cipB* alcohol dehydrogenase. Genes encoding for homologues of *M. purpureus* proteins associated with citrinin biosynthesis are shown in orange. + indicates upregulation in the mutant at a given time point, significant regulation shown in black, moderate regulation in grey.



3.2.6. CsnE is involved in regulation of genes associated with membrane transport during vegetative growth and development

The second largest group of genes whose expression is affected in *ΔcsnE* is associated with transport across membranes, accounting for up to 7% of genes misregulated mutant during vegetative growth and 4% during development. Most of the regulated transport-associated genes tend to be upregulated during vegetative growth and either downregulated or not changed during development (Table 9), although the expression changes of carbohydrate transport genes are more mixed. Transport of diverse metabolites is affected, including carbohydrates, amino acids, ions, toxins and iron / siderophores, especially via putative transporters belonging to the major facilitator superfamily (MFS). Only three of the affected genes have a known function or regulation: *mstA* is a sugar transporter (Gonzalez *et al.,* unpublished data) homologous to an *A. niger* protein regulated in response to extracellular pH (Vankuyk *et al.*, 2004), *mirB* is a siderophore triacetylfusarinine C transporter, regulated by the SreA repressor and iron (Haas *et al.*, 2003) and *alcS* belongs to *alc* ethanol metabolism cluster, dependent both on the specific transactivator AlcR and the general carbon-catabolite repressor CreA (Fillinger and Felenbok, 1996), although the expression of other genes in the cluster is not affected. Taken together,

these results suggest that CSN regulates transmembrane transport in a different way during vegetative growth as compared with developing cultures.

3.2.7. CsnE is necessary to activate the expression of cell wall degradation genes and β-glucanase activity during development

The most prominent group in the set of probes downregulated after developmental induction are genes associated with cell wall degradation. They comprise 13% of probes downregulated during asexual and 23% of probes during sexual development. This group includes autolytic enzymes, such as chitinases and glucanases, as well as enzymes involved in degradation of plant cell wall material (mostly cellobiose hydrolases). Almost all of these enzymes can be classified as glycosyl hydrolases, 82 of which have been proposed in the *A. nidulans* genome. There is a clear enrichment of glycosyl hydrolases in the CSN-regulated transcriptome; 45% (37) of them are differentially expressed at least at one developmental stage and 35% (29) of these are downregulated during either asexual or sexual development. The importance of cell wall remodelling for sexual development of *Aspergillus nidulans* has been long recognized (Zonneveld, 1972) and lead to an observation that developmental regulation of α-1,3-glucan synthesis and its subsequent degradation by α-1,3-glucanases is developmentally regulated and prerequisite for fruit body formation (Zonneveld, 1974). However, the genetic background and regulation of the cell wall glucan mobilization process is still poorly understood. A more recent study (Wei *et al.*, 2001) identified an α-glucanase *mutA* as a gene expressed during sexual development in Hülle cells, but dispensable for fruit body formation. As summarized in Table 10, *csnE* deletion leads to deficient expression of *mutA* and one more predicted α-glucanase, but surprisingly has most impact on genes encoding putative β-glucanases, with a potential to hydrolyse both β-1,3- and β-1,4-glucan. β-1,3-glucanase activity was reported to increase in developing cultures (Bagga *et al.*, 1989; Zonneveld, 1975), but there is no indication yet that it is required for development.

Table 9 (next page). Genes associated with membrane transport differentially expressed in *ΔcsnE* strain.
[a]MFS: Major Facilitator Superfamily, [b]ABC: ATP-Binding Cassette. * function validated experimentally.

Locus / Gene	Predicted function	Regulation in *ΔcsnE*			
Carbohydrate transport		**V14**	**V20**	**A48**	**S48**
AN1577.3	MFS lactose permease	+			
AN3836.3	high-affinity glucose transporter	+			
AN9295.3	MFS low-affinity glucose transporter similar to Hxt1	-			
AN2386.3	L-fucose permease	-	-		
AN4148.3	MFS monosaccharide transporter	-			
AN7774.3	L-fucose permease	-	-		
mstA	Sugar transport*		+		
AN5067.3	MFS sugar transporter		-		
AN8993.3	MFS sugar transporter			+	+
AN0233.3	MFS hexose transporter			-	
AN3115.3	MFS sugar transporter			-	
AN2475.3	Sugar transporter			-	-
AN6467.3	galactose-proton symport				+
AN1577.3	MFS lactose permease				-
AN6928.3	MFS transporter				-
AN2814.3	MFS hexose permease				-
Amino acid transport					
AN6418.3	MFS polyamine transporter	+			
AN3345.3	GABA permease	+	+		
AN3347.3	Amino acid permease	+			
AN8915.3	MFS peptide transporter, POT family	+	+		
AN2043.3	GABA permease	-			
AN3304.3	GABA permease		+		
AN8903.3	MFS peptide transporter, POT family			-	
AN9174.3	Amino acid permease			-	-
AN2781.3	Lysine-specific amino acid permease				-
Drug and toxin transport					
AN1240.3	ABC[b] multidrug transporter	+	+		
AN0732.3	putative multidrug resistance protein amr1	+			
AN0890.3	MFS multidrug transporter	-			
AN8095.3	MFS efflux pump	-	-		
AN3254.3	MFS toxin transporter, AflT-like		+	+	+
Ion transport					
AN7898.3	MFS[a] monocarboxylate permease	+	+		
AN0528.3	MFS monocarboxylate permease	+	+		
AN10782.3	mitochondrial phosphate carrier protein	+	+		
AN8366.3	MFS monocarboxylate permease		+		
AN8943.3	Cation-transporting ATPase	-	-		
AN7523.3	Cation-transporting ATPase		-		
AN8219.3	Plasma membrane ATPase			-	
AN8956.3	phosphate-repressible phosphate permease			-	
AN6782.3	Sodium:dicarboxylate symporter family				-
Others					
AN5226.3	Acetate transporter, GPR1/FUN34/yaaH family	+			
AN2822.3	Auqaglyceroporin	+	+		
AN3776.3	Allantoate permease	+	+		
AN11016.3	malic acid transport protein		+		
AN9456.3	Allantoate permease		+		
AN8018.3	Fungal auxin efflux carrier superfamily		+		
alcS	Acetate transporter		-	-	
AN4807.3	vacuolar targeting protein			-	-
Iron transport					
mirB	siderophore iron transporter*	+			+
AN5378.2	similar to siderochrome-iron transporter Sit1		-		

Table 10. Genes associated with cell wall biosynthesis, structure and metabolism differentially expressed in *ΔcsnE* strain after developmental induction.

Locus / Gene	Predicted function	Regulation in *ΔcsnE*	
A-glucanases		**asexual**	**sexual**
mutA	α-1,3-glucanase*	-	-
AN3790.3	α-1,3-glucanase		-
AN3307.3	α-1,3-glucan synthase	+	+
β-glucanases			
AN3883.3	endo-1,3(4)-β-glucanase	-	
AN0494.3	1,4-β-D-glucan-cellobiohydrolyase	-	-
AN3860.3	endo-1,4-β-glucanase	-	-
AN4852.3	exo-β-1,3-glucanase	-	-
AN1273.3	β-1,4-glucan-cellobiohydrolyase	-	--
AN4825.3	glucan-1,3-β-glucosidase	-	-
AN5282.3	β-1,4-glucan-cellobiohydrolyase	-	-
AN5176.3	1,4-β-D-glucan-cellobiohydrolyase	-	-
AN0779.3	glucan 1,3-β-glucosidase		-
AN0245.2	endo-1,3(4)-β-glucanase		-
AN0472.3	β-1,3-endoglucanase		-
eglB	endo-1,4-β-glucanase*		-
eglA	endo-1,4-β-glucanase*		-
AN6736.3	similar to Rot1, β-1,6-glucan synthesis	+	
Chitin metabolism			
AN11233.3	class V chitinase	-	-
AN0499.3	chitin binding domain protein, putative chitinase	-	
AN0799.3	chitin synthase	+	+
Uncharacterized glucanases and other cell wall-related genes			
AN3613.3	endo-1,4–β–xylanase A precursor	-	-
AN5309.3	cutinase	-	-
AN7230.3	cellobiose dehydrogenase	-	-
AN8175.3	putative endoglucanase	-	-
prtA	serine alkaline protease, subtilase family*	-	-
AN5320.3	acetylxylan esterase		-
AN10124.3	β-glucosidase		-
AN2632.3	α-L-arabinofuranosidase		-
AN2828.3	β-glucosidase		-
AN2541.3	α-L-arabinofuranosidase		-
AN3904.3	β-glucosidase		-
AN2395.3	glycosyl hydrolase		-
AN0787.3	α-1,2-mannosidase		-
AN2325.3	α-1,2-mannosidase family protein		-
AN6093.3	acetylxylan esterase		-
AN7345.3	α/β-glucosidase		-
AN1818.3	endo-1,4-β-xylanase precursor		-
AN8953.3	α-glucosidase B*	+	
AN5410.3	GPI anchored cell wall protein	+	+
Spore cell wall surface-related genes			
rodA	rodlet protein precursor*	-	-
AN1837.3	hydrophobin	-	-
dewA	spore wall hydrophobin precursor*		-
AN11612.3	putative hydrophobin	+	

* function validated experimentally, + upregulated, - downregulated

To confirm that the observed changes in gene expression result in significant changes in enzyme activity, we have tested cytoplasmic laminarinase activity of wild-type and *ΔcsnE* strains in vegetative and developing cultures. Figure 26 shows that laminarinase activity is first induced after 48 hours of development in the wt strain and that the presence of CsnE is necessary for this induction during both asexual and sexual development (panel B). Interestingly, laminarinase activity is induced most prominently in asexually inducing conditions and the effect of *csnE* deletion is stronger at this stage, in spite of the fact that a larger number of β-glucanase encoding genes is downregulated at S48 (Figure 26, panel A). This suggests that the predicted endo-1,3(4)-β-glucanase gene AN3883.3, downregulated only

