Regulators of Ubiquitin Dependent Protein Degradation in the Filamentous Fungus *Aspergillus nidulans*:

Insights into CsnB, DenA and CandA Function

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
Elke Ute Schwier
aus Herford

Göttingen 2007

Die vorliegende Arbeit wurde in der Arbeitsgruppe von Prof. Dr. Gerhard H. Braus in der Abteilung Molekulare Mikrobiologie des Instituts für Mikrobiologie und Genetik der Georg-August-Universität Göttingen angefertigt.

Teile dieser Arbeit wurden veröffentlicht in:

Lima, J.F., Malavazi, I., von Zeska Kress Fagundes, M.R., Savoldi, M., Goldman, M.H., Schwier, E., Braus, G.H., and Goldman, G.H. (2005) The *csnD*/*csnE* signalosome genes are involved in the *Aspergillus nidulans* DNA damage response. *Genetics* 171: 1003-1015.

Busch, S., Schwier, E.U., Nahlik, K., Bayram, O., Helmstaedt, K., Draht, O.W., Krappmann, S., Valerius, O., Lipscomb, W.N., and Braus, G.H. (2007) An eight-subunit COP9 signalosome with an intact JAMM motif is required for fungal fruit body formation. *Proc Natl Acad Sci U S A* 104: 8089-8094.

Schwier, E.U., Christmann, M., Nahlik, K., Westermann, M., Grond, S., Busch, S. and Braus, G.H. The split ubiquitin ligase regulator CandA is crucial for development in *A. nidulans*. Manuscript in preparation.

Schwier, E.U., Christmann, M., Busch, S. and Braus, G.H. The *Aspergillus nidulans* DenA protein represents a second deneddylase required for regulation of development. Manuscript in preparation.

D7

Referent: Prof. Dr. Gerhard H. Braus

Korreferentin: Prof. Dr. Stefanie Pöggeler

Tag der mündlichen Prüfung:

Für meinen Vater,
in Erinnerung an meine Mutter

Acknowledgements

First of all I want to thank Prof. Dr. Gerhard Braus for supervision, support during the course of my research study and optimal working conditions. I am particularly thankful to him to enable me to present parts of my work on national and international conferences.

Special thanks go to Silke Busch for supervision and a lot of helpful advice and support. I also wish to thank Martin Christmann for his contribution to this work in his diploma thesis and the good collaboration.
I am grateful to Stephanie Grond for the cooperation on the "red pigment" project, her large interest in the topic and a lot of interesting discussions. I would like to thank Mieke Westermann who performed the chemical analysis of the "red pigment" during her work for the first state exam. I also would like to thank Kay Hofmann for *candA* gene evolution analysis. I also wish to thank Prof. Dr. Gustavo Goldman for cooperation. Thanks to Verena Große for providing me an *A. nidulans* strain.

Special thanks go to Nico Vogt for a lot of helpful discussions, hints and ideas. I would also like to thank Krystyna Nahlik for cooperation in many experiments, fruitful discussions and the proofreading of many documents, including this thesis. I would like to thank the present and former members of the *Aspergillus* lab Gaby Heinrich, Kerstin Helmstaedt, Birte Koennecke, Heike Rupprecht and Andrea Grzeganek (geb. Pfeil) for being great colleagues. Another thanks go to the "11.30 mensa-group" for countless lunch breaks. Thanks also to the practical students Andrea Herrmann, Rebekka Harting, Stephanie Nagl and Caghan Kizil. I would also like to thank all other present and former members of the group for their helpfulness and the pleasant working atmosphere.

I also wish to thank my friends for giving me a lot of general support. I especially want to thank Céleo for encouragement and motivation after setbacks and in difficult times.
My deepest thanks go to my father and my sister for their love and tremendous support.

Table of contents

Acknowledgements ..... **I**

Table of contents ..... **III**

Summary **1**

Zusammenfassung **3**

1 Introduction **5**

1.1 Ubiquitin and Ubiquitin dependent protein degradation 5

1.1.1 Ubiquitin und Ubiquitin-like proteins 5

1.1.2 Ubiquitination cascade and proteasome function 7

1.1.3 Ubiquitin ligases 9

1.2 Regulation of ubiquitin ligase activity 12

1.2.1 Phosphorylation and F-box protein degradation 12

1.2.2 Nedd8 and its role in posttranslational modification of cullins 12

1.2.3 The COP9 signalosome 14

1.2.3.1 COP9 signalosome conservation and architecture 16

1.2.3.2 CSN associated proteins, deneddylation, phosphorylation and deubiquitination 17

1.2.3.3 Physiological role of CSN 19

1.2.3.4 Influence of CSN on DNA damage response and cell cycle progression in *Schizosaccharomyces pombe* 20

1.2.4 DEN1 deneddylase 20

1.2.5 The cullin-binding protein Cand1 21

1.2.5.1 Cand1 localization and interaction with cullins 21

1.2.5.2 Effect of Cand1 on protein levels of E3 targets 23

1.2.5.3 Physiological role of Cand1 24

1.2.6 Assembly and disassembly of the ubiquitin ligase SCF^{Skp2} 25

1.3 The model organism *Aspergillus nidulans* 27

1.3.1 Vegetative growth 28

1.3.1.1 Morphology of growth 28

1.3.1.2 Regulation of the cell cycle 28
1.3.2 Development 29
1.3.2.1 Asexual and sexual reproduction 30
1.3.3 Regulation of asexual and sexual development 31
1.4 Melanin and pigments 34
1.5 Scope and aim of this work 39

2 Materials and Methods 40

2.1 Growth media and growth conditions 40
2.2 Strains 41
2.2.1 *Escherichia coli* strains 41
2.2.2 *Saccharomyces cerevisiae* strains 41
2.2.3 *Aspergillus nidulans* strains 42
2.2.3.1 Construction of *csn* deletion strains of and promoter *lacZ* fusions in *Aspergillus nidulans* 42
2.2.3.2 Construction of *denA* deletion and complementation strains in *Aspergillus nidulans* 42
2.2.3.3 Construction of *Aspergillus nidulans* strains for CandA characterization 43
2.3 Genetic manipulation 45
2.3.1 Transformation 45
2.3.2 Primers and plasmids 45
2.3.2.1 Constructs for studies on CsnB in *Aspergillus nidulans* 45
2.3.2.2 Constructs for studies on DenA in *Aspergillus nidulans* 46
2.3.2.3 Constructs for studies on CandA in *Aspergillus nidulans* 47
2.3.2.4 Construction of plasmids for DenA characterization in *Saccharomyces cerevisiae* 52
2.3.2.5 Construction of plasmids used in the yeast two-hybrid tests 52
2.3.3 Sequence analysis 53
2.3.4 Recombinant DNA methods 53
2.3.5 DNA isolation and hybridisation 53
2.4 Protein methods 54

2.4.1 Protein isolation and analysis 54
2.4.2 Yeast two-hybrid analysis 55
2.4.3 Specific β-galactosidase activity assay of *Aspergillus nidulans* proteins 55
2.5 Microscopic analysis 56
2.6 Chemical analysis 56
2.7 Metabolite analysis and isolation 57

3 Results 61

3.1 Isolation and characterization of the fungal COP9 signalosome subunit CsnB 61
3.1.1 *Aspergillus nidulans csnB* encodes a PCI domain subunit of the COP9 signalosome 61
3.1.2 *Aspergillus nidulans* strains deleted for *csnB* and *csnA*/*csnB* are blocked in sexual development and produce red colored hyphae 63
3.1.3 CsnB interacts with CulD in the yeast two-hybrid test 65
3.1.4 Transcription of *csnA* and *csnE* is unaffected by DNA damaging agents 66
3.2 Isolation and characterization of the deneddylase DenA 68
3.2.1 *Aspergillus nidulans denA* encodes a putative cysteine protease 68
3.2.2 DenA is required for correct development of *Aspergillus nidulans* 69
3.2.3 *Aspergillus nidulans* DenA is unable to complement a Δ*yuh1* mutant of *Saccharomyces cerevisiae* 71
3.2.4 The *Aspergillus nidulans* deneddylase DenA is sufficient to detach yeast Nedd8/Rub1 from *A. nidulans* CulD in *Saccharomyces cerevisiae* 72
3.3 Isolation and characterization of the ubiquitin ligase regulator CandA of *Aspergillus nidulans* that is split into two genes 74
3.3.1 Cand1 is encoded by the separated genes *candA-N* and *candA-C* in *Aspergillus nidulans* 74
3.3.2 The *candA* genes are required for fungal development 76
3.3.3 A CandA-N::C fusion protein can replace the independent proteins CandA-N and CandA-C 78
3.3.4 Deletion of *candA-N* and *candA-C* as well as *csnE* leads to production of phenylethers 79
3.3.5 Production of phenylethers in the *csnE* deletion strain is *wA3* independent 82

3.3.6 A *candA-C*/*csnE* double deletion strain shows a combination of the *candA-C* and *csnE* deletion phenotypes 83
3.3.7 The nuclear localized CandA-C is expressed in vegetative mycelia and degraded upon induction of fruit body formation 83
3.3.8 CandA-C and CandA-N interact in the yeast two-hybrid system but only CandA-C interacts with cullins 85

4 Discussion 87

4.1 High sequence conservation of ubiquitin ligase interacting proteins in *Aspergillus nidulans* 87
4.2 The separation of Cand1/CandA into two genes is a specific feature of the *Aspergilli* 88
4.3 Functional conservation of the ubiquitin ligase regulators in *Aspergillus nidulans* 89
4.3.1 The deneddylases CSN and DenA are functionally conserved 89
4.3.2 The CandA proteins are functionally conserved 91
4.4 CSN, DenA and CandA in development 93
4.4.1 CandA-C is important for the onset of development 95
4.4.2 The two deneddylases DenA and CSN influence development differently 95
4.4.3 CSN and CandA function in the development of complex eukaryotic multicellular organisms is conserved 96
4.5 Expression of CSN subunits is independent of DNA damaging agents 98
4.6 CSN and CandA in secondary metabolism 98
4.6.1 Bioactivity of the isolated substances 99
4.6.2 Possible ways of orcinol formation in *csn* and *candA* deletion strains 100
4.6.3 The *Aspergillus* genome encodes several polyketide synthases 103
4.7 Outlook 105

5 References 108

Curriculum vitae 127

Summary

Ubiquitin dependent protein degradation is a central mechanism regulating multiple functions in cells. Ubiquitination of target proteins requires an enzyme cascade including ubiquitin ligases (E3s). One group of E3s contains cullins as the core component. Activity of these cullin containing E3s is modulated by the covalent modification of cullin with the ubiquitin like protein Nedd8. In this work three regulators of cullin containing E3s were studied in the fungus *Aspergillus nidulans*. (i) The COP9 signalosome (CSN) possess an enzyme activity that deneddylates cullins. Csn2/CsnB, the second subunit of the CSN, mediates the binding of the complex to ubiquitin ligases in mammals via binding to cullin. This study shows that the fungal CsnB interacts with CulD, suggesting a conserved function of the protein. Deletion of *csnB* leads, like deletion of the genes for other subunits of the CSN such as *csnA*, *csnD* or *csnE*, to a typical *csn*-deletion phenotype including red pigment formation and a block in sexual development. These results support the notion that all subunits of the complex are crucial for the deneddylase function. (ii) The putative mammalian Den1 homolog of the fungus (DenA) was proven to exhibit deneddylase activity *in vivo*. For the first time, this deneddylase was successfully deleted in an organism. Loss of *denA* in *A. nidulans* leads to a reduction of asexual development and an increased production of sexual structures. (iii) Mammalian Cand1 binds to cullins, thereby influencing the assembly of cullin containing E3s. In *Aspergilli*, the corresponding gene is split into two independent open reading frames encoding the N- and C-terminal part (*candA-N*, *candA-C*) of the mammalian homolog. Deletion of the single or both *candA* genes leads to mutants with identical phenotypes. They produce only few asexual spores, are blocked in early sexual development and appear dark red when grown under development inducing conditions. All defects of the *candA* deletion mutants can be complemented by a *candA-N*::*C* fusion construct indicating that the split is not crucial for protein functions. Only CandA-C, but not CandA-N, binds to cullins. Since both CandA proteins interact with each other, binding of CandA-N to cullin is presumably mediated by CandA-C. CandA-C is nuclear enriched and expressed in vegetative cultures but degraded at an early stage of sexual development indicating a role during onset of development. The culture filtrate of both *candA* and *csnE* deletion mutants contains orsellinic acid related substances suggesting a connection of CandA and CsnE to the regulation of secondary metabolism. This study shows that the three regulators described are crucial for fungal development, indicating that fungi are useful models to analyze the interplay between cullin containing E3 activity and differentiation.