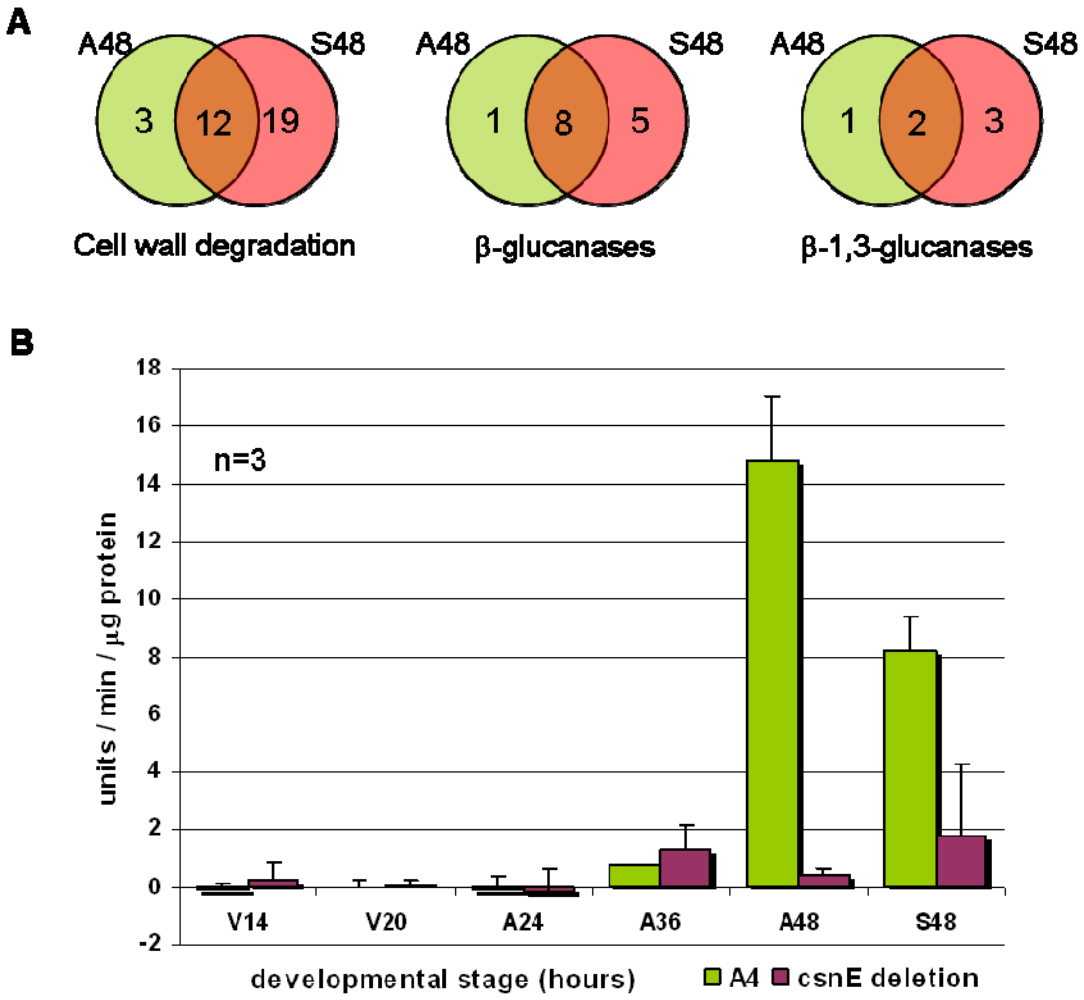


Figure 26. Appearance of developmentally regulated cytosolic laminarinase (β-1,3-glucanase) activity is abolished in *ΔcsnE* strain. (A) A diagram showing the number of genes associated with cell wall degradation in general, β-glucanases and putative laminarinases downregulated during asexual and sexual development. **(B)** Laminarinase activity is normally induced after 48 hours of development and this induction is abolished almost completely in the mutant strain during both asexual and sexual development. Activity of cytosolic enzymes was measured in dialysed protein extract using laminarinase azure as a substrate. Units are expressed in mg Remazol brilliant blue released from laminarin. The result shown is a mean of three independent measurements; error bars show standard deviation.

at A48 stage, may confer the bulk of enzymatic activity along with eight genes which are affected at both developmental conditions.

The expression of other genes involved in cell wall remodelling is also negatively affected in the mutant, including putative chitinases, α-L-arabinofuranosidases, mannosidases and *prtA*, a major extracellular protease implicated in autolysis. A large group of cellobiose hydrolases, enzymes degrading extracellular cellulose, is also downregulated. Finally, the expression of a group of hydrophobins, structural proteins conferring hydrophobicity to the spore outer layer surface, is repressed. However, the impact of hydrophobin regulation remains unclear, because *csn* mutants produce normal looking spores not showing abnormal adhesion or increased sensitivity. Interestingly, these changes are accompanied by a concomitant upregulation of a few putative genes responsible of synthesis of α- and β-glucan as well as chitin.

3.2.8. Impact of CsnE on developmental regulators and genes associated with protein degradation and defense

In the light of the developmental phenotype of *ΔcsnE*, it was interesting to see if it would show a misregulation of any major regulators of development. The expression of most known factors involved in the regulation of sexual development (such as *nsdD, steA* or *veA*) and other morphological processes do not change in the mutant, with two notable exceptions. The main transcriptional regulator of sporulation *brlA* (AN0973.3) is downregulated both during asexual and sexual development. Furthermore *ppoC,* encoding an oxylipin synthesis dioxygenase implicated in developmental balance regulation, is downregulated during vegetative growth and asexual development. *brlA* is repressed in the mutant only after initiation of development and, interestingly, the conidial yellow pigment polyketide synthase gene *wA* is also downregulated at this time. (*yA* is also downregulated, but only moderately.) This indicates a possible modulation of the intermediate developmental signalling pathway, although not on a transcriptional level and not to an extent that would result in defects in conidiation. It was shown that deletion of *ppoC* increases meiospore development and reduced mitospore production (Tsitsigiannis *et al.*, 2004a), so the repressing influence of the *csnE* deletion on *ppoC* in the early stages of development might explain the shift in balance between sexual and asexual development observed in the mutant before the developmental block. *brlA*α transcript was observed was more abundant in *ppoC* mutants (Tsitsigiannis *et al.*, 2004b), but the proportion of *brlA*α and *brlA*β transcripts in the *ΔcsnE* mutant remains to be confirmed.

The absence of CsnE also affects the expression of several genes encoding proteins directly involved in the ubiquitin-proteasome pathway, which are unregulated during asexual and sexual

development. They include *apyA*, a gene whose product interacts with carbon catabolite repression protein CreD and a HECT ubiquitin ligase HulA, presumably being involved in ubiquitination (Boase and Kelly, 2004), and three putative F-box proteins. It is unclear at this moment if this is a result of derepression or represents a compensatory response to defects in the regulation of the ubiquitin system. As mentioned in 3.2.4, a few stress and defense-related genes are influenced in the mutant strain. Besides catalase D, they include *prpA*, a gene encoding poly(ADP-ribose)- polymerase (PARP) is upregulated at A48 and S48. *prpA* activity is elevated during sporulation in *A. nidulans* (Thrane *et al.*, 2004) and it is an essential gene involved in DNA damage response and oxidative stress response (Semighini *et al.*, 2006). Finally, the gene most strongly downregulated at all growth and development stages (see Table 6) encodes a putative defensin, but shows similarities only to insect proteins. Its expression in *A. nidulans* is confirmed by Northern analysis (Figure 23), so it is not a genome sequencing artefact, but a functional gene. Interestingly, another defensin-like protein (V14), trypsin-like protein (V14) and a trypsin inhibitor-like protein (S48) similar only to insect proteins are downregulated at different time points (data not shown), although their potential role is elusive.

3.2.9. Downregulated cell wall degradation-related genes are enriched in upstream AbaA, Mcm1 and STRE binding elements

CSN-directed phosphorylation was shown to regulate the stability of transcription factors, such as JY5 in plants and p53 in mammals, by modulating their degradation by the ubiquitin system (Bech-Otschir *et al.*, 2001; Schwechheimer *et al.*, 2002). It is therefore feasible that *A. nidulans* CSN regulates the stability or activity of transcription factors involved in cellular signalling, including developmental pathways, without influencing their mRNA abundance.

To ascertain whether the co-regulated groups of genes involved in cell wall hydrolysis contained common regulatory elements, we examined their 1,000 bp upstream sequences by analysing statistically overrepresented transcription factor binding sites stored in the TRANSFAC database (Matys *et al.*, 2003) using the F-match algorithm. We have analysed separately upstream sequences of 15 genes downregulated at both A48 and S48 and 20 genes downregulated during sexual development only. Figure 27 shows the putative promoter structure of both groups of analysed genes. Upstream sequences of genes downregulated both during asexual and sexual development are enriched in consensus binding sequences specific for the developmental AbaA transcription factor (Andrianopoulos and Timberlake, 1994). Perfectly matching binding sequences are present in 12 out of 15 promoter sequences, and imperfect matches can be found upstream of the three remaining genes. On the other hand, sequences upstream of genes downregulated exclusively during sexual development are

enriched in yeast stress response elements, or STREs (Marchler *et al.*, 1993) and binding sites of the yeast mating transcription factor Mcm1 (Passmore *et al.*, 1989), suggesting a possible regulation by uncharacterized stress response transcription factors and by McmA (AN8676.3), an *Aspergillus* homologue of Mcm1. AbaA binding sites are also present although they are not statistically overrepresented. Similarly, Mcm1 sites and STREs are present, but not overrepresented in promoters of some genes from the first group.

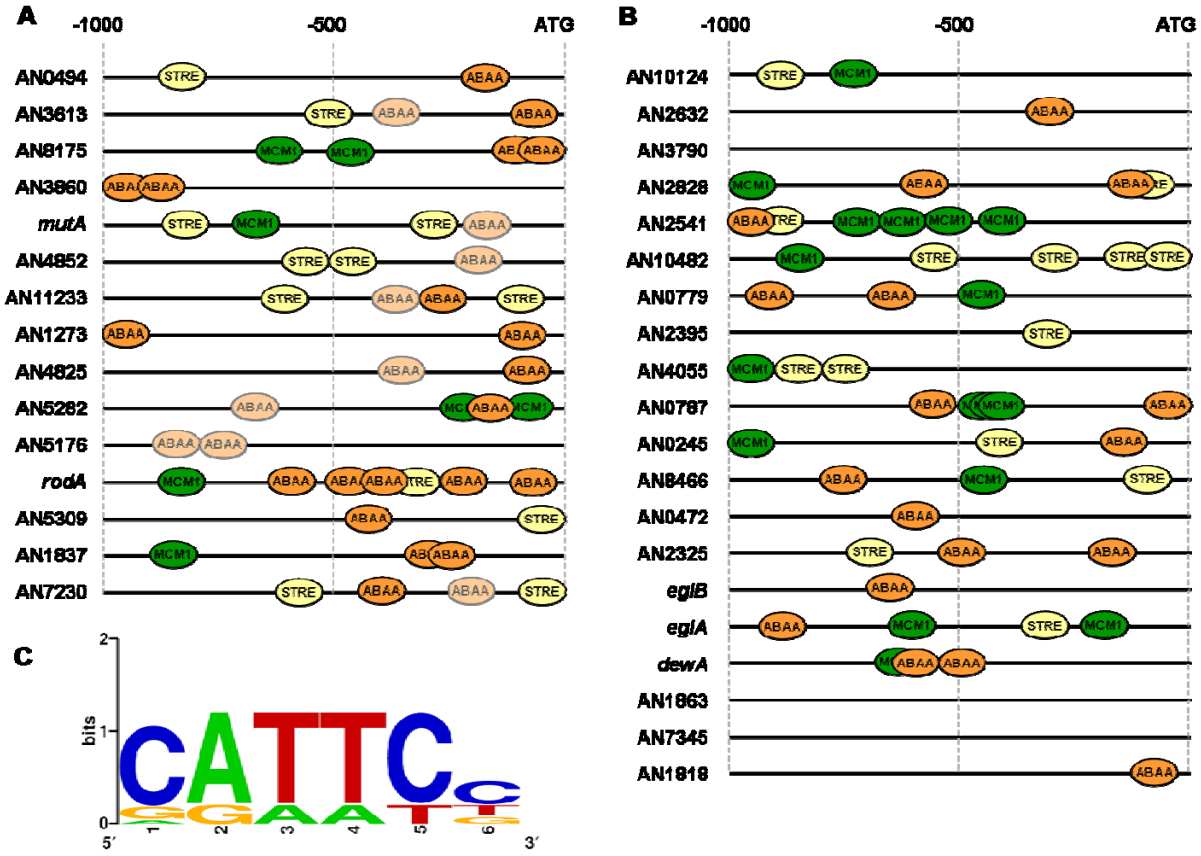


Figure 27. Several transcription factor binding sites are enriched in promoter regions of genes underexpressed in *ΔcsnE* mutant. Sequences up to 1000 bp upstream of genes assigned to "cell wall" and "cell wall degradation" categories are shown. **(A)** Sequences upstream of 15 genes downregulated both during asexual and sexual development are enriched in CATTCY consensus binding sequences of the AbaA transcription factor. Binding sites with non-perfect core matrix conservation score are shown as shaded. Mcm1 and STRE sites are also shown. **(B)** Sequences upstream of 20 genes downregulated only during sexual development are enriched in STREs (yeast stress response elements) and binding sites of the yeast Mcm1 transcription factor. AbaA binding sites are also shown. See Table 10 for functional gene annotation. **(C)** Logo of the AbaA binding site consensus sequence. Letter height corresponds to the frequency of a given nucleotide in experimentally verified binding sites.

3.2.10. Transport-related genes upregulated during vegetative growth are enriched in upstream PacC binding sites

To search for common regulatory elements across the CSN-regulated transcriptome, we have also examined statistically overrepresented transcription factor binding sites in the 1,000 bp upstream sequences of other groups genes co-regulated at various stages of development, including secondary metabolism, stress, oxidoreductases and transport-related genes, as well as the members of the novel upregulated gene cluster (Figure 25). The only group showing a significant enrichment in any binding site consensus sequences were genes associated with transmembrane transport upregulated in *ΔcsnE* at early vegetative stage (V14). Their upstream sequences are enriched in binding sites of the PacC transcription factor, which is the central regulator of the pH-dependent signalling in *A. nidulans* (Tilburn *et al.*, 1995). Figure 28 shows the putative promoter structures of these genes. Perfectly matching binding sequences are present in 10 out of 15 promoter sequences, and imperfect matches can be found upstream of 4 more genes.

The transcriptome profiling data presented here point to the role of the fungal COP9 signalosome in regulating transcription, directly or indirectly, at all stages of growth and development. The CSN seems to influence initiation of development and spore balance by

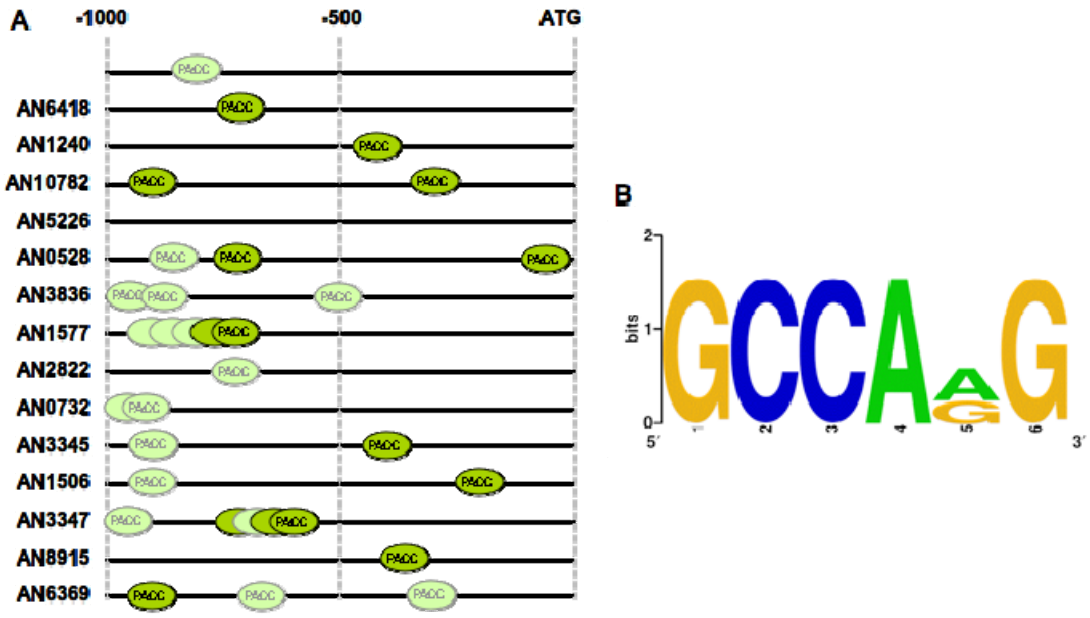


Figure 28. PacC transcription factor binding sites are enriched in promoter regions of genes associated with membrane transport overexpressed in *ΔcsnE* mutant. (A) Sequences up to 1000 bp upstream of 15 genes assigned to "transport" category upregulated at V14 are shown. Upstream sequences are enriched in GCCARG consensus binding sequences of the PacC transcription factor. Binding sites with a non-perfect core matrix conservation score are shown as shaded. **(B)** Sequence logo of the PacC binding site consensus. Letter height corresponds to the frequency of a given nucleotide in experimentally verified binding sites. See Table 9 for functional gene annotation.

regulating the expression of *ppoC* and *brlA*, as well as plays a role in activation of developmentally-induced cell wall degradation as shown by β-glucanase activity. Additionally, a role in regulation of oxidative balance, secondary metabolism and transport emerges. The results of the promoter enrichment analysis suggest that CSN might coordinate interplay between asexual and sexual regulatory pathways. They do not rule out regulation of CsnE targets by other transcription factors, especially considering the scarcity of experimental information about *A. nidulans* transcription factor binding sites, but suggest the points of main interest for further analysis.

The studies on CsnE function as a non-essential deneddylase and its relevance to gene regulation in the filamentous fungus *A. nidulans* provide new insights into the conserved mechanism and biological function of the CSN complex in eukaryotic organisms.

CHAPTER 4: DISCUSSION

4.1. The COP9 signalosome of filamentous fungi

The PCI+MPN architecture and activities of the COP9 signalosome are highly conserved in higher eukaryotes, as is its essentiality for early stages of development (Figure 29). In mammals, disruption of individual CSN subunits results in early embryonic lethality, characterized by disrupted cell proliferation, cell cycle arrest, increased apoptosis and accumulation of cyclin E and p53 (Lykke-Andersen *et al.*, 2003; Tomoda *et al.*, 2004; Yan *et al.*, 2003). Plant embryos defective in CSN display a photomorphogenic phenotype accompanied by accumulation of the HY5 photomorphogenesis activator in the dark (Schwechheimer and Deng, 2000) and arrest growth at seedling stage (Dohmann *et al.*, 2005; Kwok *et al.*, 1996; Wei *et al.*, 1994). Similarly, *Drosophila* csn mutant embryos exhibit lethality at the late larval or pupal stages, being a result of numerous pleiotropic defects in oocyte and embryo patterning, including eye development (Freilich *et al.*, 1999; Oron *et al.*, 2002). On the other hand, the complexes in budding and fission yeast are not essential for cell proliferation, although they retain their deneddylating activity. Incomplete CSN of fission yeast is important in DNA repair and S-phase progression, but different *csn* mutants do not share common phenotypes, suggesting divergent roles for the subunits (Mundt *et al.*, 2002). The

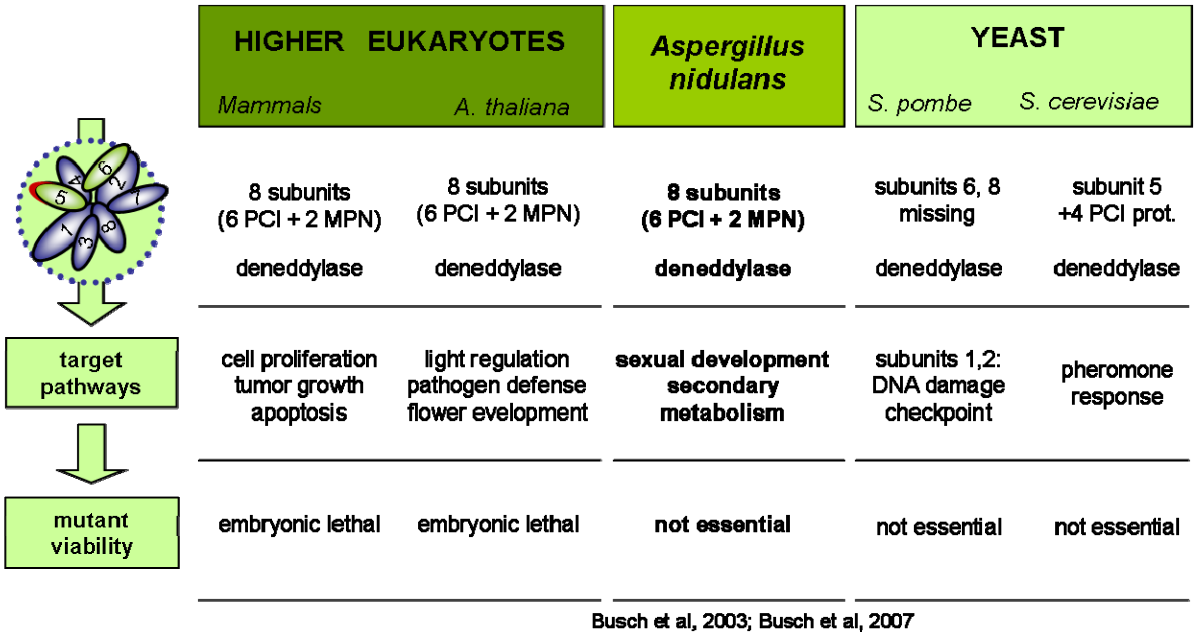


Figure 29. Conservation, architecture, cellular functions and essentiality of the COP9 signalosome in higher eukaryotes, yeast and *Aspergillus nidulans*. The COP signalosome is divergent in lower eukaryotes where it is not necessary for growth and development, while the conserved 8 subunit complex is essential in plants and animals and dispensable in filamentous fungi.

divergent budding yeast signalosome plays a role in pheromone signaling response (Maytal-Kivity *et al.*, 2002). The conserved CSN complexes of filamentous fungi provide a unique opportunity for studying the effects of their disruption in a multicellular organism. Whereas *Neurospora crassa* signalosome, which plays a role in circadian clock regulation is missing subunit 8 (He *et al.*, 2005), *Aspergillus nidulans* contains an eight subunit COP9 complex architecturally comparable to that of the higher eukaryotes (Busch *et al.*, 2007). Considering that all characterized *A. nidulans csn* mutants are viable while exhibiting pleiotropic developmental phenotypes suggesting similarity of function wither eukaryotes, this fungus is ideally situated for studying the CSN function in an easily amenable organism. This study focuses on two aspects of the *A. nidulans* signalosome, the conserved deneddylase motif of the CsnE subunit and the transcriptional effects of CSN on different stages of growth and development.