Zusammenfassung

Ubiquitin-abhängiger Proteinabbau ist ein zentraler Mechanismus zur Regulation verschiedenster Zellfunktionen. Ubiquitinierung von Zielproteinen benötigt eine Enzymkaskade, zu der die Ubiquitin-Ligasen (E3s) gehören. Eine Gruppe der E3s enthält Cullin als zentrale Komponente. Die Aktivität dieser E3s wird durch kovalente Modifikation des Cullins mit dem Ubiquitin-ähnlichen Protein Nedd8 beeinflusst. In dieser Arbeit wurden drei Regulatoren von Cullin-enthaltenden E3s in *Aspergillus nidulans* untersucht. (i) Das COP9 Signalosom (CSN) ist in der Lage, Culline zu deneddylieren. Csn2/CsnB bindet an Culline und vermittelt in Säugerzellen den Kontakt zu E3s. Diese Arbeit zeigt, dass pilzliches CsnB mit CulD interagiert, was eine konservierte Funktion des Proteins nahelegt. Die Deletion von *csnB* führt, wie auch die Deletion von anderen CSN Untereinheiten wie *csnA*, *csnD* oder *csnE,* zu einem für Δ*csn*-Mutanten typischen Phänotyp. Dieser zeichnet sich durch die Bildung eines roten Farbstoffs und eine unvollständige sexuelle Entwicklung aus. Diese Ergebnisse unterstützen die Annahme, dass alle Untereinheiten für ein intaktes CSN benötigt werden. (ii) Es wurde gezeigt, dass DenA, das *A. nidulans* Homolog des Den1 aus Säugetieren, *in vitro* Deneddylaseaktivität besitzt. *A. nidulans* ist der erste Organismus in dem *denA* deletiert wurde. Die Deletionsmutante zeigt eine verminderte asexuelle Entwicklung und die vermehrte Produktion von sexuellen Strukturen. (iii) Cand1 aus Säugern bindet an Cullin, wodurch es den Zusammenbau der E3s beeinflusst. In *Aspergillen* ist das entsprechende Gen in zwei unabhängige offene Leserahmen geteilt, die den N- und C-terminalen Teil (*candA-N*, *candA-C)* des menschlichen Homologs codieren. Die Deletion von einem oder beiden *candA* Genen führt zu Mutanten mit identischen Phänotypen. Sie produzieren nur wenig asexuelle Sporen, sind in der frühen sexuellen Entwicklung blockiert und sind dunkelrot wenn sie unter Entwicklungs-induzierenden Bedingungen angezogen werden. Da alle Defekte der Δ*candA* Mutante durch die Integration eines *candA-N*::*C* Fusionskonstrukts komplementiert werden können, ist die Trennung der CandA Proteine für ihre Funktion offensichtlich nicht essentiell. Nur CandA-C, aber nicht CandA-N bindet an Culline. Da beide CandA Proteine miteinander interagieren, wird die Bindung von CandA-N an Cullin vermutlich durch CandA-C vermittelt. CandA-C sammelt sich im Zellkern an und hat vermutlich eine Funktion am Beginn der Entwicklung, da es in vegetativen Kulturen exprimiert aber zu Beginn der sexuellen Entwicklung abgebaut wird. Das Kulturfiltrat der Δ*candA* und Δ*csnE* Mutanten enthält Substanzen die sich von Orsellinsäure ableiten lassen, so dass sich eine Verbindung von CandA und CsnE zu der Regulation des Sekundärmetabolismus vermuten lässt. Diese Arbeit beschreibt die Wichtigkeit der drei untersuchten E3 Regulatoren für die pilzliche Entwicklung und zeigt *Aspergillus* als nützliches Modell zur Analyse der Wechselwirkung zwischen E3-Aktivität und Differenzierung.

1 Introduction

1.1 Ubiquitin and Ubiquitin dependent protein degradation

1.1.1 Ubiquitin und Ubiquitin-like proteins

Ubiquitin is the central protein in the ATP-dependent, ubiquitin-mediated protein degradation system discovered in 1980 (Ciechanover *et al.*, 1980; Hershko *et al.*, 1980). Ubiquitin was originally isolated as a polypeptide with lymphocyte-differentiating properties, was thought to be ubiquitous and universally represented in living cells and therefore called ubiquitin (Goldstein *et al.*, 1975). Already two years later ubiquitin was found to be conjugated to histone H2A by formation of an isopeptide bond between the carboxyl terminus of ubiquitin and a lysine side-chain amino group (Goldknopf and Busch, 1977; Hunt and Dayhoff, 1977). About the same time, research on the non-lysosomal intracellular proteolysis was performed with rabbit reticulocytes, which lack lysosomes.

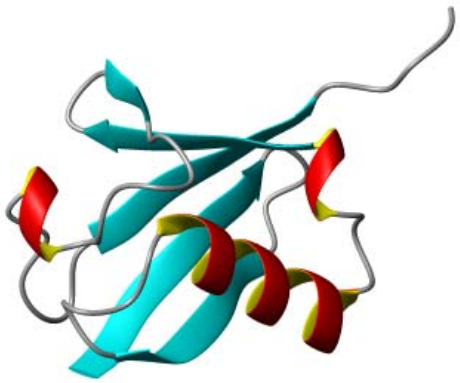


Fig. 1: Crystal structure of human erythrocytic ubiquitin (Vijay-Kumar *et al.*, 1987).

Multi-ubiquitination is a term used to describe ubiquitination of several different lysines of the target protein while the formation of an ubiquitin chain, covalently linked through its C-terminal Gly76 to an ε-NH_2 group of an internal lysine residue of the substrate, is called poly-ubiquitination. The following ubiquitin molecules are attached with their C-terminus to a lysine of the preceding ubiquitin molecule building a chain of at least four ubiquitin molecules. Another form of this multi-ubiquitination is the N-terminal ubiquitination (reviewed by Ciechanover and Ben-Saadon, 2004). In this case, the first ubiquitin moiety is fused linearly to the alpha-NH_2 group of the N-terminal residue of the substrate.

From the fractionated reticulocyte lysate, an active factor was obtained from fraction I, called APF-I that was covalently conjugated to proteins in the presence of ATP and fraction II (Ciechanover *et al.*, 1980; Hershko *et al.*, 1980). Ubiquitin was then shown to be identical to APF-1 (Wilkinson *et al.*, 1980). Ubiquitin is a 76 aa long protein, highly conserved in evolution (Fig. 1). It turned out that there are different types of ubiquitin modification: multi-ubiquitination, poly-ubiquitination and mono-ubiquitination. The ubiquitin molecule contains seven internal lysines, which have all been found to be involved in ubiquitin chain formation. The ubiquitin chain formation via linkage of the C-terminal Gly-76 of one ubiquitin molecule to Lys-48 or, less often Lys-29 of another ubiquitin, results in a compact ubiquitin chain that targets the proteins for degradation. Ubiquitination on Lys-63 results in a less compact ubiquitin chain and plays a role in DNA repair, endocytosis, stress response and activation of the IκBα signaling complex (reviewed by Welchman *et al.*, 2005). This form of ubiquitination influences the activation and inactivation of the target proteins rather than tagging them for degradation. In addition, enzymes and enzyme pairs have been identified that catalyze the formation of ubiquitin chains via Lys-6, Lys-11, Lys-27 and Lys-33 of ubiquitin (Baboshina and Haas, 1996; Kim *et al.*, 2007). Mono-ubiquitination describes the modification of target proteins with a single ubiquitin. This kind of ubiquitination has no proteolytic function but is involved in endocytosis, transcriptional regulation and DNA repair (reviewed by Hicke, 2001; Welchman *et al.*, 2005).

Following the discovery of ubiquitin, several distinct ubiquitin-like proteins (UBLs) have been found to function as protein modifiers as well. One of them is SUMO, also named UBL1 or sentrin (Shen *et al.*, 1996). The protein has been found to be important in transcriptional regulation, apoptosis, protein stability, cell cycle and response to stress (reviewed by Kroetz, 2005). Another UBL, Nedd8, is ligated to the core component of cullin containing E3 ubiquitin ligases and therefore plays a role in the regulation of protein degradation. For a detailed description of Nedd8 and its function as an E3 regulator see 1.2.2. All UBLs share the same three-dimensional structure with ubiquitin, the ubiquitin or β–grasp fold (Hochstrasser, 2000). The UBLs and their roles in cell function are summarized in Tab. 1 and were reviewed by Kerscher *et al.* (2006) and Welchman *et al.* (2005).

Tab. 1: Ubiquitin-like proteins.

Ubiquitin-like protein*	substrate(s)	attributed function(s)	selected reference(s)
SUMO1-4 (Smt3)	many	nuclear localization, transcriptional regulation, mitosis, antagonizing ubiquitination	Gill (2004); Mahajan *et al.* (1997)
Nedd8 (Rub1)	cullins, p53, MDM2	Regulation of E3s, transcriptional regulation of p53	Hori *et al.* (1999); Xirodimas *et al.* (2004)
ISG15/UCRP**	PLCγ1, JAK1, STAT1, ERK1/2, serpin 2a	May act in transcription and pre-mRNA splicing during IFN response; induced by IFN-α/β	Hamerman *et al.* (2002); Malakhov *et al.* (2003)
ATG8	phosphatidylethanolamine	Autophagy, cytoplasm-to vacuole targeting	Ichimura *et al.* (2000)
ATG12	Atg5	Autophagy, cytoplasm-to vacuole targeting	Mizushima *et al.* (1998)
URM1	Ahp1	Oxidative-stress response, nutrient sensing (through the TOR signaling pathway)	Goehring *et al.* (2003)
FAT10**	unknown	Ubiquitin-independent substrate degradation; induced by IFN-γ and TNF-α	Raasi *et al.* (2001)
FUB1/MNSFβ	TCR-α-like protein, Bcl-G	T cell activation	Nakamura *et al.* (1995)
UBL5 (Hub1)	Unknown	Pre-mRNA splicing	Dittmar *et al.* (2002); Luders *et al.* (2003)

* the names of the *S. cerevisiae* proteins are shown in brackets when they differ from the mammalian protein name. ** ISG15/UCRP and FAT10 both contain two ubiquitons each. Ahp1, alkyl hydroperodide reductase-1; Atg/ATG, autophagy; Bcl, B-cell lymphoma-G; C/EBPβ1, CCAAT/enhancer-binding protein-β1; E3, enzyme-3 (ubiquitin-protein ligase); ERK, extracellular signal-regulated kinase; FUB1, Fau ubiquitin-like protein-1; Hub1, homologous to ubiquitin-1; ISG15, interferon-stimulated gene-15; JAK, Janus kinase; MDM2, mouse double minute-2; NEDD8, neuronal-precursor-cell-expressed developmentally downregulated protein-8; PLCγ1, phospholipase Cγ1; Rub1, related to ubiquitin-1; Smt3, suppressor of *MIF2* mutations; STAT1, signal transducer and activator of transcription-1; SUMO, small ubiquitin-like modifier; TCR, T-cell receptor; TOR, target of rapamycin; UBL5, ubiquitin-like protein-5; UCRP, ubiquitin cross-reactive protein; URM1, ubiquitin-related modifier-1. Modified from Welchman *et al.* (2005).

1.1.2 Ubiquitination cascade and proteasome function

A general mechanism in eukaryotic cellular and developmental regulation is the precise and controlled protein turnover mediated by the ubiquitin-proteasome system. It ensures the degradation of cellular proteins including key cellular factors in a highly complex, temporally controlled and tightly regulated manner. In addition the ubiquitin-proteasome system is able to degrade misfolded proteins while energy is released that can be used for cellular and developmental processes. The ubiquitin-proteasome system is involved in the regulation of cell cycle progression, differentiation, stress response, secretion,

transcriptional regulation, DNA repair, circadian rhythms, immune response and biogenesis of organelles. For targeting a protein for degradation by the ubiquitin-proteasome system, it has to be modified with ubiquitin. The covalent attachment of multiple ubiquitin molecules involves a three step cascade mechanism (Fig. 2). First, the ubiquitin is activated by the ubiquitin activating enzyme E1 in an ATP-requiring reaction generating a high-energy thiol ester E1-S~ubiquitin intermediate. The activated ubiquitin is then transferred to one of several E2 ubiquitin conjugating enzymes (UBCs) to form an E2-S~ubiquitin high energy intermediate. From the E2 the ubiquitin is subsequently transferred to the substrate that is bound by a member of the protein ligase family, E3. The protein ligase is responsible for the specific recognition of the substrate. The mechanism of how the ubiquitin is passed to the substrate depends on the type of E3 ligase. A direct transfer of the activated ubiquitin from the E2 onto the substrate is mediated by the RING finger-containing E3 ligases, while the activated ubiquitin builds another intermediate with an active site Cys residue of HECT (homologous to the E6-AP COOH terminus) domain E3s, before it is attached to the substrate. The ubiquitin molecule is covalently bound to an ε-NH_2 group of an internal Lys residue of the substrate. By attachment of the C-terminal residue of the following ubiquitin to a lysine (K48) residue of the preceding ubiquitin molecule a polyubiquitin chain is formed. The ubiquitin chain is recognized by a specific receptor on the 26S proteasome (reviewed by Glickman and Ciechanover, 2002; Hochstrasser, 1996). The 26S proteasome holoenzyme is highly conserved among all eukaryotes and specifically degrades ubiquitinated proteins to short peptides of approximately 3-22 amino acids (Kisselev *et al.*, 1999) while the ubiquitin is reused.

The 26S proteasome consists of the 20S core particle and the 19S regulatory particle, displaying degradation activity. The 20S core particle (CP) of approximately 700 kDa displays a cylindrical structure with a proteolytic center for degradation of the substrates. The way into the CP via 13 Å wide pores, located at either end of the chamber, can be occluded by peptides of core particle subunits. For degradation activity, the 19S regulatory particle (RP) has to attach to the CP. It is composed of two eight-subunit subcomplexes. The base contains ATPase and chaperon activity, while the lid complex (LID) encompasses eight subunits, at least one of them displaying deubiquitination activity. The RP mediates proteasome function by removing the peptides occluding the pore and by destabilizing the tertiary structure of protein substrates.

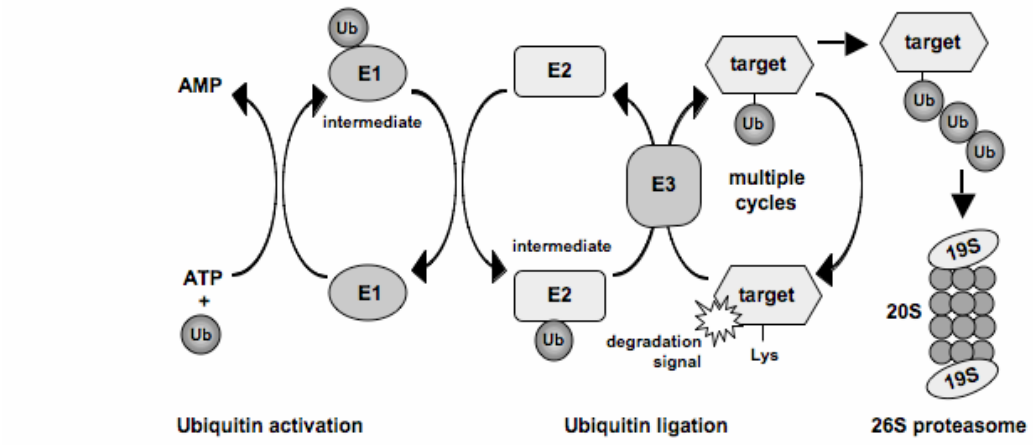


Fig. 2: Ubiquitin-dependent protein degradation pathway.

Ubiquitin is activated by an E1 ubiquitin activating enzyme and transferred to an E2 ubiquitin conjugating enzyme. The E3 ubiquitin ligase transfers ubiquitin to a lysine residue of the substrate. Multiple cycles result in polyubiquitinated substrate that is transported to the proteasome and degraded. For details see Glickman and Chiechanover (2002).

During the last years, the proteasome has been found to be a highly dynamic, heterogeneous complex whose abundance is responsive to cell needs and stress conditions. Its subunits are subject to a variety of posttranslational modifications and the subunit composition alters. One example are the "immunoproteasomes" that are formed during the immune response. Upon interferon-γ treatment, three specific proteasomal subunits are upregulated. They replace three components of the 20S proteasome, resulting in different peptide cleavage specificity. The resulting peptides are better bound to the major histocompatibility complex (MHC) class I molecules, which leads to an enhanced antigen presentation. Several reviews deliver insight into this field (Bochtler *et al.*, 1999; DeMartino and Gillette, 2007; Glickman and Maytal, 2002; Glickman and Adir, 2004; Glickman and Raveh, 2005).

1.1.3 Ubiquitin ligases

The question how the ubiquitin system achieves its high specificity and selectivity is the focus of ongoing research. It appears that the ubiquitin ligases are the main factors for specificity. The ubiquitin conjugation machinery displays a hierarchical structure. The single E1 enzyme can transfer the activated ubiquitin onto one of several E2s. Each E2 in

turn transfers the ubiquitin to one of several E3s that can on their part modify a multitude of proteins. Thus, E3 ubiquitin ligases are the key factors in substrate recognition. Ubiquitin ligases are proteins or protein complexes that bind both the E2 ubiquitin conjugating enzyme and the substrate. The group of E3s is heterogeneous including, beside several minor groups, two major groups, the HECT domain- and RING finger-containing E3s.

The HECT (homologous to E6-associated protein C-terminus) domain proteins are characterized by a 350 aa sequence homologous to the C-terminal domain of the first enzyme described in this family, E6-AP (E6-associated protein). This E3 ligase mediates rapid degradation of the target protein p53 in the presence of the HPV oncoprotein E6. The activated ubiquitin is transferred from the E2 to a conserved Cys residue in the HECT domain while the NH_2-terminal domain is probably involved in substrate recognition.

The RING finger-containing E3s do not bind the ubiquitin itself but mediate the transfer of the activated ubiquitin from the E2 to the substrate. Characteristic for this group is a pattern of conserved Cys and His residues: $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$. Dependent on whether a Cys or His occupies the fifth coordination site, the domains are classified as RING-HC or RING-H2. Some of the ubiquitin ligases are single subunit ubiquitin ligases, such as Mdm2, containing the RING finger and the substrate-binding site in the same molecule, while the majority of the E3s are multisubunit complexes. Well-investigated representatives are the anaphase promoting complex (APC) and the cullin RING finger ubiquitin ligases (CRL). The CRLs are classified according to the cullin they contain, the core component of the complexes (Tab. 2). Cullins are elongated proteins that consist of a long stalk and a globular domain, serving as a rigid scaffold.

Among the CRLs are the well-investigated Skp1-Cullin/Cdc53-F-box protein (SCF) - RING-H2 finger complexes, containing Cul1 (Fig. 3). Their target proteins include mainly signal- and cell cycle induced proteins that become a target when phosphorylated. Beside their function, also the structure of the four subunit SCF is known (Zheng *et al.*, 2002b). The RING finger protein Rbx1/Roc1/Hrt1 binds to the C-terminal globular domain of Cul1, forming the binding site for E2 ubiquitin conjugating enzymes. The substrate recognition complex consists of the Skp1 protein, mediating binding to the N-terminal stalk of Cul1 and one of several F-box proteins.

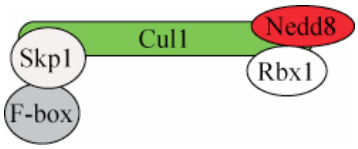


Fig. 3: Scheme of the SCF complex of higher eukaryotes.

The core component cullin (Cul1) can be Nedd8-modified (Nedd8) and binds the RING finger protein (Rbx1) on its C-terminal globular domain. The adaptor protein (Skp1) binds to the N-terminal stalk. The F-box protein (F-box) in turn binds to Skp1 via its F-box domain while the substrate specific binding site of the protein mediates the binding of the substrate to the complex. For a more detailed description see text.

Tab. 2: Human cullin-RING-ligase family.

Cullin*	putative homolog in *A. nidulans* (Busch *et al.*, 2007)	Subunit organization	Names
CUL1 (Cdc53)	CulA (AN1019.3)	F-box protein/SKP1/CUL1/RING	SCF, CDL1
CUL2		SOCS/BC-box protein/elongin BC/CUL2/RING	VBC, CBC, ECS, SCF2, CDL2
CUL3 (Cul3)	CulC (AN3939.3)	BTB-domain protein/CUL3/RING	BCR3, SCF3, CDL3
CUL4A	CulD (AN10008.3)	Receptor/DDB1/CUL4A/RING	VDC, DCX, SCF4, CDL4
CUL4B		?/CUL4B/RING	None
CUL5		SOCS/BC-box protein/elonginBC/CUL5/RING	SCF5, CDL5
CUL7		FBX29/SKP1/CUL7/RING	SCF7, CDL7

*putative homologs in *S. cerevisiae* are shown in brackets, Cdc53, cell division cycle 53 protein; BCR, `BTB protein, CUL3, RING´; BTB, `Broad complex, Tramtrack, Bric-a-brac´; CBC, `cullin, elongin BC´; CDL, cullin-dependent ligase; CUL, cullin; DDB1, DNA-damage-binding protein-1; DCX, `DDB1, CUL4A, X (for unknown) box´; ECS, `elongin BC, CUL2, SOCS/BC box´; FBX29, F-box protein-29; SCF, `SKP1, CUL1, F-box protein´; SCF2-5 and SCF7, SCF-like complexes-2-5 and -7; SOCS/BC, suppressor of cytokine signaling/elongin BC; VBC, `von Hippel-Lindau (VHL), elongin BC´; VDC, V-dependent complex of virus-degradation complex or `V-DDB1-CUL4A´. Modified from Petroski and Deshaies (2005).

The F-box protein binds to Skp1 by an N-terminal approximately 60 aa long F-box motif and is responsible for substrate recognition and binding via its protein-protein interaction motif. The potential number of SCFs is highly variable depending on the number of F-box proteins. To distinguish between the different SCF-complexes, the name of the F-box protein is added to the name of the ubiquitin ligase in superscript. The human genome encodes for about 68 F-box proteins (Jin *et al.*, 2004) and approximately 700 F-box proteins have been identified in the genome of *A. thaliana* (Gagne *et al.*, 2002; reviewed by Glickman and Ciechanover, 2002).

1.2 Regulation of ubiquitin ligase activity

1.2.1 Phosphorylation and F-box protein degradation

A variety of ubiquitin ligases target a multitude of proteins for degradation. The number of target proteins identified increases continuously, including transcriptional regulators, cyclins, cyclin dependent kinase inhibitors and tumor suppressors. To ensure specificity, the activity of the ubiquitin ligases towards its substrates is regulated on different levels. Several substrates are phosphorylated, which is a well-known regulatory mechanism, changing their affinity to the ubiquitin ligase. Examples are the cyclin-dependent kinase (CDK) inhibitor Sic1 (Nash *et al.*, 2001) and the central transcription activator Gcn4 of *S. cerevisiae* that becomes a substrate of the ubiquitin ligase SCF^{Cdc4} after phosphorylation by the nuclear Pho85p cyclin-dependent protein kinase (Meimoun *et al.*, 2000). It is suggested that the kinase is inactivated upon amino acid starvation by the dissociation of the Pho85p/Pcl5p complex (Bömeke *et al.*, 2006). In humans the CDK inhibitor p27(Kip1) is targeted to SCF^{Skp2} upon phosphorylation (reviewed by Kaldis, 2007).
Another regulatory mechanism is the alteration of F-box protein stability by cellular localization. In budding and fission yeast the half-life of F-box proteins Cdc4, Met30 and Grr1 is about 5-30 minutes after assembly with Skp1 (Galan and Peter, 1999; Zhou and Howley, 1998).

1.2.2 Nedd8 and its role in posttranslational modification of cullins

The 81 aa Nedd8/Rub1 (neural precursor cell expressed developmentally downregulated gene 8)/(ubiquitin-related protein) protein, first discovered in the mouse, shows approximately 60% identity to ubiquitin being the highest conserved ubiquitin-like protein investigated throughout all eukaryotes (Kumar *et al.*, 1993; Welchman *et al.*, 2005) (Fig. 4). Nedd8 was supposed to play a role in cell-cycle progression, because it is expressed in proliferating cells and is down-regulated upon cellular differentiation (Podust *et al.*, 2000). Nedd8/Rub1 was later found to be conjugated as a single protein to the cullin components of ubiquitin ligases, first discovered for CDC53p in *S. cerevisiae* (Lammer *et al.*, 1998). Like ubiquitin, Nedd8 is synthesized in a precursor form.

Fig. 4: Alignment of eukaryotic Nedd8/Rub1 sequences.

Accession numbers of the corresponding Nedd8/Rub1 sequences are: *H. sapiens* (hs, NP_006147); *A. thaliana* (at, NP_564379, annotated sequence was shortened N-terminal for 77 aa); *D. melanogaster* (dm, NP_609919); *S. pombe* (sp, NP_595955); *S. cerevisiae* (sc, NP_010423); *N. crassa* (nc, XP_330745); *A. nidulans* (an, XM_658691); *A. fumigatus* (af, XM_750058); *A. niger* (ani, XM_001390617). Amino acid identities to human Nedd8 are given in brackets. Arrows indicate conserved residues potentially involved in chain formation (K) and conjugation (G). Residues with consensus value ≥ 90% are indicated by black boxes.

To open access to the active glycine residue, the amino acids following this residue are removed by the hydrolases UCH-L3 and Den1 in mammals (Gan-Erdene *et al.*, 2003; Wada *et al.*, 1998; Wu *et al.*, 2003) or the UCH-L3 homolog Yuh1 in *S. cerevisiae* (Linghu *et al.*, 2002).

An enzyme cascade of E1 and E2 analogous to the ubiquitin conjugation system performs conjugation of Nedd8/Rub1 to the target protein in a process called neddylation (Gong and Yeh, 1999; Liakopoulos *et al.*, 1998). The proteins needed for activation of Nedd8/Rub1 are Ula1 (APP-BP1 in mammals) and UBA3, related to the N- and C-terminal domains of E1 ubiquitin activating enzyme. The Nedd8 conjugating enzyme UBC12 is related to E2 ubiquitin conjugating enzyme while the proteins DCN-1/Dcn1p of *C. elegans* and *S. cerevisiae* have been identified as putative Nedd8 E3 ligases (Kurz *et al.*, 2005; Yang *et al.*, 2007). They catalyze the formation of an isopeptide bond, linking the carboxyl-end of NEDD8, Gly-76 to the ε-amino group of a conserved Lys residue of the cullin molecule. Nedd8 modification of cullins in a cycle of neddylation and deneddylation is thought essential for activity and correct function of all cullin containing ubiquitin ligases (see 1.2.6). Posttranslational Nedd8 modification of cullins has been shown first by Hori *et al.* (1999) but the exact function of cullin modification with Nedd8 has been revealed only recently for the Cul1 containing SCF complex. Nedd8 forms an active platform on the SCF complex together with Rbx1. This increases ubiquitin ligase activity because the ubiquitin-charged E2s bind selectively to this area while Nedd8 E2s do not (Sakata *et al.*, 2007).