4.2. The neddylation cycle in *A. nidulans*

4.2.1. CsnE deneddylation activity is crucial for sexual development and response to different stress factors

The Jab/MPN+ deneddylase motif was shown to be critical for CSN function in higher eukaryotes, as CSN5 alleles mutated in JAMM are not able to rescue developmental lethality and defects in eye development in *Drosophila melanogaster* (Cope *et al.*, 2002). On the other hand, the catalytic motif does not seem to be essential for folding of the MPN domain and CSN complex formation, as shown in *S. pombe* (Cope *et al.*, 2002) and *A. thaliana* (Gusmaroli *et al.*, 2004). Furthermore, binding of at least some interacting proteins by CSN5 seems to be mediated by the MPN domain, but independently of the JAMM motif (Burger-Kentischer *et al.*, 2005). The deneddylase activity is therefore not necessary for CSN complex assembly and protein interactions. Although there is no data available showing whether defects in deneddylation influence directly the associated phosphorylating and deubiquitinating activities of CSN, the lethal phenotypes of JAMM mutants in higher eukaryotes indicate that a coordination of all these activities is necessary for function of the complex in development. To see if this coordination and the crucial role of deneddylation are conserved in *A. nidulans* we investigated the effects of JAMM deficiency on CSN developmental function.

Four *A. nidulans csn* deletion mutants characterized up to date share an identical phenotype. They are blocked in fruit body formation at the level of primordia, while initiating preferentially the sexual cycle and producing a red pigment (Busch *et al.*, 2003). Both mutant strains constructed in this study, one defective in the codon for Asp and other in codons for Asp

and His residues of the JAMM deneddylase motif mimic this phenotype, as the mutated alleles are not able to complement any of its aspects. This indicates that the deneddylase activity of the COP9 signalosome is essential for fruit body formation as well as for the regulation of secondary metabolism and initiation of sexual development balance in response to light. The shortened, hyperbranched cells initially observed in *csnD* and *csnE* deletion strains (Busch *et al.*, 2003) are not apparent only sometimes in the point mutants (data not shown), suggesting that some aspects of CSN-dependent growth regulation might be JAMM-independent. However, this aspect of *csn* phenotype is not very penetrant and is not always clearly distinguishable in the deletion mutants either.

It is not known if the *A. nidulans* CSN associates with protein kinases or deubiquitinating enzymes. Accordingly, the conservation and role of phosphorylation and deubiquitination CSN activities in *A. nidulans* is not known. The non-lethal phenotype raises the question if all of the functions of the complex in higher eukaryotes are conserved in fungi. It seems to be the case, considering the role of CSN in stabilisation of the FWD-1 F-box protein in *N. crassa* (He *et al.*, 2005) and an apparently deneddylation-independent role in the yeast pheromone response (Maytal-Kivity *et al.*, 2002). Purification of the *A. nidulans* complete CSN using a TAP tag approach did not reveal any co-purifying proteins (Busch *et al.*, 2007). It would be nevertheless interesting to investigate potential interactions of the complex on both genetic and biochemical levels with the homologues of CSN-associated protein kinases (see 1.2.1) or Ubc12 in this model organism, as the binding with putative kinases or deubiquitinating enzymes might be transient or too weak to be detected by co-purification. However, our experiments indicate that the deneddylase motif is the primary effector of the developmental COP9 function in this organism.

Sexual development seems to be correlated with stress response in *A. nidulans*. MAPK kinase SakA is activated in response to osmotic and oxidative stress and *ΔsakA* mutant displays premature steA-dependent sexual development and produces stress-sensitive asexual spores, indicating that SakA is involved in stress signal transduction and repression of sexual development (Kawasaki *et al.*, 2002). Previous studies have also shown that *csnD* and *csnE* play a role in *A. nidulans* DNA damage response and are upregulated, probably by stabilisation of mRNA, in the presence of DNA-damaging agents (Lima *et al.*, 2005). In agreement with this data, additional analyses of the *csnE* deletion and point mutants reveal an increased sensitivity to temperature and stress factors, especially involving oxidative stress, DNA damage response and microtubule dynamics. The JAMM-defective mutants are as sensitive to all these agents as the *csnE* deletion strain. These results are furthered by an observed influence of CSN on the

expression of *prpA*, encoding poly-(ADP-ribose)-polymerase (PARP) involved in DNA repair (see 3.2.8). These observations also support a proteomic study of the *ΔcsnE* strain, which identified proteins associated with redox balance of the cell and response to oxidative stress as differentially expressed in the *ΔcsnE* strain (Busch, personal communication). Overall this data strongly suggest an interdependence of COP9 signalosome developmental functions with oxidative and DNA damaging stress. The mechanism of impact of CSN on microtubule assembly is not clear and merits further investigation. It is not crucial enough to prevent cell cycle progression and mitotic decisions, but might influence both the meiotic divisions required for ascospore formation and morphological changes depending on microtubule transport during cleistothecia development.

Surprisingly, the results *in vitro* interaction studies between JAMM-mutated CsnE and CsnF subunit suggest a wakening of the binary protein-protein binding between these two subunits. Considering the limitations of the two hybrid method it is possible that the JAMM mutation reduces the ability to interact with a single partner subunit without affecting the complex formation as a whole, especially in the light of a suggestion that the other PCI containing subunits might form a primary scaffold for the assembly of the whole complex (Busch *et al.*, 2007; Kim *et al.*, 2001). Furthermore, TAP-tagged CsnE is not able to recruit CsnF or other subunits when CsnA or CsnD are missing. It is also important to bear in mind that fusion of mammalian CSN5 to the yeast two hybrid binding domain used in this study results in strong background activity (Nordgard *et al.*, 2001), which might indicate that the JAMM mutation affects this unspecific activity and not the real binding, which would explain why the binding in the other orientation is not affected (Figure 18). Determining the effects of the point mutations on complex formation *in vivo* would require biochemical investigation, such as TAP-tag purification of the JAMM-defective complex. Nevertheless, considering that the binary interaction between CsnE1/CsnE2 and CsnF subunits is not abolished and that this interaction might not be crucial for complex assembly, we tentatively conclude that the JAMM mutant phenotype stems from the lack of deneddylase activity and not from the malfunctions of the whole complex.

4.2.2. Neddylation is essential for *A. nidulans* growth

Nedd8/Rub1, the ubiquitin-related modifier of cullins, is extremely conserved in eukaryotes, with the *A. nidulans* RubA characterised in this study sharing 75% identity with the human Nedd8 orthologue. It is also most similar to ubiquitin among all Ubls, the mature forms sharing 53% identity in yeast and 55% in *A. nidulans*. Nedd8 is essential for growth and survival of

numerous organisms from *S. pombe* to mice (Osaka *et al.*, 2000; Tateishi *et al.*, 2001), with the notable exception of *S. cerevisiae*, whose *Δrub1* mutants are "distressingly healthy" (Lammer *et al.*, 1998). The first attempts to create a *rubA* deletion strain in *A. nidulans* were unsuccessful, both in a wild-type background and in strains facilitating an increased frequency of homologous recombination. Using the heterokaryon rescue method we were able to show that *rubA* is indeed essential for *A. nidulans* growth and that *ΔrubA* spores fail to germinate and undergo cell division, similar to the situation in higher eukaryotes. The phenotype of the primary deletion transformants during germination and thus the exact role of RubA on a cellular level remain to be investigated. The components of Nedd8/Rub1 conjugation pathway, which is similar to that of ubiquitin with the exception of a lack of an E3 enzyme (see 1.1.4), are also essential in higher eukaryotes. There are multiple genes in *A. nidulans* genome encoding possible homologues of all these components. Interestingly, the closest homologue of yeast Ubc12 is UvsJ (AN5344.3), playing a role in DNA damage repair (Jang, unpublished data). The identification of the neddylation pathway components would be the next logical step in characterizing regulation of protein degradation in *A. nidulans*.

Another protein important in the neddylation and deneddylation cycles of cullins is Cand1 (see 1.1.4), whose binding to cullins is mutually exclusive with the CSN and neddylation. Surprisingly, *A. nidulans* CandA is encoded by two separate genes whose deduced products are homologous to the C and N termini of Cand1 (Schwier, personal communication) and result in phenotypes similar but not identical to *ΔcsnE*, showing severely hindered, but not abolished cleistothecia production accompanied by an even higher production of red pigment.

The essentiality of neddylation as compared to dispensable COP9-dependent deneddylation in *A. nidulans* raises interesting questions as to the regulation of the ubiquitin ligases in this model organism as compared with higher eukaryotes and yeasts, respectively. Nedd8 has to be proteolytically processed before its attachment to cullins, freeing the N-terminal glycine for the conjugation. This processing is performed by DEN1, a dual function protease also able to remove Nedd8 from hyper-neddylated cullins *in vitro* (Gan-Erdene *et al.*, 2003; Wu *et al.*, 2003). DenA, an *A. nidulans* homologue of this protease was characterized and seems to function in repressing sexual development, as *ΔdenA* strains produce very dense cleistothecia also in the presence of light (Christmann, personal communication). It is possible that DenA is able to deneddylate cullins *in vivo*, although it remains to be investigated. Construction of a double *ΔcsnE*/*ΔdenA* mutant and analysing possible synthetic lethality should answer the question whether there really is an asymmetry in the importance of neddylation and deneddylation for *A. nidulans.*

It is conceivable that AN4492.3, a proposed Rpn11 homologue (see 3.1.1) also containing the MPN/JAMM domain and known to be responsible for deubquitination, exhibits cross-activity in this fungus, although it would have no precedence in other organisms. Alternatively, the influence of cullin neddylation on its activity, adaptor stability and E3 complex reassembly could differ. *A. nidulans* encodes 69 putative adaptors of SCF E3 ubiquitin ligases (F-box proteins). This is more than in *S. cerevisiae*, which has 18 putative F-box proteins, but still a much smaller number than in higher eukaryotes, especially plants, and suggest that a rapid adaptor reassembly might not be as crucial to the E3 ligase activity in filamentous fungi as it is in higher eukaryotes. A detailed analysis of multiple *A. nidulans* F-box proteins is currently