The observation that neddylation promotes activity of SCF *in vitro* but diminished deneddylase function inhibits the turnover of SCF targets *in vivo* lead to a model of

cyclical attachment of Nedd8 to cullins (Lyapina *et al.*, 2001). Consistently with this cycling model, also deneddylation of cullins is crucial for ligase activity and only a part of the total cullin pool exists in the neddylated, active form. Until now only two enzymes, the COP9 signalosome (CSN) and a protein called Den1, are known to catalyze this deneddylation process specifically (see 1.2.3 and 1.2.4). Nedd8 is a crucial regulator of all cullin containing ubiquitin ligases and hence the deletion of the corresponding gene leads to lethality of all organisms investigated from *S. pombe* to mice (Osaka *et al.*, 2000; Tateishi *et al.*, 2001) with *S. cerevisiae* as the only exception (Lammer *et al.*, 1998). Cullins were the only known Nedd8 modified proteins for a long time. Recently, new studies identified p53 tumor suppressor as an additional Nedd8 target. p53 is neddylated by the RING finger ligase Mdm2, inhibiting its transcriptional activity (Xirodimas *et al.*, 2004). Thereby, the F-box protein FBXO11 acts as an adaptor protein, mediating neddylation of the non-cullin substrate (Abida *et al.*, 2007). These findings suggest that there might be a more global role for neddylation and a great diversity of E3 ligases as regulators of protein function.

1.2.3 The COP9 signalosome

The COP9 signalosome (CSN) of higher eukaryotes is a multiprotein complex consisting of eight subunits. Initially the CSN has been described in *Arabidopsis thaliana* as a signaling complex mediating light control of development (Wei *et al.*, 1994). The complex was named after the mutant gene *cop9* that has been discovered in a screen for mutants displaying a constitutive photomorphogenic (cop) phenotype (Wei and Deng, 1992). The *cop* mutants together with the *fusca* (latin word referring to a dark purple color) (Misera *et al.*, 1994) and the *det* (*de-etiolated*) mutants (Chory *et al.*, 1989; Chory *et al.*, 1991) found in other screens, are now known as the pleiotropic *cop*/*det*/*fus* mutants displaying a light grown phenotype when grown in the dark (Quail, 1994; Schwechheimer and Deng, 2001; Wei and Deng, 1999). Six of the *cop*/*det*/*fus* loci encode subunits of the COP9 signalosome (Wei and Deng, 2003). The mammalian CSN, also called JAB1- containing signalosome was isolated independently by two groups, both as a human ortholog of the *Arabidopsis thaliana* COP9 signalosome and as a co-purifying byproduct of the 26S proteasome (Seeger *et al.*, 1998; Wei and Deng, 1998; Wei *et al.*, 1998). The independent discovery of

CSN subunits in different organisms led to several historical gene names for the CSN subunits. For this work the unified nomenclature of the COP9 signalosome subunits (Deng *et al.*, 2000) is used. The original and unified nomenclature is summarized in Tab. 3.

Tab. 3: Unified and original nomenclature of the COP9 signalosome proteins.

unified	*H. sapiens*[a]	*M. musculus*[b]	*D. melanogaster*[c]	*A. thaliana*[d]	*A. nidulans*[e]	*N. crassa*[f]	*S. pombe*[g]	*S. cerevisiae*[h]
CSN1	Sgn1, GPS1	COPS1, Mfh	DCH1	COP11, FUS6	CsnA	CSN-1	Caa1, Sgn1[sp]	PCI domain proteins: Csn9p Csn10p/Rri2p Csn11p/Pci8p Csn12p
CSN2	Sgn2, TRIP15, hAlien	COPS2	DCH2, Alien	subunit 2	CsnB	CSN-2	Sgn2 [sp]	
CSN3	Sgn3	COPS3	DCH3	subunit 3	CsnC	CSN-3	Csn3	
CSN4	Sgn4	COPS4	DCH4	COP8, FUS4	CsnD	CSN-4	Sgn4 [sp]	
CSN5	Sgn5, JAB1	COPS5, Jab1	DCH5	AJH1, AJH2	CsnE	CSN-5	subunit 5	MPN domain protein: Rri1p (Csn5)
CSN6	Sgn6, HVIP	COPS6	DCH6	subunit 6	CsnF	CSN-6	-	
CSN7	Sgn7	COPS7a, COPS7b	DCH7	FUS5	CsnG	CSN-7	subunit 7	
CSN8	Sgn8, hCOP9	COPS8	DCH8	COP9	CsnH	-	-	

[a] (Chamovitz and Deng, 1995; Claret *et al.*, 1996; Lee *et al.*, 1995; Mahalingam *et al.*, 1998; Seeger *et al.*, 1998; Spain *et al.*, 1996); [b] (Schaefer *et al.*, 1999; Tomoda *et al.*, 1999; Wei *et al.*, 1998); [c] (Freilich *et al.*, 1999; Goubeaud *et al.*, 1996); [d] (Castle and Meinke, 1994; Chamovitz *et al.*, 1996; Karniol *et al.*, 1998; Kwok *et al.*, 1998; Serino *et al.*, 1999; Wei *et al.*, 1994); [e] (Busch *et al.*, 2007) [f] (He *et al.*, 2005) [g] (Mundt *et al.*, 1999), Genebank; [h] (Maytal-Kivity *et al.*, 2003). Modified from: http://www.tau.ac.il/~chamd/csn.html.

1.2.3.1 COP9 signalosome conservation and architecture

The COP9 signalosome belongs to the PCI family of multiprotein complexes consisting of the regulatory lid of the 26S proteasome, the COP9 signalosome and the translation initiation factor eIF3. Subunits of these complexes contain either a PCI or a Mpr1- Pad1 N-terminal (MPN) domain. While the PCI domain is characterized by a conserved α-helical secondary structure of about 200 aa and presumably stabilizes protein-protein interactions in the complex, the MPN domain comprises about 140 aa and forms an α/β fold (Aravind and Ponting, 1998; Glickman *et al.*, 1998; Hofmann and Bucher, 1998). The CSN and the lid, both containing six PCI and two MPN domain proteins appear to share a common evolutionary origin while the eIF3 seems to be a degraded copy of an ancient lid-like complex (Kim *et al.*, 2001; Scheel and Hofmann, 2005) (Tab. 4).

The architecture of the COP9 signalosome subunits in the complex has been described in a model created on the basis of electron microscopy and subunit interaction studies (Freilich *et al.*, 1999; Fu *et al.*, 2001; Kapelari *et al.*, 2000; Tsuge *et al.*, 2001) (Fig. 5). Phosphorylation of subunits CSN2 and CSN7, presumably by the associated kinases (see 1.2.3.2), may be responsible for conformational changes of the complex (Kapelari *et al.*, 2000). In addition to the eight subunit CSN of approximately 450 kDa, small 100 kDa subcomplexes containing CSN4-CSN8 can be found in the cytoplasm of mammalian cells (Tomoda *et al.*, 2002; Tomoda *et al.*, 2005).

Tab. 4: Relationship of CSN, proteasome and eIF3 subunits.

	similar to		
COP9 signalosome	proteasome lid	eIF3[a]	domains
Csn1	Rpn7	eIF3a (p170) eIF3c (p110) eIF3e (p48) eIF3l (p69)	PCI
Csn2	Rpn6		PCI
Csn3	Rpn3		PCI
Csn4	Rpn5		PCI
Csn7	Rpn9		PCI
Csn5	Rpn11	-	MPN+
Csn6	Rpn8	eIF3f (p47) eIF3h (p40)	MPN
Csn8	Rpn12	eIF3k	PCI

[a]general groupings are shown. Modified from Glickman *et al.* (1998); Hofmann and Bucher (1998); Scheel and Hofmann (2005).

1.2.3.2 CSN associated proteins, deneddylation, phosphorylation and deubiquitination

The CSN regulates ubiquitin-dependent protein degradation on various levels and CSN subunits interact with a multitude of other non-CSN subunit proteins (Fig. 5) (reviewed by Wei and Deng, 2003). It has been reported to build large supercomplexes with the LID of the proteasome and cullin-RING E3 ligases (Huang *et al.*, 2005; Peng *et al.*, 2003). The existence of these complexes has been supported by the ability of the proteasomal subunit AtS9/RPN6 of plants and mammals to directly interact with CSN3 (Kwok *et al.*, 1999; Seeger *et al.*, 1998) The only known intrinsic enzyme activity of CSN is a deneddylase activity. It is located in subunit 5 (Csn5), the only subunit of the COP9 signalosome conserved in all eukaryotes. Csn5 contains a MPN+ domain conserved in archaea, bacteria and eukaryotes containing a His-X-His-X_{10}-Asp motif accompanied by an upstream conserved Glu, termed the JAMM motif (Jab1/MPN domain metalloenzyme). This motif confers the metalloprotease (deneddylation) activity (Cope *et al.*, 2002; Maytal-Kivity *et al.*, 2002). CSN is able to deneddylate cullins, first described for Cul1 (Lyapina *et al.*, 2001; Zhou *et al.*, 2001). By deneddylating the cullins, CSN influences E3 activity. To be active as a deneddylase, CSN5 needs to be incorporated into the complex like its relative Rpn11 of the proteasomal lid (Cope *et al.*, 2002; Lundgren *et al.*, 2003; Maytal-Kivity *et al.*, 2002; Verma *et al.*, 2002). Binding of CSN to the ubiquitin ligase SCF is mediated both by interaction of CSN2 with the SCF core component Cul1 and by interaction of CSN6 with the RING-domain Rbx1 (Lyapina *et al.*, 2001; Yang *et al.*, 2002). Besides the intrinsic deneddylation activity, CSN also has associated enzyme activities. CSN counteracts the ubiquitination activity of ubiquitin ligases by recruiting the deubiquitination enzyme Ubp12 (Zhou *et al.*, 2003). Thereby CSN deneddylation and Ubp12 deubiquitination activities work together in counteracting autocatalytic instability of cullin containing RING ubiquitin ligase specific adaptors (Wee *et al.*, 2005).

The second CSN-associated enzyme activity is a kinase activity. CSN mediates phosphorylation of substrates of the protein degradation machinery. It associates with inositol 1,3,4-trisphosphate 5/6-kinase that interacts with CSN1, protein kinase CK2 (CK2) and protein kinase D (PKD) (Sun *et al.*, 2002; Uhle *et al.*, 2003). The CSN associated kinases phosphorylate proteins with binding capacity to CSN5, such as the tumor

suppressor p53 (Bech-Otschir *et al.*, 2001) and the mammalian transcription factor c-Jun (Claret *et al.*, 1996; Naumann *et al.*, 1999), changing their susceptibility to ubiquitination. CSN5 interacts with the cyclin-dependent-kinase (CDK) inhibitory protein $p27^{Kip1}$, whose proteolysis triggers entry into S phase (Amati and Vlach, 1999; Tomoda *et al.*, 1999), and the hypoxia-inducible factor-1, HIF-1α (Bae *et al.*, 2002) (Fig. 5). The overall functions of the CSN are not yet fully understood. Two hypotheses of CSN function are discussed. The first one points to the overall structure similarity that CSN shares with the 19S lid sub-complex and competition with it for binding to the 26S proteasome, suggesting that CSN might act as an additional or alternative proteasome lid (Huang *et al.*, 2005; Wei and Deng, 1999). The second hypothesis interprets CSN as a scaffold protein acting as a platform for a multitude of proteins connecting signaling with proteolysis (Bech-Otschir *et al.*, 2002).

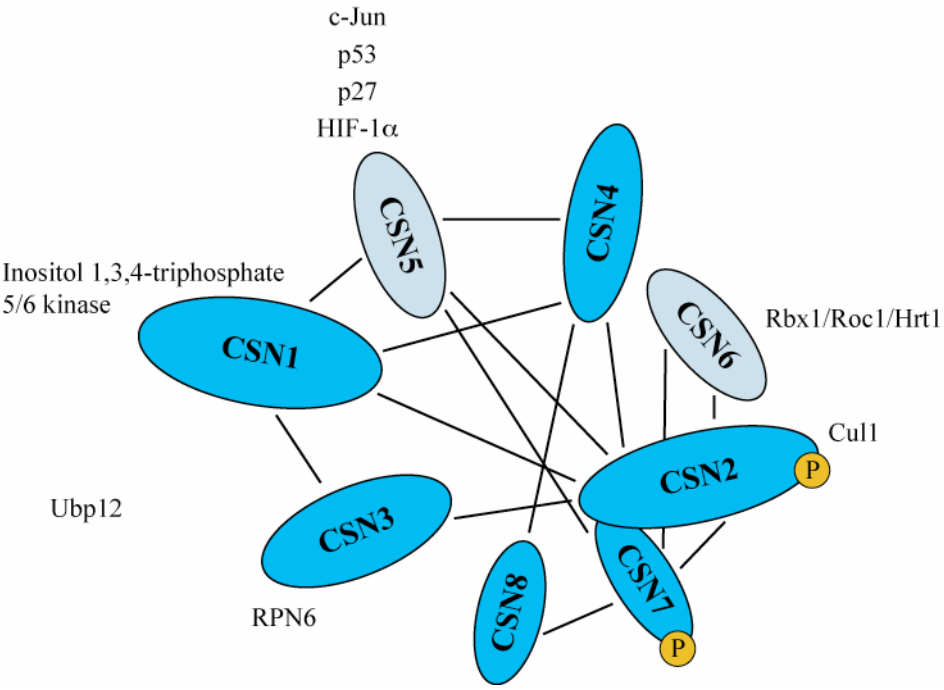


Fig. 5: Proposed COP9 signalosome architecture with selected interacting proteins.
Phosphorylated COP9 signalosome subunits are indicated (P). Modified from Kapelari *et al.* (2000).