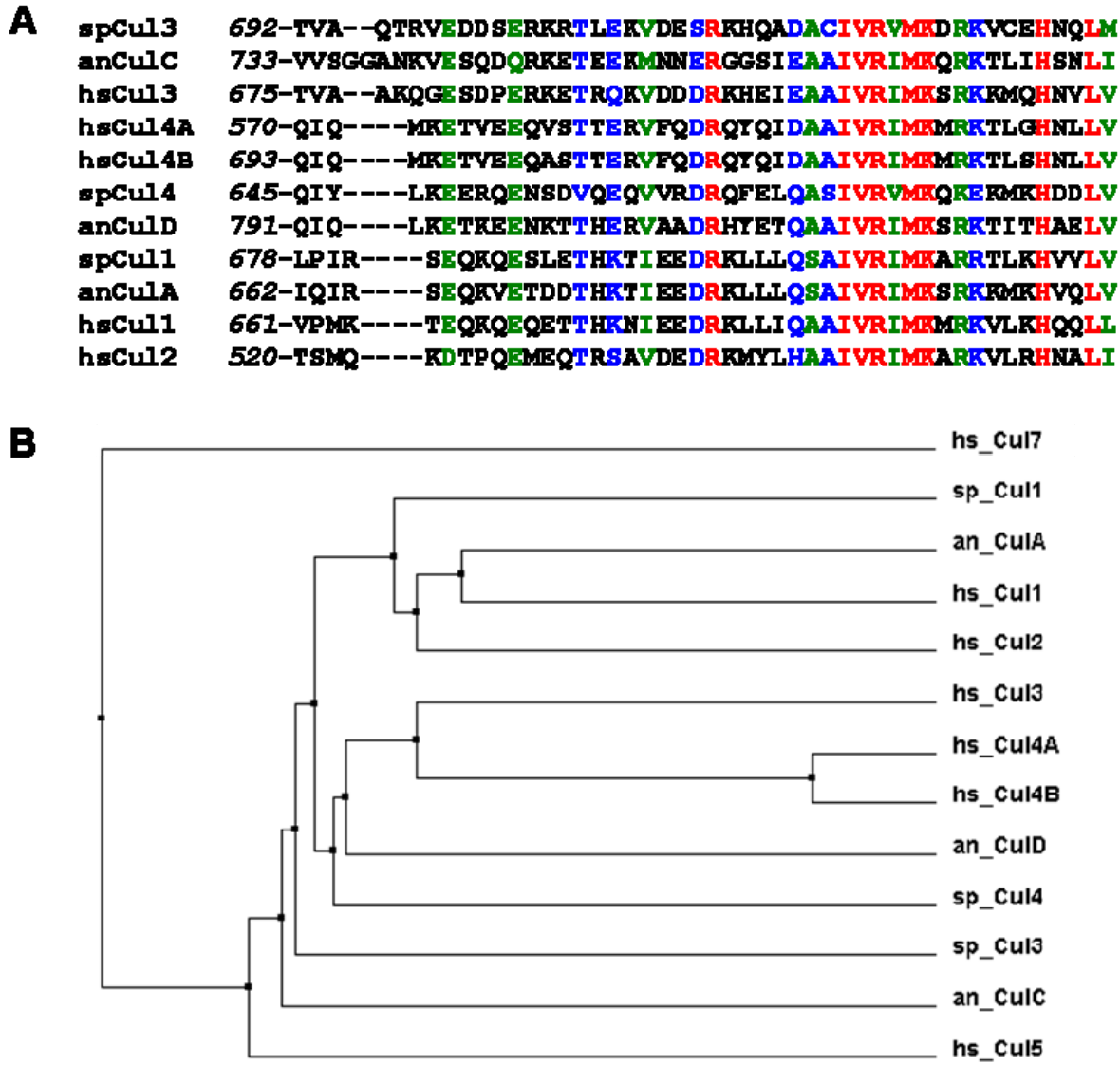


Figure 30. *A. nidulans* genome encodes 3 cullins homologous to mammalian Cul1, Cul3 and Cul4. **(A)** alignment of fungal and human cullins shows high conservation of the IVR(V/I)MK neddylation site. Identical residues are marked in red, strongly similar in green and slightly similar in blue. an - *Aspergillus nidulans*, hs - *Homo sapiens*, sp - *Saccharomyces pombe*. **(B)** Phylogenetic tree shows conservation within cullin families and reveals that *A. nidulans* cullins are more closely related to *S. pombe* proteins, with the exception of CulA, which is more similar to human Cul1.

underway and should reveal more facts about their function in fungal development and the mechanism of their regulation.

4.2.3. *A. nidulans* encodes three putative cullins

Neddylation affects the activity of E3 ubiquitin ligases by covalent cullin modification. We have searched the *A. nidulans* genome for cullin homologues using the tblastn algorithm and found that it encodes three putative cullins. *culA* (AN1019.3), one of these genes encoding a homologue of mammalian Cul1, was identified previously in a REMI mutagenesis screen for acleistothecial strains (Eckert, 2000, PhD thesis), although it was not further characterised. *culC* (AN3939.3) and *culD* (AN10008.3) are homologous to mammalian an yeast Cul3 and Cul4, respectively. There is also one protein similar to the anaphase promoting complex (APC) subunit 2 (AN10258.3, not shown). Phylogenetic analysis reveals that they share more similarity with the mammalian cullin proteins, with the exception of CulD, which is more related to its yeast counterpart (Figure 30, panel B).

Activity of cullins in RING E3 complexes influences various aspects of cellular regulation. Cul1 as a component of the SCF complex plays a critical role in the control of the cell cycle by promoting the degradation of several regulatory proteins. In particular, the budding yeast SCF^{CDC4} and the mammalian SCF^{SKP2} (the name of the F-box protein being indicated in superscript) are required to destroy the cell cycle-dependant kinase inhibitors SIC1 and p27, respectively (Deshaies and Ferrell, 2001; Pagano, 2004), and thus promoting the entry into S-phase. Cul3, Cul4. There are fewer targets of Cul3-containing E3 ligase known, but it plays an important role in transition from meiosis to mitosis in *C. elegans* (Pintard *et al.*, 2003). Components of the Cul4A E3 ligase complex were recently identified and may be critical for the regulation of DNA repair (Groisman *et al.*, 2003).

Several attempts to delete the *culA* gene using the same system as for *rubA* were unsuccessful. It is presumably also essential in *Aspergillus*, as suggested by formation of wild-type/deletion diploids by the transformants analogous to the *rubA*, although this remains to be proven experimentally. Alignment of putative cullins with their mammalian and yeast orthologues (Figure 30) shows a complete conservation of the neddylation sites in all three proteins. A mutational analysis of the conserved neddylation site lysines (K697 of CulA, K772 of culC and K826 of culD) of the three *A. nidulans* cullins should therefore reveal more details about their individual cellular and developmental functions, potentially resulting in viable strains. Comparison of these defective cullin phenotypes will allow distinguishing the different

aspects of the fungal COP9 signalosome function through the regulation of different E3 ubiquitin ligases.

4.3. Regulation of *A. nidulans* transcriptome by CSN

Because of the variety of interactions and influences that CSN exerts on the ubiquitin-proteasome system, protein stability and thus numerous downstream cellular processes, it has recently become a focus of systems biology research, investigating its impact on the genomic and transcriptomic scale (Ma *et al.*, 2003; Oron *et al.*, 2007). Analysis of COP9 signalosome in *Arabidopsis* and *Drosophila* described in the literature revealed a difference in the function of the complex between higher eukaryotes; in plants the phenotypes of the mutants of individual CSN subunits are indistinguishable (Dohmann *et al.*, 2005), while in animals the functions of different subunits in development are overlapping but not identical (Freilich *et al.*, 1999). These differences were reasserted in transcriptional level by microarray analysis (Ma *et al.*, 2003; Oron *et al.*, 2007). In *Aspergillus*, the influence of different subunits on development is identical and seems to depend primarily on the deneddylase activity of CsnE. Therefore the analysis of a strain lacking this subunit provides a representative overview of the developmental functions of the CSN on transcriptional level.

The deletion of *csnE* affects a distinct portion of the transcriptome, leading to differential expression of 5% of genes represented on the array. Functional categorization of these genes identified oxidoreductases, as well as genes associated with transport, secondary metabolism and cell wall dynamics as the main differentially expressed groups across development. P450 cytochromes appear to be enriched among the regulated oxidoreductases and glucanases among the developmentally downregulated cell wall-related genes. Still, the vast majority of identified genes have not been experimentally validated in *A. nidulans* and about a third encodes hypothetical or conserved hypothetical proteins with unknown function (Figure 24, Table 7). Our profiling data and the advent of genome-wide oligo arrays should improve existing automated gene annotation. In the course of this study we have identified several unannotated regulated genes with homologies in other organisms, as well as several misannotated genes and a novel co-regulated genomic cluster (see 3.2.5). Comparison of datasets from different transcriptome profiling experiments should contribute to more confident assignment of *A. nidulans* gene functions in the future.

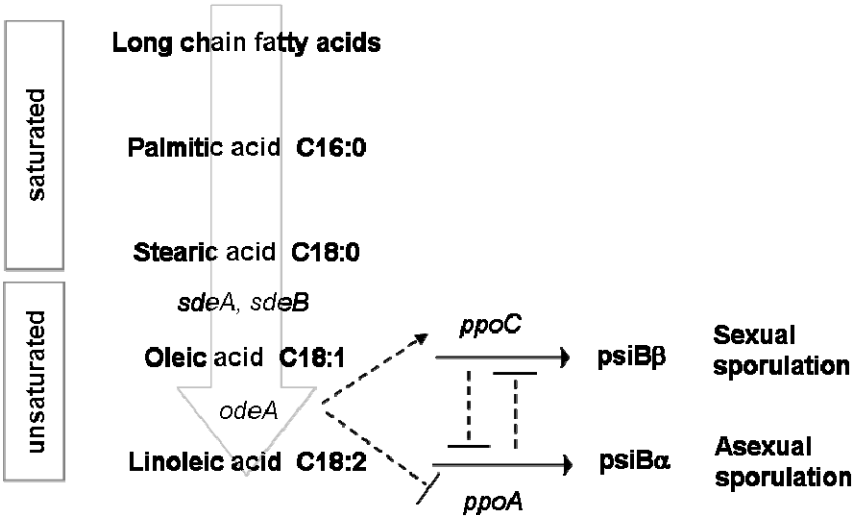
4.3.1. Influence of CSN on secondary metabolism and oxidative balance

Secondary metabolite production in fungi is connected both with asexual sporulation and sexual fruit body formation (reviewed by Calvo *et al.*, 2002). Melanins are often associated with developmental structures, while synthesis of sterigmatocystin and other *Aspergillus* toxins is connected with the asexual development via a G-protein-mediated signalling pathway. In *A. nidulans*, secondary metabolites derived from linoleic acid, the psi factors, have direct sporogenic effects and regulate the balance in asexual and sexual spore formation. Three monooxygenase-encoding *ppo* genes have been identified that control their synthesis (Tsitsigiannis *et al.*, 2005). *ppoA* and *ppoC* act antagonistically in spore development, *ppoC* promoting asexual and *ppoA* - sexual sporulation (Tsitsigiannis *et al.*, 2004b). These signals cross talk with the main developmental signalling pathway and *brlA*α transcript is more abundant in *ppoC* mutants (see Figure 9) (Tsitsigiannis *et al.*, 2004b). CSN is necessary for *ppoC* expression before the onset of development, which is presumably responsible for the partially light-insensitive phenotype of the mutant when induced from pregrown cultures. This sexual induction phenotype is not as strong in colonies grown on directly inoculated plates, used for the transcriptome profiling experiment, which suggests that the changes in *ppoC* expression might actually be more pronounced when the phenotype is observed. The upstream signals in this pathway remain to be investigated, although an AbaA binding site present in the *ppoC* promoter (Figure 33) suggests that this transcription factor might play a role at this early stage as well, integrating asexual pathways with establishing the sexual/asexual spore ratio.