1.2.3.3 Physiological role of CSN

CSN is directly involved in the regulation of and interacts with multiple cellular regulatory factors like transcription factors, cell cycle regulators, hormone receptors and tumor suppressors (reviewed by Wei and Deng, 2003). Thereby CSN has a strong influence on several cellular and developmental processes including cell cycle and checkpoint control, hormone signaling and MAPK kinase signaling as was shown in several organisms including mammals, insects, plants and fungi (Doronkin *et al.*, 2003; Harari-Steinberg and Chamovitz, 2004; Liu *et al.*, 2003; Mundt *et al.*, 1999). In higher eukaryotes like plants and animals, CSN is an essential regulator of development, influencing cellular homeostasis, growth and differentiation. Defects of CSN or deletion of single subunits result in a pleiotropic mutant phenotype leading to embryonic lethality (Freilich *et al.*, 1999; Lykke-Andersen *et al.*, 2003; Serino *et al.*, 2003; Tomoda *et al.*, 2004; Wei and Deng, 1999; Yan *et al.*, 2003).

In contrast, in lower eukaryotes the composition of CSN is less conserved. In budding yeast, Csn5 and four additional proteins containing PCI domains mediate control of cullin neddylation, suggesting the existence of a COP9 signalosome-like complex (Wee *et al.*, 2002). In the fission yeast *Schizosaccharomyces pombe* only six subunits are conserved. CSN1 and CSN2 are essential for cell cycle progression in S-phase, *csn1* and *csn2* mutants show slow S-phase progression and a modest sensitivity to DNA damaging agents, while deletions of *csn3*, *csn4* and *csn5* do not display a mutant phenotype. Nevertheless, all *S. pombe csn* mutants accumulate the cullin Pcu3/Cul3 in a neddylated state (Mundt *et al.*, 1999; Mundt *et al.*, 2002; Zhou *et al.*, 2001). The filamentous fungus *Neurospora crassa* has a seven subunit CSN and disruption of subunit 2 leads to a defect in circadian clock regulation (He *et al.*, 2005). The first complete, eight subunit CSN of the kingdom of fungi has recently been identified and isolated in *Aspergillus nidulans*, where deletion of subunits leads to the accumulation of secondary metabolites and developmental defects (Busch *et al.*, 2007).

1.2.3.4 Influence of CSN on DNA damage response and cell cycle progression in *Schizosaccharomyces pombe*

DNA replication and repair is influenced by the COP9 signalosome via indirect activation of ribonucleotide reductase (RNR). RNR is a key enzyme responsible for the biosynthesis of deoxyribonucleotide triphosphates (dNTPs). Two small subunits (Suc22 in *S. pombe*) and two large Cdc22 subunits assemble to form the four subunit active complex. During growth phase, Suc22 is anchored in the nucleus by Spd1, while Cdc22 resides in the cytoplasm. In S phase and in response to checkpoint activation, ubiquitin dependent degradation of Spd1 (S-phase delayed) allows export of Suc22 into the cytoplasm and thereby activation of the RNR, providing nucleotides for DNA replication and repair. CSN promotes degradation of Spd1 by regulating the putative Pcu4/Cul4-containing E3 ubiquitin ligase, which at least partially explains the S-phase delay observed in the *csn1* and *csn2* mutants of *S. pombe* (Mundt *et al.*, 1999). Deletion of the genes encoding for Csn1 or Ddb1, a component of a putative complex related to the human Cul4-containing ubiquitin ligase DCX, leads to Spd1 accumulation, preventing nuclear export of Suc22 and activation of RNR. When *S. pombe* cells differentiate into meiosis Ddb1/Csn1/Cul4-mediated Spd1 degradation becomes essential (Bondar *et al.*, 2004; Holmberg *et al.*, 2005; Liu *et al.*, 2003; reviewed by Nielsen, 2003; Schwechheimer, 2004).

1.2.4 DEN1 deneddylase

The human Deneddylase, Den1/NEDP1, belongs to the family of Ubl-specific proteases (ULPs), responsible for processing ubiquitin-like proteins (Ubls). Because of its homology to the Ulp1/SENP cysteinyl SUMO-deconjugating enzyme family, it was first annotated as SENP8. The 212 aa protein contains the signature motif of cysteine proteases, consisting of the highly conserved catalytic triad His (H), Asp (D) and Cys (C) in addition to an invariant glutamine (Q) residue (Wilkinson and Hochstrasser, 1998). The Nedd8 specific protease processes hyper-neddylated cullin and is able to deconjugate Nedd8 from cullins. In addition, it efficiently processes the C-terminus of Nedd8 to expose the diglycine motif needed for the Nedd8-substrate binding (Gan-Erdene *et al.*, 2003; Mendoza *et al.*, 2003; Wu *et al.*, 2003). The ability of human Den1 and its homologues in other species to discriminate between Nedd8 and ubiquitin is mediated by a seven amino acid loop

(residues 93-99) that swings over the C-terminus of Nedd8 when the two proteins bind to each other. Thereby, the alanine at position 72 in Nedd8, which is an arginine at the corresponding position in ubiquitin, is crucial for the recognition by Den1 (Shen *et al.*, 2005). Until now, Den1 and the COP9 signalosome are the only enzymes known specific for Nedd8 deconjugation. In budding yeast, no direct homolog of Den1 is known but the genome encodes for one member of the ubiquitin C-terminal hydrolase (UCH) family, the protein Yuh1p. It processes both ubiquitin and Rub1 and yeast strains deleted for Yuh1 fail to neddylate/rubbylate the yeast cullin Cdc53 (Linghu *et al.*, 2002).

1.2.5 The cullin-binding protein Cand1

Another factor, which has been connected to cullin containing ubiquitin ligases, is the protein CAND1 (cullin-associated Nedd8-dissociated protein 1). It was first discovered as TIP120A (TBP (TATA binding protein) interacting protein) in a screen for TBP binding proteins, associating with TBP in nuclear extracts of mammalian cells (Yogosawa *et al.*, 1996). Makino *et al.* (1999) reported that TIP120A facilitates the binding of RNA polymerase II to DNA *in vitro*, activates the basal level of RNA polymerase II transcription and stimulates RNA polymerase I- and III driven transcription in a mouse cell line although it is not stably incorporated into a TBP-DNA complex. Later the ability of TIP120A to associate specifically with unneddylated cullins was discovered and TIP120A was renamed Cand1 (Zheng *et al.*, 2002a). Cand1 is encoded by a single gene in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana* and less conserved in *Schizosaccharomyces pombe* but has not been found in budding yeast.

1.2.5.1 Cand1 localization and interaction with cullins

Several studies in plants and mammalian cells have shown that Cand1 forms a complex with Cul1 and Rbx1/Roc1, while it does not coexist in a complex with neddylated cullins, Skp1 or any F-box proteins (Chuang *et al.*, 2004; Feng *et al.*, 2004; Hwang *et al.*, 2003; Liu *et al.*, 2002; Min *et al.*, 2003; Oshikawa *et al.*, 2003; Zheng *et al.*, 2002a). Consistent with that, TIP120A/Cand1 and Cul1 co-localize and are mainly in the nucleus in mammalian cells (Oshikawa *et al.*, 2003; Yogosawa *et al.*, 1996). Cand1 contains 27

HEAT (huntingtin-elongation-A subunit-TOR) repeats that are tandemly arranged, bihelical structures (Goldenberg *et al.*, 2004). The crystal structure of the protein in a complex with Cul1 and Rbx1 revealed that Cand1 binding blocks the neddylation site and the Skp1 binding site on Cul1, impeding assembly of the SCF complex (Fig. 6). Skp1 binding to Cul1 is significantly spatially impeded by a 25 Å long, highly conserved hsCand1 β-hairpin at the C-terminus of the protein. It consists of two antiparallel β-strands connected by a four amino acid β-turn (M1068, Gly1069, Pro1070, and Phe1071). It makes, in addition to Val1066 and His1073 van der Waals contacts with Cul1 amino acids. At the C-terminus of Cul1, the neddylation site Lys720 forms a tridentate interaction with Asp19 and Asp21 of the first HEAT repeat of Cand1 and Cul1 Tyr776. This way Cand1 mostly buries Cul1 Lys720 so that neddylation is impeded (Goldenberg *et al.*, 2004). Beside association with Cul1, Cand1 also interacts with all other mammalian cullins tested (Cul1, Cul2, Cul3, Cul4a, Cul4b and Cul5) (Liu *et al.*, 2002; Min *et al.*, 2003; Zheng *et al.*, 2002a).

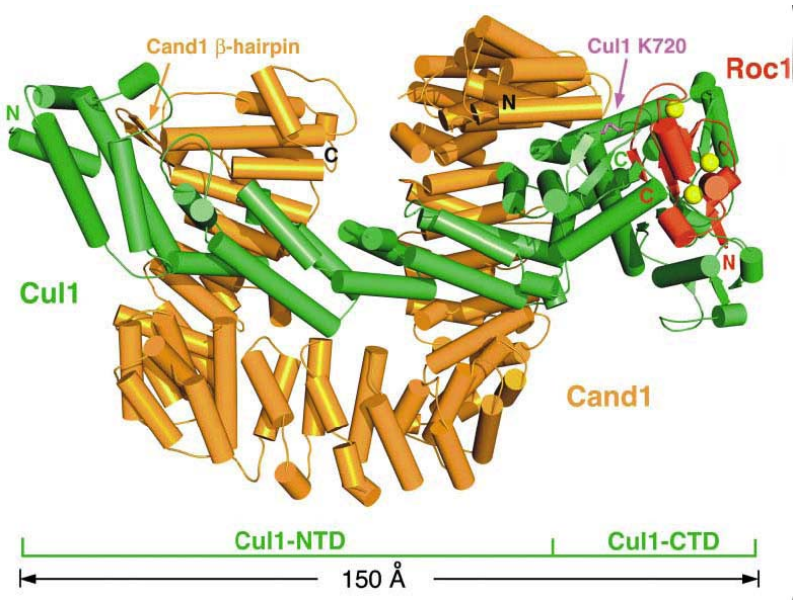


Fig. 6: Crystal structure of Cand1 in complex with Cul1 and Rbx1/Roc1 (Goldenberg *et al.*, 2004).

N-terminal (N) and C-terminal (C) ends of the proteins as well as Cand1 β-hairpin and the part blocking the neddylation side on cullin are indicated.

Deletion of the codons for 53 residues from the Cul1 N-terminus abolishes binding with Skp1 and Cand1. In addition Cul1 shortened for 31 or 22 residues at the C-terminus is not able to bind to Cand1. Deletion of 100 residues at the N-terminus of CUL4A disrupts its association with CAND1. Additionally, the interaction of Cand1 with Cul1 is weakened when the neddylation site on Cul1 is mutated (K720A) (K720R) (Liu *et al.*, 2002; Zheng *et al.*, 2002a). On the contrary, investigation of the *Arabidopsis thaliana* Cand1-cullin association in a yeast two-hybrid test revealed an even stronger interaction of atCul1 with atCand1 when the Cul1 lysine was substituted for arginine (K682R), probably because Cul1 is indeed modified by Nedd8/Rub1 in yeast (Feng *et al.*, 2004).

1.2.5.2 Effect of Cand1 on protein levels of E3 targets

As an effector of ubiquitin ligase activity, Cand1 has influence on protein levels of several regulatory proteins. In mammalian cells, siRNA mediated silencing of Cand1 leads to stabilization of p27 (Zheng *et al.*, 2002a) and Cand1 inhibits IκBα ubiquitination *in vitro* (Liu *et al.*, 2002; Min *et al.*, 2003). Similar effects on proteins regulated by the ubiquitination system can be observed in *A. thaliana*. The protein Hy5 is stabilized in *cand1* mutants and there is a higher level of gibberellic acid (GA) pathway repressor ga1-3 (RGA), usually degraded by SCF^{SLY1} in the wild-type (Feng *et al.*, 2004). The deletion phenotype of *A. thaliana* Cand1 mutants was only partially rescued by the Cand1 transgene from *A. thaliana* (Feng *et al.*, 2004) and in mammalian cells both ectopic overexpression and siRNA-mediated knockdown of Cand1 decreased the ability of the BTB-Kelch protein Keap1, part of Cul3 containing ubiquitin ligases, to target the transcription factor Nrf2 for ubiquitin-dependent degradation. This resulted in stabilization of Nrf2 and activation of Nrf2-dependent gene expression (Lo and Hannink, 2006) suggesting that for efficient regulation of ubiquitin ligases most likely an appropriate level of Cand1 is required.

1.2.5.3 Physiological role of Cand1

Most studies about Cand1 have been performed *in vitro* or in mammalian cells. Expression patterns of Cand1 have been analyzed in mice. TIP120A/Cand1 was expressed in all samples of development stages of mouse embryos from 7 to 17 days and transcripts were expressed in heart, liver, brain, skeletal muscle, and slightly in the spleen and lung (Aoki *et al.*, 1999; Yogosawa *et al.*, 1999). Upon retinoic acid (RA) treatment, which induces differentiation, TIP120A expression was elevated and TIP120A overexpression resulted in the growth arrest of mammalian cells. This suggests that TIP120A/Cand1 is at least temporally required for differentiation in mammalian cells, presumably for arresting cell growth and promoting cell differentiation (Yogosawa *et al.*, 1999). In plants, atCAND1 is ubiquitously expressed and can already be detected in young seedlings (Cheng *et al.*, 2004; Chuang *et al.*, 2004).

The first *cand1* mutants have been described for *A. thaliana* and named *Atcand1*, *eta2* and *cand1* respectively by three different groups (Cheng *et al.*, 2004; Chuang *et al.*, 2004; Feng *et al.*, 2004). These mutants are collectively referred to as *cand1* mutants below. All plants with an apparent *cand1* null allele or a mutated *cand1* are viable. Mutations do not have an effect on germination and seedling stage growth and the plants develop all organs correctly. However, the plants develop much smaller rosette leaves with a wavy morphology. The plants flower later than the wild-type with an increased number of rosette leaves, indicating that the vegetative to reproductive growth transition of the primary shoot apical meristem is affected (Feng *et al.*, 2004). Mutant plants also show a severe defect in fertility. Producing less than one seed on average per silique, the mutant strains are almost completely sterile (Cheng *et al.*, 2004; Chuang *et al.*, 2004; Feng *et al.*, 2004). Dominant mutations affecting genes for components of the Nedd8/RUB-conjugation or deconjugation pathway of Cul1/AXR6 (Hellmann *et al.*, 2003) all result in decreased SCF^{TIR1} activity and a dramatic reduction in auxin response (reviewed by Schwechheimer and Deng, 2001). Actually, also in Cand1 loss of function mutants the auxin response is reduced while the ETA2/Cand1 protein levels are not affected by mutations in the gene encoding for the F-box protein TIR1 or ASK1/Skp1. Furthermore *cand1* transcription is not regulated by auxin. Like *csn* mutants, *cand1* mutants are highly anthocyanic and although the defect is weaker than in the *csn* mutants, they display a partial constitutive

photomorphogenic phenotype. Interestingly, double mutants of *cand1* with the gene encoding for the Ring finger protein COP1 or the E2 ubiquitin-conjugating enzyme COP10 show synergistic effects; the HY5 level in *cand1*/*cop1-6* mutants is higher than in their parental single mutants. In addition, *cand1*/*tir1-1* seedlings were significantly more resistant to auxin than either single mutant line, suggesting that ETA1 and TIR1 interact synergistically (Cheng *et al.*, 2004; Chuang *et al.*, 2004; Feng *et al.*, 2004).

1.2.6 Assembly and disassembly of the ubiquitin ligase SCF^{Skp2}

Recently, a model of how assembly and disassembly of the SCF complex is regulated has been established (Bornstein *et al.*, 2006) (Fig. 7). *In vitro* experiments showed that Skp1 is able to dissociate Cand1 from Cul1 and vice versa. This is also true for the BTB-Kelch protein Keap1 that functions as a substrate adaptor protein for the Cul3-dependent E3 ubiquitin ligase complex. Association of Keap1 with Cul3 was decreased by ectopic expression of Cand1 and was increased by small interfering RNA-mediated knockdown of Cand1 (Lo and Hannink, 2006). The assembled complex is probably protected from deneddylation and degradation to a certain extent by the F-box proteins. This is supported by the observation that F-box protein Skp2 in a complex with Skp1 impedes deneddylation of cullins by the CSN, while Skp1 alone does not have this effect. In the absence of an appropriate F-box protein, CSN can deneddylate the cullin, thereby initiating disassembly. Because binding of CSN and Cand1 to Cul1 seem to be mutually exclusive, CSN has to leave the complex before Cand1 can bind (Bornstein *et al.*, 2006; Min *et al.*, 2005). Accordingly, Cand1 presumably has only an indirect influence on the ratio of neddylated to deneddylated cullins. And indeed, a reduction or loss of Cand1 by either siRNA in mammalian cells or mutation in *A. thaliana* leads neither to a modified ratio of neddylated to unneddylated cullins nor to an alteration of Skp1 or Cul1 protein levels (Liu *et al.*, 2002; Zheng *et al.*, 2002a).

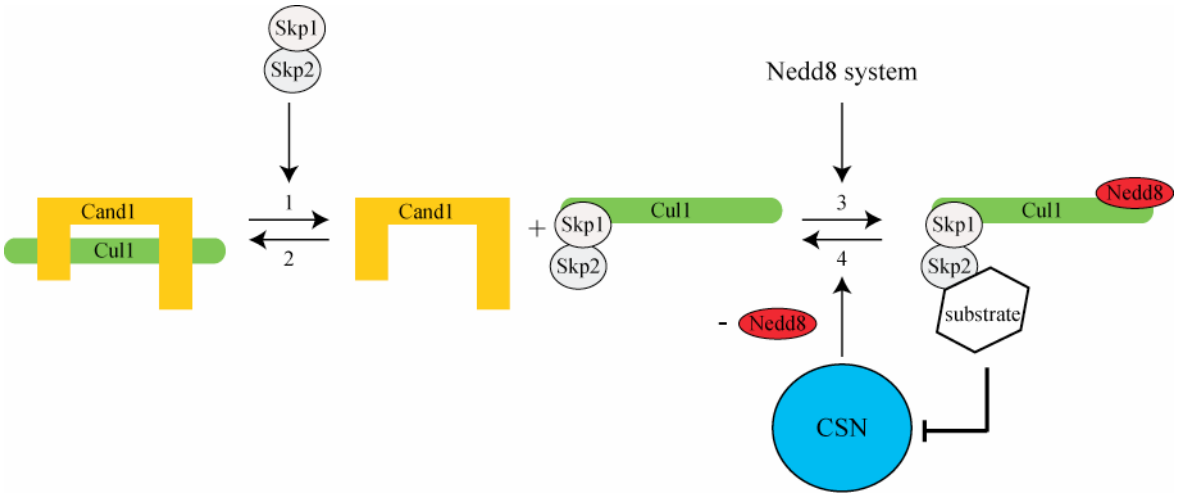


Fig. 7: Proposed sequence of events of neddylation and assembly of SCF^{Skp2} complex. Modified from Bornstein *et al.* (2006).

1. Due to an increased level of F-box protein (Skp2), Cand1 is displaced from Cul1 by the adaptor protein Skp1 and Skp2. 2. This reaction is concentration dependent and reversible. 3. The neddylation system neddylates Cul1 in complex with Skp1 and Skp2 and the complex becomes active as long as the substrate prevents CSN activity 4. Without substrate, Cul1 is the target of the COP9 Signalosome (CSN) and is deneddylated.

1.3 The model organism *Aspergillus nidulans*

Fungi of the genus *Aspergillus* can be found worldwide as about 185 species. They belong to the order Eurotiales and are heterogeneous according to the benefits and disadvantages they bring to mankind. Most *Aspergilli* are non-pathogenic saprophytic soil organisms. Nevertheless, inhaling the spores can lead to different types of respiratory hypersensitivity disorders and the fungi can infect wounds of otherwise healthy individuals. In recent years, mainly three *Aspergillus* species gained notoriety as human pathogens in immunocompromised patients. These are *A. terreus*, *A. flavus* and *A. fumigatus*. *A. fumigatus* can be found mainly in temperate climates while *A. flavus* favors hot, tropical climate. These fungi cause invasive pulmonary aspergillosis leading to death in over 90% of the cases. *A. flavus* and *A. parasiticus* are often found on crops and represent a severe problem in food industry because they produce aflatoxins that are potent mutagenic and carcinogenic substances (reviewed previously by De Lucca, 2007). In contrast, other species are highly beneficial. *A. niger* is used for citric acid fermentation (Papagianni, 2007) and *A. oryzae* is extensively used for industrial production of enzymes, metabolites and food like sake (rice wine), shoyu (soysauce) and miso (soybean paste) (Abe *et al.*, 2006). Up to now, the genomes of nine related filamentous fungi, *A. nidulans*, *A. fumigatus*, *A. oryzae*, *A. clavatus*, *A. flavus*, *A. niger*, *A. parasiticus*, *A. terreus* and *Neosartorya fischeri* have been sequenced and published (reviewed by Jones, 2007). *A. nidulans* has been introduced into science as a model organism by the Italian Guido Pontecorvo in 1953. It is a haploid, homothallic fungus displaying a sexual cycle. The 30,6 Mb genome of the model organism has been sequenced (Galagan *et al.*, 2005) and the fungus has been extensively studied with respect to genetic and metabolic regulation, development, cell polarity and cell cycle control. Today it is a well-characterized genetic system and the findings on the molecular basis can be transferred to its pathogenic and industrial relatives as well as to higher eukaryotic organisms.

1.3.1 Vegetative growth

1.3.1.1 Morphology of growth

A. nidulans is a fast growing, filamentous fungus. From one spore, a haploid mycelium develops, capable to grow as vegetative hyphae and able to form mitotically and meiotically derived spores, a process of high morphological complexity. To germinate, the G1-arrested spores break dormancy and enter the cell cycle. By isotropic swelling, the spore increases its volume before it switches to polarized growth. A germ tube is formed that grows by addition of new cell wall material to the tip. Simultaneously to the apical extension, nuclei undergo mitosis leading to multinucleate vegetative mycelium. Perforated septae are formed resulting in 40 µm long cells, arrested for growth and mitosis, harboring 3-4 nuclei. Later on, lateral branch formation can be initiated from these cells by the establishment of new polarity axes under the breakdown of the cell wall material at these positions (McGoldrick *et al.*, 1995; Momany *et al.*, 1999).

1.3.1.2 Regulation of the cell cycle

Progression of the cell cycle is controlled by cyclin-dependent protein kinases that regulate transition from one cell cycle phase to the other. According to their important function the kinases are highly conserved throughout the eukaryotes. By phosphorylating their target proteins, these kinases regulate expression and degradation of a multitude of cyclin subunits. Subsequently, a defective cell cycle control leads to growth or developmental defects. Several kinases involved in cell cycle control have been described for *A. nidulans*, including the cyclin-dependent kinase pair PhoA and PhoB. Although deletion of one kinase is not lethal for *A. nidulans*, *phoA*/*phoB* double deletion leads to a decreased nuclear division frequency (Dou *et al.*, 2003). The kinase NimA (never in mitosis) is an essential mitotic regulator, required for progression form G2 into mitosis (Lu *et al.*, 1993) while NimO, similar to the regulatory subunit of Cdc7p kinase in *S. cerevisiae*, is required for efficient progression through S phase (James *et al.*, 1999). Cyclins regulate a multitude of processes. One well characterized representative important for conidia production is the cyclin homologue PclA. *pclA* gene expression is cell cycle dependent with peak transcription levels in S phase (Schier *et al.*, 2001; Schier and Fischer, 2002).

1.3.2 Development

Each germinated spore of *A. nidulans* has the potential to form an independent developmental unit. Hyphae grow outwards, form a colony and take up the available nutrients from the surroundings. After 16-20 hours of growth the fungus establishes developmental competence (Axelrod *et al.*, 1973). From this time point on, it is able to produce tremendous amounts of spores. As a homothallic (self-fertile) fungus, *A. nidulans* is capable of two distinct ways of propagation: formation of asexually derived conidiospores or sexually derived ascospores (Fig. 8). A prerequisite for spore formation is growth on an air-medium interface. In submerged cultures only vegetative hyphae are formed. Just under extreme growth conditions of carbon or nitrogen starvation stress the spore formation can be induced also in liquid cultures (Axelrod *et al.*, 1973; Skromne *et al.*, 1995).

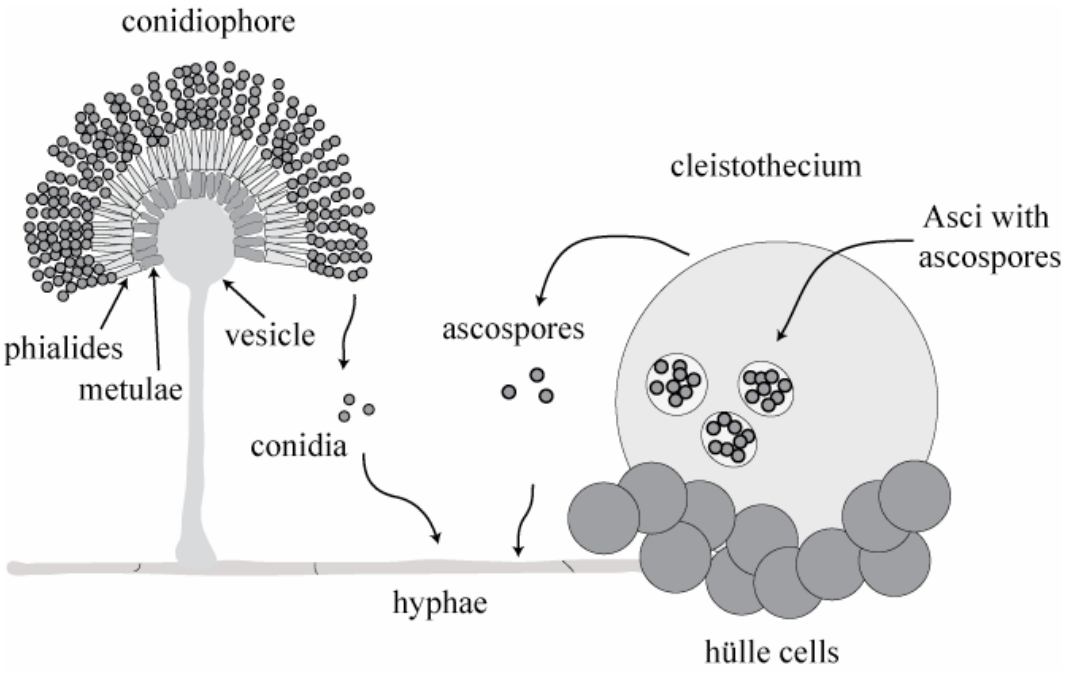


Fig. 8: *Aspergillus nidulans* life cycle.

Starting from vegetative hyphae, asexual development results in conidiophores producing conidia and sexual development results in ascospore containing cleistothecia. Modified from Tsitsigiannis *et al.* (2004).