Another important gene related to the oxylipin synthesis pathway is *odeA* which encodes an oleate delta-12 desaturase, catalyzing the conversion of oleic acid into polyunsaturated linoleic acid (Figure 31). Deletion of *odeA* leads to changes in the fatty acid profile, including the composition of psi factors, and leads to delayed but increased over time ascospore production as well as downregulation of *ppoA* and upregulation of *ppoC*. *odeA* transcription is light-induced, but only in the absence of functional *veA* (Calvo *et al.*, 2001). Interestingly, AN7204.3, a gene very closely related to *odeA*, but not *odeA* itself is also downregulated in *ΔcsnE* strain during vegetative growth. *A. nidulans* genome encodes only two putative oleate delta-12 desaturases and it would be therefore interesting to investigate the role of this novel gene in psi factor biosynthesis and developmental balance.

In contrast to most genes involved in primary metabolism, genes encoding secondary metabolite biosynthetic enzymes exist in contiguous clusters within the genome (Brown *et al.*, 1996). The influence of CSN on the expression of other secondary metabolites seems to focus on such a single genomic cluster, which is strongly upregulated in the mutant across all growth

Figure 31. Known genes involved in the synthesis of *A. nidulans* psi factors and their mutual regulation. *sdeA* and *sdeB* are stearate δ-9 desaturases (Wilson *et al.*, 2004), *odeA* is an oleate δ-12 desaturase, *ppoA* and *ppoC* are oxylipin synthesis monooxygenases. Solid arrows indicate biochemical processes and dotted arrows indicate regulation of genes. Modified from (Tsitsigiannis *et al.*, 2004a)



development time points. The cluster contains 13 genes, 12 of which are upregulated in the *ΔcsnE* strain on at least one growth or developmental stage, and encodes mostly putative secondary metabolism genes. Four of them encode homologues of *M. purpureus* citrinin biosynthesis enzymes. Citrinin is a nephrotoxic and bacteriocidal toxin produced by the polyketide pathway in fungi belonging to several *Penicilium*, *Aspergillus* and *Monascus* species. It is synthesised from a polyketide precursor in the course of many reactions including condensation, reduction and oxidation (Hajjaj *et al.*, 1999b) and the enzymes responsible are only partially known. Interestingly, the production of secondary metabolites including citrinin is light-regulated in *Monascus* species (Miyake *et al.*, 2005), which provides a potential link with the process of establishing the developmental balance by phytochrome sensing and VeA-mediated signal transduction. Additionally, citrinin biosynthesis appears to be coupled to a production of a red pigment in several *Monascus* species.

All of the genes in the CSN-regulated cluster seem to be regulated in the same or similar fashion (Figure 25), suggesting that the cluster as a whole might be functional or at least actively regulated in *A. nidulans.* However, it might be responsible for synthesis of another yet unknown secondary metabolite, as this fungus is not known to produce this toxin, or represent a citrinin biosynthesis unit inactivated in the course of evolution. The genetics of *Aspergillus* citrinin production is not well studied and further investigation is needed to determine if *A. nidulans csnE* mutants are indeed able to synthesise citrinin and if the red pigment observed in *Δcsn* strains is similar to the one produced by *Monascus* in a pathway associated with citrinin biosynthesis (Hajjaj *et al.*, 1999a) and is a side effect of citrinin biosynthesis derepression.

The analysis of cis-regulatory elements in the promoters of genes belonging to the cluster as well as other differentially regulated genes associated with secondary metabolism did not reveal an enrichment of any characterized binding sites. However, it is theoretically possible that the cluster could be under epigenetic control of LaeA, a protein methyltransferase involved in general secondary metabolite regulation (Bok and Keller, 2004). A recent transcriptional profiling of *A. fumigatus laeA* mutant revealed its role in regulation of multiple gene clusters (Perrin *et al.*, 2007) and a detailed comparative analysis of CSN and LaeA transcriptomes could shed more light on this topic.

The role of monooxygenases in metabolic conversions is of considerable interest. These enzymes are involved in both the catabolism and anabolism of many toxic compounds. Several fungal secondary metabolite biosynthetic pathways require monooxygenase activities (reviewed by Keller *et al.*, 2000). Detailed studies of the aflatoxin pathway had assigned five monooxygenase steps up to sterigmatocystin synthesis and an additional monooxygenase step. The sterigmatocystin gene cluster in *A. nidulans* includes five putative monooxygenase genes, four of them encoding P-450 monooxygenases, and one a probable flavin-requiring monooxygenase. In the light of the sheer number genes encoding P450 oxygenase family proteins which are misregulated in the *ΔcsnE* mutant and the genes similar but not identical the ones involved in ST biosynthesis (see Table 8), it is probable that the impact of CSN on fungal secondary metabolism is much broader and extends to yet uncharacterised metabolic pathways.

4.3.2. Role of cell wall plasticity in *A. nidulans* morphogenesis

The best-investigated group of cell wall biosynthesis genes with respect to their role in morphogenesis are chitin synthases, divided into six classes. *chsC* and *chsA*, genes encoding class I and II chitin synthases are involved in septum formation, but are also important in conidiation and reciprocally affect the asexual regulator *abaA* (Fujiwara *et al.*, 2000; Ichinomiya *et al.*, 2005a; Ichinomiya *et al.*, 2005b). *chsB* gene encodes a class III chitin synthase and plays a role in organized hyphal growth and conidiation (Borgia *et al.*, 1996), as well as *chsD*, encoding a class IV chitin synthase (Ichinomiya *et al.*, 2002). Class V and VI chitin synthases *csmA* and *csmB* are important for hyphal tip growth (Takeshita *et al.*, 2006).

Cell wall degradation and remodelling though, seems to be more specifically connected with developmental processes, especially the energy-costly fruit body formation. Early studies by Zonneveld indicated that α-1,3 glucan synthesis and its subsequent mobilization by an α-1,3 glucanase activity are both related to and required for cleistothecia development and that expression of α-1,3 glucanase is repressed by glucose (Zonneveld, 1974). Presence of α-glucan

and fruit body formation seem to be correlated with melanin synthesis (Polacheck and Rosenberger, 1977) β-1,3-glucanase and amylase, but not α-1,3 glucanase were found attached to hyphal walls (Polacheck and Rosenberger, 1978). An α-1,3-glucanase gene *mutA* was recently investigated in detail, revealing specific induction of the gene during sexual development in Hülle cells. Although degradation of α-1,3-glucan is greatly affected in the *mutA* deletion strain, it is still able to form wild-type numbers cleistothecia, suggesting that additional carbon sources are available during sexual development (Wei *et al.*, 2001).

Production of endo-β-1,4-glucanases also seems to be induced during development. Analysis of developmental *aco* mutants showed a correlation between cleistothecial development and the level of endoglucanases and one of three detected isozymes could be observed only during cleistothecial development (Bagga *et al.*, 1989). The requirement for β-glucanase activity in fruit body formation remains to be investigated, but interestingly Ssg1, one of three exoglucanases in *S. cerevisiae,* is expressed only in sporulating diploids and its disruption leads to delayed asci formation, pointing to a more wide-spread role of β-glucanases in fungal sexual development (Larriba *et al.*, 1995).

Ace2 transcription factor and Cbk1 serine-threonine kinase have emerged as important regulators of chitinase and glucanase expression in yeast during daughter cell separation after budding (Racki *et al.*, 2000). Protein kinase C is also implicated in regulation of cell wall hydrolase activities in yeast and its deletion results in drastic reduction of the alkali-and acid-insoluble glucan in the cell wall (Shimizu *et al.*, 1994). However, little is known about the molecular mechanisms underlying regulation of wall hydrolase expression in filamentous fungi.

4.3.3. CSN and the regulation of cell wall degradation

The accumulation and subsequent degradation of cell wall α-glucan was identified as necessary for fruit body formation in *A. nidulans*. It is suggested that α-1,3-glucan accumulated during vegetative growth serves as the main reserve material for developing cleistothecia. However, the deletion of *mutA*, an α-1,3-glucanase does not impair sexual development, suggesting that multiple enzymes are involved in this process (Wei *et al.*, 2001). β-glucans belong to the alkali-insoluble fraction of *Aspergillus* cell wall, which is responsible for its rigidity. This study provides evidence that β-1,3-glucans and possibly also β-1,4-glucans are mobilized along with α-glucans during sexual development. The extent of cell wall remodelling necessary for fruit body formation might be therefore much greater than previously thought and CSN appears to be a crucial regulator of this process. Although β-glucanase activity is highest

when *A. nidulans* is induced to develop asexually, it seems to be critical only for sexual development, as the *ΔcsnE* mutant is not impaired in conidiation. It was observed earlier that production of endo-β-1,4-glucanases is induced during cleistothecial development (Bagga *et al.*, 1989), although the requirement for β-glucanase activity in fruit body formation, hinted at by the sexually defective *S. cerevisiae* Ssg1 mutant (see 4.3.2), remains to be investigated. Interestingly, one predicted endo-1,3(4)-β-glucanase gene AN3883.3, which is downregulated only during sexual development only, seems to exert a large effect on the overall laminarinase activity, along with three genes that are affected at both developmental conditions. This gene could prove an interesting target for further genetic studies.

Based on the cis-element enrichment analyses we propose that the specific influence of CSN on the expression of genes related to cell wall plasticity might be mediated at least in part by the AbaA transcription factor (see 4.4.1 for more details). Further analysis of transcription factor stability and activity is required to confirm this hypothesis, but preliminary data from further transcriptome profiling experiments indicate that many of the β-glucanases influenced by CSN are indeed upregulated during development in a coordinate way (Dumkow, personal communication), supporting a hypothesis of regulation by a common transcription factor.