1.3.2.1 Asexual and sexual reproduction

Asexual development starts with the formation of an approximately 70 µm long stalk that swells at its tip to form a vesicle. From these multinuclear sterigmata metulae are formed by budding. From the metulae, a second row of mononuclear sterigmata, the phialides, originate by budding. Subsequently, long rows of conidia arise from the phialides by asymmetrical cell division (Fig. 9).

Fig. 9: Conidiospore production of *A. nidulans*.

A. A stalk-like structure develops from hyphae and B. swells to a vesicle. C. metulae and D. phialides arise via budding. E. Conidiospores emerge by symmetrical cell division. (Sewall *et al.*, 1990).

Conidial pigments protect the genetic material inside the spores. The asexual spores are only about 10 µm in diameter and highly suitable for dispersal by air.

Aspergillus nidulans is also capable of undergoing a sexual cycle. The sexual form of the fungus has been named *Emericella nidulans*. About 100 hours after germination, differentiation of meiotically derived ascospores is initiated. At first, specialized vegetative hyphae start budding, producing so-called Hülle cells characterized by a thick wall. They contain the enzyme phenoloxidase (laccase II) that is specific for this cell type (Hermann *et al.*, 1983; Scherer and Fischer, 1998). The Hülle cells surround the fruit body during the complete developmental process and are suspected to be nurse cells for the developing cleistothecium (Zonneveld, 1977). After about three days the primordium becomes visible in a nest like structure that matures to a micro-cleistothecium. In the center of the bulk of Hülle cells specialized ascogenous hyphae fuse and grow out to form a dikaryotic mycelium. At the tip of these hyphae, two haploid nuclei fuse to induce formation of asci. One meiotic and one mitotic nuclear division lead to the formation of eight haploid,

uninucleate spores that undergo another mitosis without cell division. This way, binucleate ascospores are formed that are colored red by the substance asperthecin (see 1.4). A network of flat and highly branched hyphae surrounds the fertile hyphae in the micro-cleistothecium from which a stable, dark cleistothecial wall derives. A mature cleistothecium can measure up to 200 µm in size and contains about 80,000 ascospores. Sexual development has been reviewed by Braus *et al.* (2002) and Champe *et al.* (1994).

1.3.3 Regulation of asexual and sexual development

Development of reproduction units that harbor mature, germinable spores is a complex event and requires exact spatial and temporal regulation of many regulatory and structural factors involved. The results of extensive research shed light on the regulatory cascade underlying development in *A. nidulans*. Fig. 10 summarizes selected regulatory factors acting in the regulation of development.

Aeration and light are triggers for asexual spore formation. When the vegetative mycelium is exposed to red light during a specific time frame, conidia-formation is induced while it is repressed by an immediate shift to far red light (Mooney and Yager, 1990). This effect is reminiscent of the behavior of phytochromes known from plants and bacteria. And indeed, *A. nidulans* does possess a fungal, cytoplasmatic phytochrome, FphA. It is similar to the bacterial ones and acts as a red-light sensor (Blumenstein *et al.*, 2005). In UVA light and blue light the nuclear localized photolyase-like protein CryA is responsible for the repression of sexual development (Bayram, 2007).

A well-known factor of light regulation, connecting the light signal with the downstream developmental response, is the gene product of *veA*. Expression of this positive regulator of sexual development is significantly increased during sexual development. While in the light VeA is found abundantly in the cytoplasm, the protein localizes to the nucleus in the dark (Stinnett *et al.*, 2007). In the dark VeA supposedly forms a heterotrimeric *velvet* complex with VelB and LaeA in the nucleus that influences development (Bayram, 2007). Deletion of *veA* leads to strains producing solely asexual spores while overproduction leads to enhanced sexual development, indicating that VeA represses initiation of asexual spore production (Kim *et al.*, 2002). Many of the laboratory strains carry the *veA1* mutant

allele that encodes a truncated, less active form of VeA. These strains favor asexual spore production in a light independent manner (Kim *et al.*, 2002; Mooney and Yager, 1990).
Another environmental factor influencing development is the availability of nutrients. Either nitrogen or carbon starvation initiates asexual spore production (Skromne *et al.*, 1995). Also phosphate deficiency leads to the induction of asexual development, probably by a signaling cascade including the gene product of *phoA*. The cyclin–dependent kinase is involved in the sensing of environmental signals like phosphate, inoculation density and pH (Bussink and Osmani, 1998).
Asexual development is controlled by a central regulatory cascade including two main transcription factors, specifically required for conidia formation but not for vegetative growth: BrlA (bristle) and AbaA (abacus) (Clutterbuck, 1969). The zinc finger transcription factor BrlA is crucial for the switch from apical growth to the swelling of the vesicle and the sterigmata budding. Mutation of the gene results in the formation of conidiophore stalks. Expression of BrlA is the major and essential control point of asexual development (Han *et al.*, 1993; Prade and Timberlake, 1993), because it activates several developmentally regulated genes. Among these are the genes *ivoB* and *yA,* encoding for enzymes responsible for spore pigmentation (see 1.4) as well as *abaA* and *wetA* (Clutterbuck, 1990; Miller *et al.*, 1992).
abaA is transcribed at the switch from sterigmata budding to conidiospore formation, approximately 24 hours after germination. It encodes a transcription factor that activates its own transcription in a positive feedback-loop as well as transcription of *wetA* (wet). *wetA* is a regulatory gene expressed in developing conidia. WetA regulates spore-specific gene expression and is essential for formation of cell wall components as well as conidia maturation (Marshall and Timberlake, 1991). Another transcription factor influencing conidiophore formation is VosA (Ni and Yu, 2007). Deletion of the *vosA* gene results in uncontrolled activation of asexual development even in liquid culture and altered expression of *yA*, *wA* and *brlA*. For a more detailed description of asexual sporulation and its regulation see Adams *et al.* (1998).
Fruit body development is impaired by amino acid deficiency (Eckert *et al.,* 1999) but induced by a deficiency of nutrients like glucose, nitrate and phosphate, although cleistothecia formation is an energy-consuming process. To ensure energy availability for sexual development, polysaccharide α-1,3-glucan is stored in the hyphal cell wall. Upon

consumption of the external carbohydrates and induction of sexual development, α-1,3-glucanase is expressed and cleaves the α-1,3-glucan, thereby reactivating the glucose (Zonneveld, 1972).

Selected factors influencing development of *A. nidulans* are summarized in Fig. 10. Two well-characterized transcription factors, essential for sexual development exclusively, are NsdD (never in sexual development) and SteA (sterile 12-like). NsdD is a GATA transcription factor with a zinc-finger DNA binding domain expressed in early phase of vegetative growth and sexual development. During asexual sporulation its mRNA levels decrease. Deletion of *nsdD* impairs cleistothecia formation while overproduction leads to a highly increased number of sexual fruit bodies (Han *et al.*, 2001).

SteA is the homolog of the zinc-finger transcription factor Ste12 from *S. cerevisiae*. *steA* deletion mutants are not able to form cleistothecia but formation of conidiophores and Hülle cells is unaffected (Vallim *et al.*, 2000). Another putative zinc-finger transcription factor that has its function in repressing sexual development is RosA. In *rosA* deletion strains transcription of the sexual development regulators *nsdD*, *veA* and *stuA* is increased. RosA also represses NosA, another putative zinc-finger transcription factor essential for primordia maturation (Vienken *et al.*, 2005; Vienken and Fischer, 2006). An interesting,

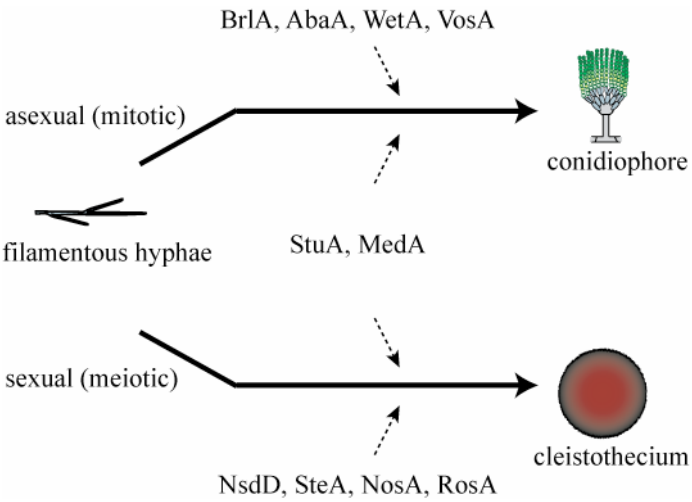


Fig. 10: Summary of selected transcription factors in connection to the development of *A. nidulans*.

Transcription factors described to regulate asexual (mitotic) development (BrlA, AbaA WetA, VosA), sexual (meiotic) development (NsdD, SteA, NosA, RosA) or both (StuA, MedA) are indicated. For explanations of transcription factor abbreviations and a description of their functions see text. Dashed arrows indicate the developmental way affected by the respective transcription factors.

not well-characterized group of mutants are the ones defective in ascosporogenesis. They display defects in crozier formation, karyogamy, meiosis or postmeiotic mitosis. These strains are characterized by the lack of germinable ascospores in otherwise wild-type like cleistothecia (Swart *et al.*, 2001). The F-box protein GrrA is one factor controlling ascospore-formation. The yeast GrrA homolog (Grr1p) acts as the adaptor protein of E3 ubiquitin ligases. Deletion of *grrA* leads to a strain characterized by the disability of ascosporogenesis due to a block in meiosis (Krappmann *et al.*, 2006).

The two factors MedA (medusa) and StuA (stunted) are developmental modifiers of sexual and asexual development (Clutterbuck, 1969). *stuA* mRNA is upregulated upon developmental competence. Asexual structures of *stuA* mutants are disorganized and the spores bud directly from the vesicle. The mutants totally lack Hülle cells and are acleistothecial (Miller *et al.*, 1991; Vallim *et al.*, 2000). *medA* mutants are able to form conidia, but they are located on a conidiophore harboring four or more rows of sterigmata, while the wild-type has only two. The strains produce unorganized Hülle cells but no cleistothecia or ascospores (Clutterbuck, 1969). Both factors influence asexual development by regulating *brlA* and *abaA*. While MedA controls the correct temporal expression of *brlA* and is a coactivator of *abaA* expression, StuA controls the correct spatial distribution of BrlA and AbaA (Busby *et al.*, 1996; Miller *et al.*, 1992).

1.4 Melanin and pigments

Melanins are macromolecular pigments that can be found ubiquitous in animals, plants and fungi. They derive via oxidative polymerization of phenolic or indolic compounds resulting in a heterogeneous group of substances with similar characteristics. They protect the organism from environmental stress conditions such as UV light, extreme temperatures, chemical and biochemical stresses. Some melanins and their biogenetic byproducts show antibiotic action against other microorganisms (Bell and Wheeler, 1986). In the case of the opportunistic pathogen *A. fumigatus*, the absence of melanins can lead to decreased virulence due to a higher sensitivity of the spores to hydrogen peroxide and NaOCl (Jahn *et al.*, 1997).

Due to the size, heterogeneity and insolubility of melanins not much is known about their structures. According to Plonka and Grabacka (2006), melanins can be classified into three

major groups. The black or brown eumelanins arise by oxidation of tyrosine (and/or phenylalanine) to o-dihydroxyphenylalanine (DOPA) and DOPAquinone (Fig. 11). DOPA is subsequently converted to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) that is in turn converted to eumelanins by oxidative polymerization. The yellow-red pheomelanins derive also from DOPAquinone that undergoes cysteinylation resulting in cysteinylDOPA. This polymerizes into derivatives of benzothiazines forming pheomelanins.