Recently, comparative *in silico* studies indicated that a broad range of inducer-dependent genes including cell wall lytic enzymes are upregulated at the onset of fungal development (Prade *et al.*, 2001). There is a clear overlap between these transcripts and the gene categories downregulated in *ΔcsnE* mutant during development, including chitinases, cellobiose hydrolases and a few proteases, glucose derepression-like and stress-response genes in addition to glucanases. This overlap suggests that CSN controls many aspects of developmentally regulated transcription. Interestingly, *eglC* gene encoding a putative β-1,3-endoglucanase, whose expression was shown to be dependent on *nsdD* but dispensable for development (Choi *et al.*, 2005), is not affected, suggesting that CSN signalling in regulation of cell wall degradation might be independent of NsdD transcription factor.

In addition to glucanases, other genes involved in cell wall remodelling are downregulated affected in the *ΔcsnE* mutant, including putative chitinases, mannosidases and cellobiose hydrolases, enzymes degrading extracellular cellulose. This indicates that other aspects of cell wall plasticity except for glucan mobilization, possibly associated with the morphological changes at different stages of sexual development, might be also controlled directly and indirectly by CSN. The concomitant upregulation of a several putative genes responsible of synthesis of α- and β-glucan as well as chitin suggests that the signalosome might be

controlling cell wall recycling during development on many levels, either via different pathways or by differential regulation of the same signalling networks.

4.4. Coordination of transcriptional networks by CSN

Arabidopsis COP9 is a negative regulator of transcriptional factor stability (Schwechheimer *et al.*, 2002), so it was quite surprising to find that in general more genes are up- than downregulated in *ΔcsnE*, indicating that *Aspergillus* CSN might have a different mode of action. There are many groups of genes, which are both up-, and down regulated across development, but only a few genes potentially involved in ubiquitin-dependent degradation seem to be exclusively upregulated in the mutant. They include 3 out of 69 putative predicted F-box proteins in this fungus, one of them potentially involved in the regulation of sexual development (Rupprecht, personal communication). This group of genes might represent a compensating response of the protein degradation system when CSN function is compromised, but their significance remains unclear.

An additional link between sexual development and secondary metabolism regulation recently emerged, which could be connected with the CSN-dependent signalling. The slight-dependent regulator VeA was shown to form a part of a trimeric complex including LaeA (Bayram, personal communication). The complex presumably assembles in the nucleus in the dark, where it controls light-dependent gene expression and production of secondary metabolites, although it was not shown to bind directly to DNA. *veA* has little effect on the expression of known sexual transcription factors *nsdD* and *steA* (Kato *et al.*, 2003), but regulates the expression of the asexual transcription factor *brlA* (see 1.4.1) by modulating the α/β transcript ratio. *brlA*, but not the other two factors is downregulated during development in *ΔcsnE* mutant, pointing to the possible connection between CSN and VeA as well as to the importance of crosstalk between metabolic and developmental light-dependent signalling.

Conspicuously missing from the transcriptional regulation picture is CpcA, an *Aspergillus* homologue of mammalian c-Jun (Claret *et al.*, 1996) and yeast Gcn4 (Hinnebusch, 1984), although the fungal factors play seemingly different roles in the cell, being responsible for cross-pathway control of amino acid biosynthesis during starvation. Under normal growth conditions, Gcn4p is a highly unstable protein, resembling many other eukaryotic transcription factors and is stabilised under starvation conditions. Gcn4p is degraded by ubiquitin-dependent proteolysis mediated by the Skp1/cullin/F-box (SCF) ubiquitin ligase and is destabilised primarily in the nucleus (Bomeke *et al.*, 2006; Kornitzer *et al.*, 1994). Nutritional status plays a role in initiating *A. nidulans* developmental programs, but the analysis of statistically

overrepresented transcription factor binding sites did not reveal any CPREs (cross-pathway control response elements) to which Gcn4 is known to bind. The CpcA consensus site of *A. nidulans* has not been characterised yet, so it is both possible that it is divergent and that CSN does not influence the cross pathway control system.

4.4.1. AbaA binding sites

The enrichment of AbaA binding sites in promoters of genes encoding glucanases downregulated during development led to a conclusion that if AbaA is involved in their transcriptional control, it should be either destabilized in the absence of CSN or act as a repressor of transcription of these genes. AbaA is a transcription factor involved in asexual development signalling cascade downstream of BrlA. *wA* transcript accumulation during conidiation requires AbaA (Mayorga and Timberlake, 1990) and its promoter contains an AbaA binding site, suggesting direct binding. CSN influences only a subset of abaA-dependent genes: *brlA* and *wA* are affected, as well as *rodA* and *prpA* (Semighini *et al.*, 2006), but not other genes described to be AbaA-dependent: cyclin-encoding *pclA*, *yA* (which is downregulated, but only to a moderate degree) and *abaA* itself (Aramayo and Timberlake, 1993; Schier *et al.*, 2001).

AbaA is a homologue of well characterized *S. cerevisiae* Tec1, a transcription factor functioning in co-operation with Ste12p (a homologue of *A. nidulans* SteA) to induce mating and pseudohyphal development (Gavrias *et al.*, 1996). Tec1 is phosphorylated by a MAP kinase to achieve regulation of these two pathways (Bruckner *et al.*, 2004), which influences its stability (Brückner, personal communication). Tec1 and Ste12 regulate the developmental processes by cooperative binding to combined enhancer elements consisting of a Tec1-binding site (TCS) and an Ste12-binding site present in the promoter regions of target genes, although Tec1 alone is able to activate gene expression via TCS elements, suggesting that the regulation of invasive and pseudohyphal growth by Stel2p and Tec1p is executed by many levels of combinatorial control (Kohler *et al.*, 2002)

It is therefore tempting to speculate that an interaction of AbaA with another factor could be hindered in the absence of CSN. Considering the presence of Mcm1 binding sites in some of the AbaA enriched promoters, the most obvious candidate would be McmA, an *A. nidulans* close homologue of the yeast Mcm1 factor encoded by AN8676.3 ORF. There is not much known about function of McmA up to date, but it seems to be involved both in arginine catabolism and sexual development (see below). There is a similar co-localisation of Aba binding sites with yeast stress response elements (STREs, see 4.4.3), but no STRE-binding

transcription factors have been identified in *Aspergillus* up to date and it is not known if their function in stress signalling is conserved.

4.4.2. Mcm1 binding sites

The *S. cerevisiae* Mcm1 protein is a transcription factor necessary for expression of mating-type-specific genes, also required for the maintenance of minichromosomes and implicated in arginine metabolism. It binds to the upstream regulatory sequences of both a- and α-specific genes and functions as a pheromone/receptor transcription factor (PRTF) and a general regulator of mating type (GRM) (Ammerer, 1990; Passmore *et al.*, 1989). Mcm1 acts as a heterodimer, binding cooperatively with the α1 and α2 proteins to achieve transcriptional activation and repression, respectively (Elble and Tye, 1991). The protein contains striking homology to the DNA-binding domain of the human serum response factor (SRF) and can bind to the human *c-fos* serum response element (Passmore *et al.*, 1989). This indicates that Mcm1 and SRF, as well as their DNA recognition sequences, have been conserved over large evolutionary distances and have a general eukaryotic function in transition from resting to proliferative state.

One homologue of the *S. cerevisiae mcm1* gene can be found in the *A. nidulans* genome. *mcmA* encodes a 224 amino acid SRF-type transcription factor with a 46% identity to the yeast Mcm1p, but with 86% identity within the MADS-box domain of the SRF transcription factor type. The N-terminal part of the protein required for DNA binding is highly conserved, but not the acidic patch involved in transcriptional activation. The fact that one of three C-terminal glutamine-rich regions necessary for repression of a-specific genes in α cells (Elble and Tye, 1991) is conserved in McmA additionally suggests that it might act mainly as a transcriptional repressor. The morphological phenotypes of the *ΔmcmA* mutant, which is able to produce conidiophores and Hülle cells, but not cleistothecia, indicate that McmA is involved in sexual development (Kobayashi, personal communication). This phenotype is strikingly similar to the *ΔcsnE*, suggesting that if CSN indeed regulates McmA activity or stability, it should contribute greatly to its developmental function. Furthermore, McmA was shown to bind to the promoter of *eglA* that carries a yeast Mcm1p binding consensus (see Figure 27), confirming that the MCM binding sites identified in the enrichment analysis in the upstream sequences of genes related to cell wall degradation which are downregulated during sexual development are indeed functional in *A. nidulans* and are recognized by McmA (Kobyashi, personal communication). It would be interesting to investigate whether *A. nidulans* McmA also acts cooperatively with other proteins, for example with AbaA, in regulation of gene expression.

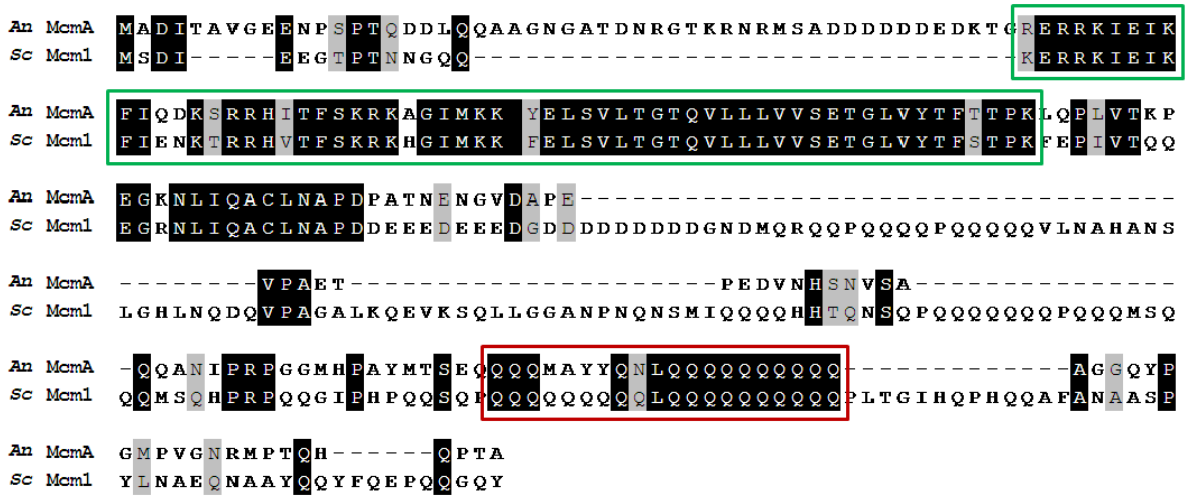


Figure 32. Alignment of *A. nidulans* McmA and *S. cerevisiae* Mcm1 proteins. Identical residues are shaded in black, similar residues in grey. The MADS-box transcription factor domain, spanning residues 52-111 (green frame) is very conserved in McmA. One of three glutamine-rich regions (red frame), but not the acidic patch (D/E) (Elble and Tye, 1991), is conserved in the *A. nidulans* protein.

4.4.3. STRE elements and PacC binding sites

One more type of cis-acting element, STRE, is enriched in the promoters of cell wall degradation-related genes downregulated during sexual development. Two transcription factors are known to bind to the STRE elements in yeast, Msn2p (Martinez-Pastor *et al.*, 1996) and Msn4p (Marchler *et al.*, 1993). *A. nidulans* encodes two proteins with high similarity to Msn2p and three proteins with high similarity to Msn4p. One of them is the SteA zinc-finger transcription factor important for the sexual cycle regulation, which possesses homology to both of these proteins, although it is more closely related to Msn4p. Because SteA is primarily a homologue of yeast Ste12, it is conceivable that it could integrate in *A. nidulans* the functions normally performed by several yeast transcription factors. SteA was recently shown to form a complex with McmA *in vitro* (Kobayashi, personal communication), reinforcing the possibility that McmA might bind cooperatively with another transcription factor in regulation of the genes associated with cell wall degradation during development. The mechanism of this binding remains open and it would be especially interesting to investigate whether the regulation of glycosyl hydrolases occurs primarily via the AbaA binding sites, McmA binding sites, STRE elements, or a combination of these.

The broad scope of CSN action in transcriptional control is demonstrated by the enrichment of PacC binding sites in the upstream element of genes related to membrane transport, which are upregulated in the *ΔcsnE* strain. PacC is a central regulator of pH-dependent regulation of gene expression in *A. nidulans,* ensuring among others that enzymes are expressed at a pH that allows for their activity. The expression of a number of genes is regulated by ambient pH via

this transcription factor, including *prtA*, xylanases, secondary metabolite biosynthesis genes and multiple permeases (reviewed by Arst and Penalva, 2003; Denison, 2000). It seems quite feasible that genes associated with transmembrane transport of metabolites should be regulated by this factor, although the connection with sexual development remains unclear. Perhaps the link might be the impact of pH signalling on secondary metabolism, as aflatoxin and sterigmatocystin production, like that of penicillin, appears to be influenced by growth medium pH and the expression of all these metabolites is also related to sporulation (see 1.5.3). Further research is necessary to elucidate the complex interactions between pH and other environmental factors that influence morphological and chemical differentiation in *Aspergillus*.

4.5. Conclusions and outlook

Only a small number of transcription factors binding sites and control element are known for filamentous fungi in general, including *Aspergillus*. An analysis of sequences conserved across three *Aspergillus* species in the sequences up- and downstream of genes identified 69 conserved patterns showing an enrichment for co-occurrence which are likely to correspond to novel transcription factor binding sites (Galagan *et al.*, 2005). This study identified a stress response element as an additional putative mechanism of gene regulation with impact on sexual development, but our knowledge about *A. nidulans* transcriptional networks and the binding specificity of factors involved in them is still fragmentary. The question of whether the COP9 signalosome acts in *A. nidulans* by affecting a few targets with wide-spread effects or many targets acting in specific pathways still remains open to discussion. However, the results of the transcriptome profiling study presented here constitute an important step towards identifying these networks and their connections. The cis-analysis results indicate that CSN might be involved in the interplay between asexual and sexual signalling, which is still poorly understood and might influence a balance of these two developmental cycles. A biochemical analysis of the stability and activity of transcription factors such as AbaA, McmA, SteA and PacC in *A. nidulans* strains defective in CSN function should at least provide tangible evidence for some of the mechanisms of transcriptional control exerted by the COP9 signalosome during development of filamentous fungi.

Furthermore, CSN probably exerts its function by controlling the activity of three putative *A. nidulans* cullins independently at different stages of growth and development. It is conceivable that affected functions of CulA, culC and CulD could differentially contribute to the *ΔcsnE* phenotype, presumably by controlling the stability of different substrates, including

transcription factors (see Figure 33). Mutational and biochemical analysis of *A. nidulans* cullins should provide more insight into this process.

The transcriptome profiling reveals that CSN exerts both distinct and overlapping effects at different stages of *A. nidulans* development. On one hand, genes associated with oxidative reactions, secondary metabolism (including a novel gene cluster) and membrane transport are differentially expressed at all times and the mechanism of this regulation is very hypothetical. On the other hand, CSN positively regulates *ppoC* and the establishment of spore balance during vegetative growth and at the early stages of development, as well as *brlA* expression and the coordinated expression of cell wall degrading enzymes during asexual and sexual development (Figure 33). These effects might be mediated by regulation of activity of different cullins affecting stability of target proteins, including transcription factors. The upstream signals regulating CSN activity at different time points are not known and could include both external signals, such as light or nutritional status, and internal signals, such as developmental G-protein signalling. Overall, our results not only reassert the COP9 signalosome as a central regulator of fungal development, but also hint at the importance of crosstalk between asexual and sexual signalling during development and suggest a much greater extent of cell wall remodelling prior to fruit body formation than previously thought.

The group of molecules and genes associated with the virulence of *Aspergillus fumigatus* includes many cell wall components, such as β-(1-3)-glucan, galactomannan, galactomannan proteins and chitin synthetases. Some genes and molecules have been implicated in evasion from the immune response, such as the rodlets layer (rodA/hyp1 gene) and the conidial melanin-DHN (pksP/alb1 (PKS) gene) (reviewed by Rementeria *et al.*, 2005). The reactive oxygen species (ROS) detoxifying by catalases and superoxide dismutases, had also been pointed out as essential for virulence (Paris *et al.*, 2003; Rementeria *et al.*, 2005). It is striking that many of the *A. nidulans* genes whose expression was affected by the absence of CSN in this study had been identified as important in *A. fumigatus* virulence. It raises the possibility that the *A. fumigatus* COP9 signalosome could constitute a promising target for antifungal therapy, as its inhibition might allow influencing multiple virulence factors at the same time.

In this work, we have confirmed that the importance of the deneddylase motif of the COP9 signalosome extends to a model filamentous fungus. The control of transcriptional networks by CSN observed in this organism and the possible interplay of different conserved transcription factors might provide new insight also into the function of the complex in higher eukaryotes, especially mammals, where the genome-wide impact of CSN activity has not been investigated.

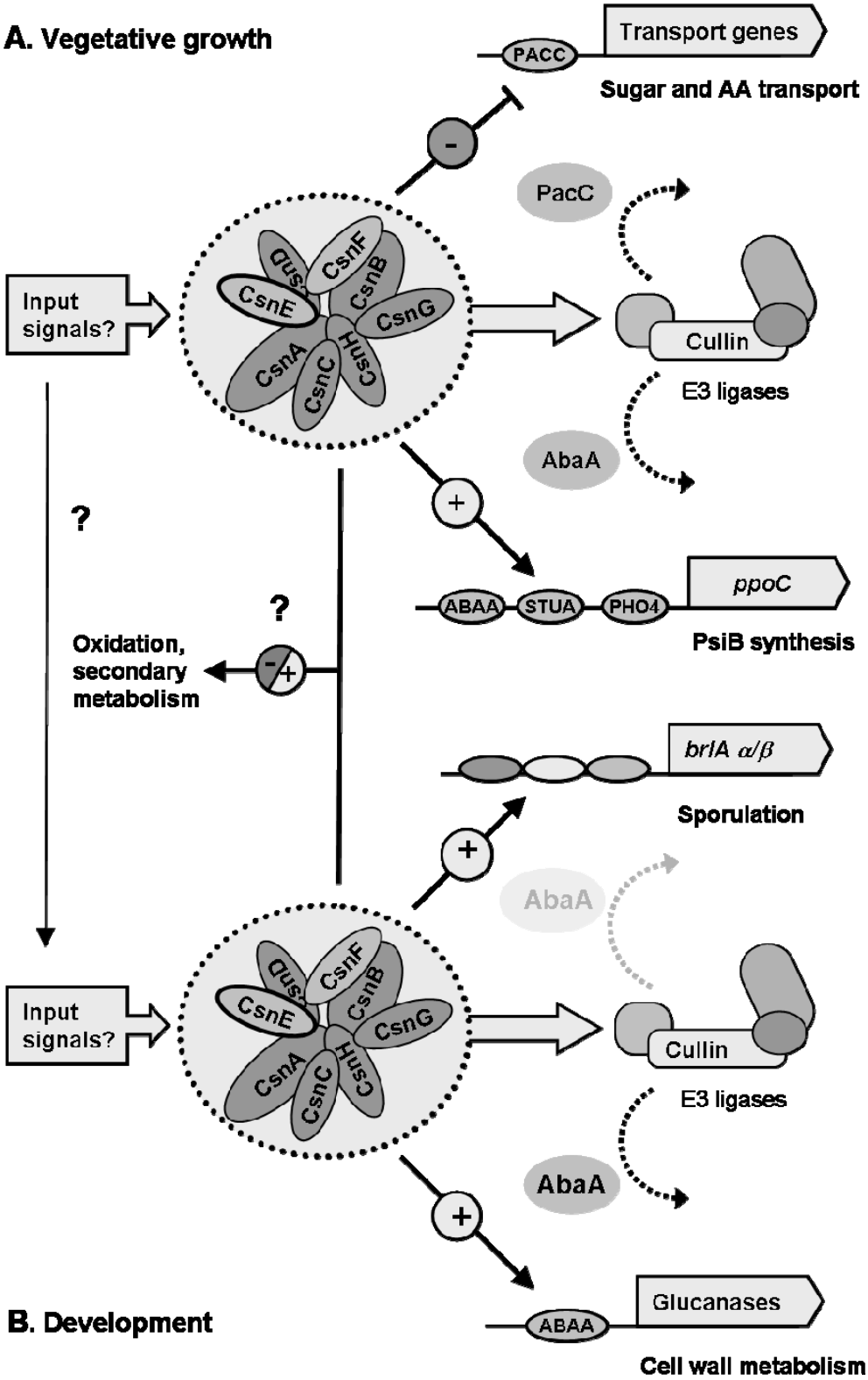


Figure 33. A hypothetical model of CSN function in A. nidulans development based on the mutagenesis and transcriptome profiling data. The effect of CSN on developmental regulation triggered by yet undetermined input signals is dependent on the JAMM motif of CsnE (outlined in bold). **(A)** During vegetative growth CSN represses transcription of genes encoding transport proteins and during initiation of sexual development is needed for *ppoC* transcription and regulating asexual to sexual reproduction ratio. **(B)** During sexual development CSN impacts activation of cell wall degradation genes, mainly β-glucanases, as well as expression of the transcriptional activator *brlA*. Oxidation and secondary metabolism is influenced at all times. These effects might be mediated by regulation of one or more E3 ubiquitin ligases containing any of the 3 putative *A. nidulans* cullins and the subsequent stabilisation or destabilisation of transcription factors.

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