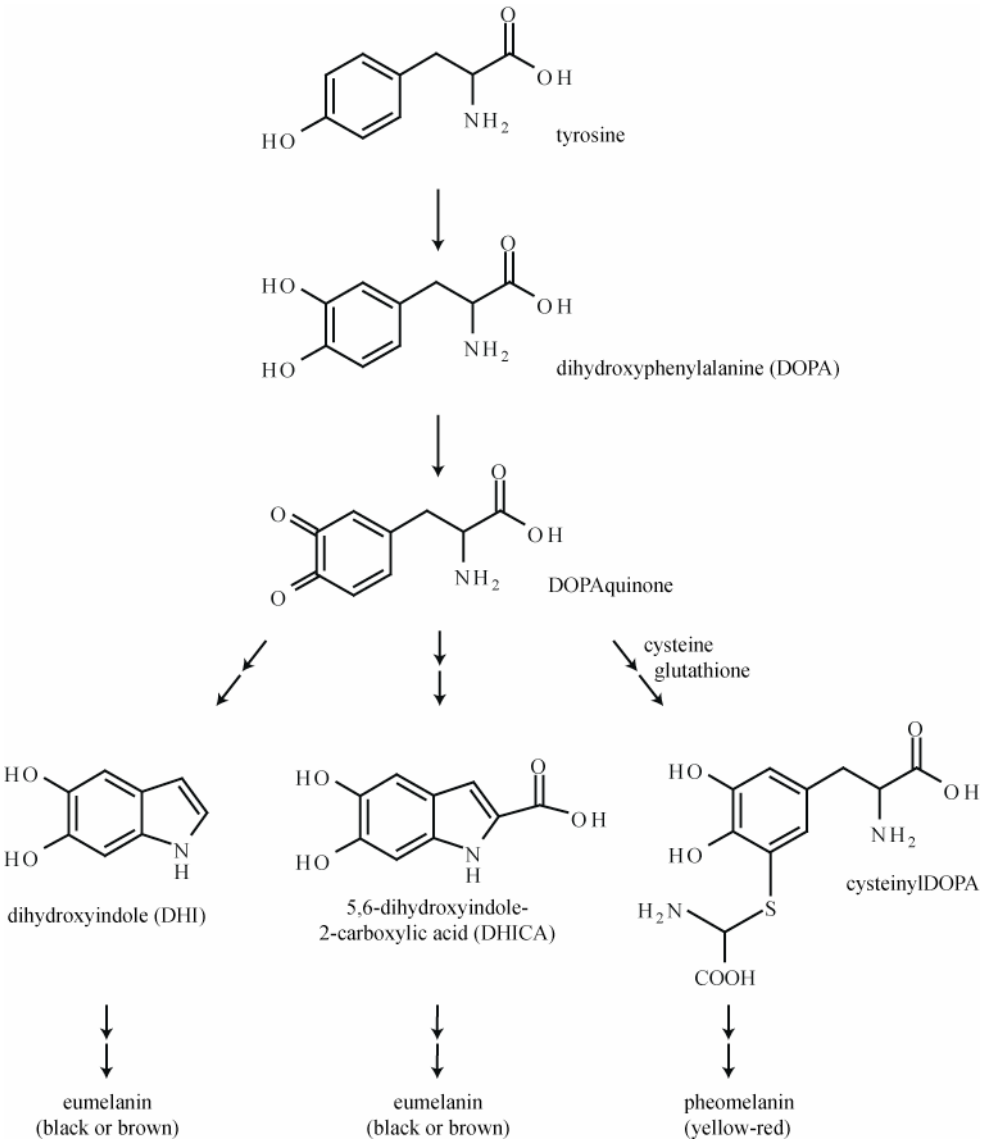


Fig. 11: Eumelanin and pheomelanin biosynthesis pathway. Modified from Plonka and Grabacka (2006).

The various colored polymers in the third, very heterogeneous group are allomelanins. This group includes, among others, the dihydroxynaphthalene-melanins (DHN-melanins). They derive from malonyl-CoA via PKS-biosynthesis and final oxidative polymerization of 1,8-dihydroxynaphthalene (DHN) (Fig. 12). The first step of the DHN-melanin biosynthesis, which is the formation of polyketides, is catalyzed by multidomain enzymes, polyketide synthases (PKS). For example the PKS1 of *Colletotrichum lagenarium* has been found to produce the pentaketide 1,3,6,8-THN when expressed in the heterologous host *Aspergillus oryzae*. It has been suggested that this PKS produces also the tetraketide orsellinic acid as an artificial byproduct in the course of heterologous expression.

Only little is known about the spore color formation, pigmentation and melanins of *Aspergilli* and the substance or substances causing the color of asexual spores have not been identified yet. However, a biosynthetic route similar to that of the DHN-melanin biosynthesis known from *Colletotrichum lagenarium* and *Magnaporthe grisea* has been suggested to be involved in the formation of the grey-green pigment of *A. fumigatus* asexual spores (Wheeler and Bell, 1988) (Fig. 12). Deletion of the PKS *pksP*/*alb1* of *A. fumigatus* or the homologous PKS *wA* of *A. nidulans* results in white conidia (Langfelder *et al.*, 1998; Mayorga and Timberlake, 1990). Both PKS produce the heptaketide naphthopyrone (YWA1) (Fujii *et al.*, 2000; Fujii *et al.*, 2001). For *A. fumigatus* it was shown that naphthopyrone is subsequently converted to 1,3,6,8-THN by the serine protease-type hydrolytic enzyme AYG1 (Fujii *et al.*, 2004; Tsai *et al.*, 2001) and 1,3,6,8-THN is a precursor of DHN melanin. Deletion of the gene encoding the scytalone dehydrating enzyme ARP1 of *A. fumigatus* influences the pigmentation of the conidia resulting in reddish-pink conidia (Tsai *et al.*, 1997). Tricyclazole, a specific inhibitor of the reductases in the DHN-biosynthesis pathway (Tsai *et al.*, 1999), inhibits conidial pigmentation of *A. fumigatus* (Wheeler and Bell, 1988). For *A. nidulans* it is known that the spore pigmentation comprises a white and yellow state and that it requires the gene products of the *p*-diphenol-oxidase encoding gene *yA* in addition to the PKS WA (Fig. 13B). Deletion of *wA* results in white spores while deletion of *yA* results in yellow spores (Aramayo and Timberlake, 1993).

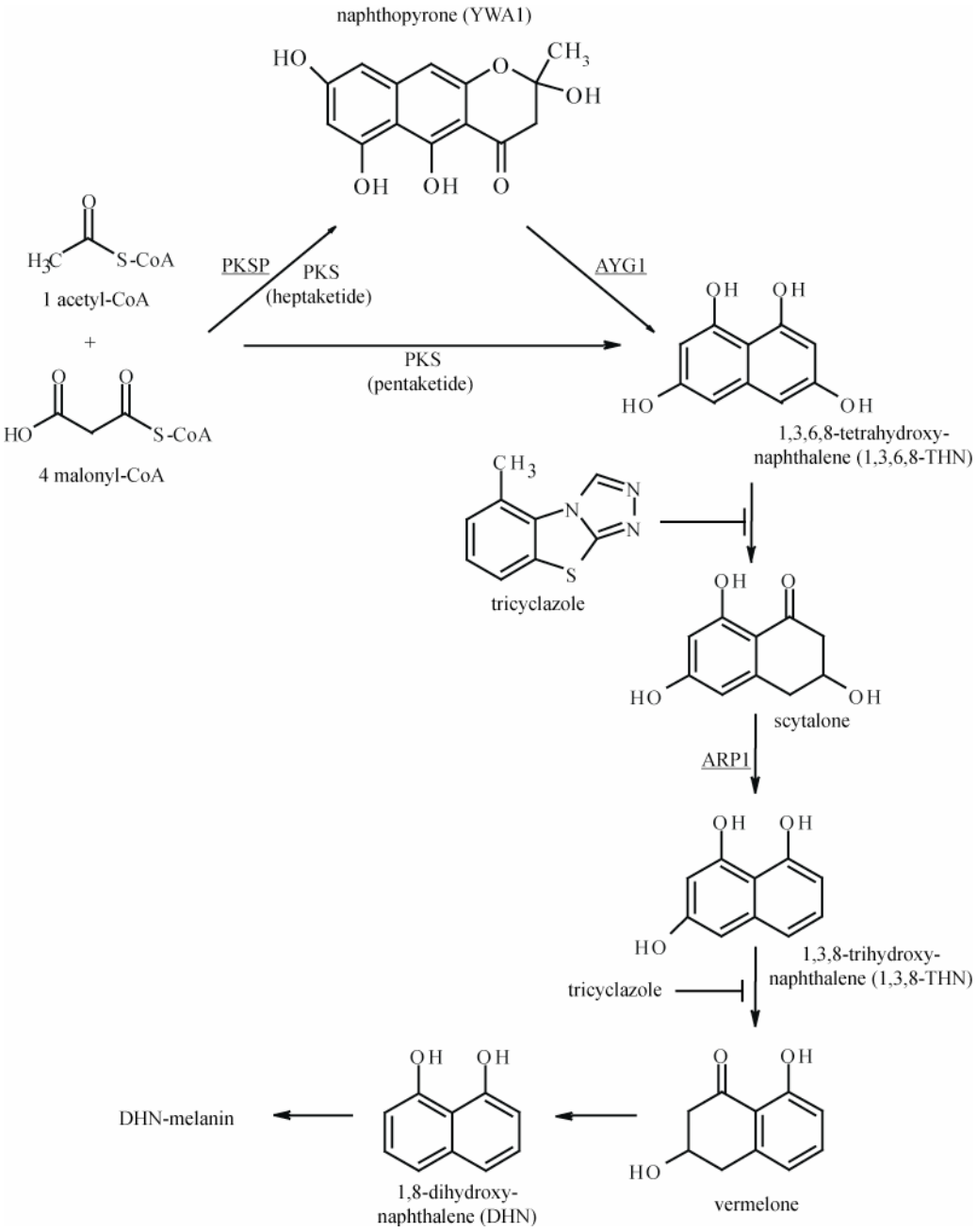


Fig. 12: General model for DHN-melanin biosynthesis. Modified from Langfelder *et al.* (2003).

From one acetyl-CoA and four malonyl-CoA the polyketide synthase (PKS) synthesizes 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) or naphthopyrone (YWA1), which can be converted to 1,3,6,8-THN. This is reduced to scytalone that is dehydrated to 1,3,8-THN that in turn is reduced to vermelone. Vermelone is dehydrated to 1,8-dihydroxynaphthalene (DHN). Melanin supposedly derives via DHN dimerization and polymerization. The reduction steps are specifically inhibited by tricyclazole. Selected enzymes of the corresponding steps in *A. fumigatus* are underlined. AYG1, serine protease-type hydrolytic enzyme, ARP1, scytalone dehydrating enzyme, PKSP, polyketide synthase P (PKSP/ALB1).

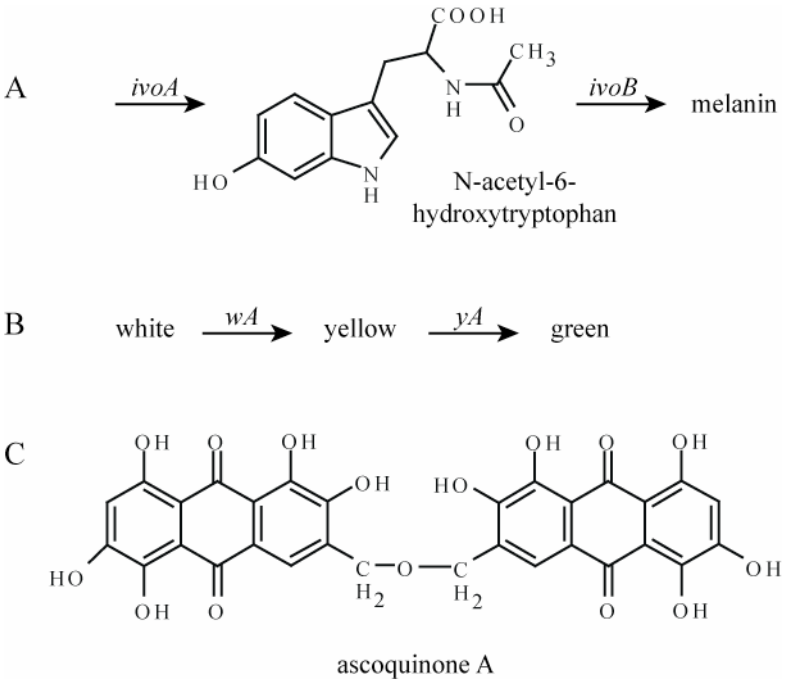


Fig. 13: Enzymatic activities and substances involved in *A. nidulans* pigment formation.

A. Enzymes necessary for conidiophore color formation, *ivoB* codes for a phenoloxidase, B. enzymes necessary for green color formation of conidia, *wA* codes for a polyketide synthase, C. pigment isolated from *A. nidulans* ascospores, ascoquinone A. Arrows show putative (multiple) enzymatic steps.

The sexual spores of *A. nidulans* are dark red in contrast to the green asexual spores. The red spore pigment of the ascospores has been identified as the anthraquinone ascoquinone A (Brown and Salvo, 1994) (Fig. 13C). However, only little is known about the biosynthesis pathway of these substances.

Melanins have also been found in vegetative hyphae and the conidiophores of *A. nidulans* wild-type strains. The fungus accumulates a small amount of dark brown melanin in elder hyphae of vegetative cultures, which is also released into the medium. This melanin has been suggested to be a co-polymer of dihydroxyphenylalanine (DOPA) residues and non-nitrogenous monomers (Pirt and Rowley, 1969). It has antioxidant activity which has been shown using a highly melanized *A. nidulans* mutant Mel1 (de Cassia *et al.*, 2005). In *A. nidulans* pigments are also formed in a developmentally specific way in the conidiophores that are pigmented grey-brown. This pigmentation is not formed in *ivo* (ivory- unpigmented conidiophores) mutants. The *ivoB* gene encodes for a developmental specific phenoloxidase that has N-acetyl-6-hydroxytryptophan (AHT) as a substrate while

ivoA deficient strains are not able to form AHT (Birse and Clutterbuck, 1990; Clutterbuck, 1990) (Fig. 13A). However, the exact structure of the melanins is not determined yet.

1.5 Scope and aim of this work

Ubiquitin dependent protein degradation and its regulators are highly conserved throughout higher eukaryotes. Important interaction partners of E3 ligases involved in the neddylation-deneddylation regulatory circuit like CSN, CandA and DenA are conserved in the filamentous fungus *A. nidulans*, the only fungus so far shown to express a complete COP9 signalosome. Deletion of CSN components in *A. nidulans* showed that the complex, in contrast to higher eukaryotes, is only essential for sexual spore formation but not for growth and reproduction (Busch *et al.*, 2003; Busch *et al.*, 2007). These findings qualify *A. nidulans* as an outstanding model organism for investigating neddylation/deneddylation-dependent regulation of protein degradation in this fungus.

This work directs the focus on the proteins CsnB, DenA and CandA, their mode of action in the model organism *A. nidulans* and the roles these putative ubiquitin ligase regulators play in growth, development and secondary metabolism of the fungus. The first part of this work focuses on the COP9 signalosome and addresses the question whether different CSN subunits work solely together in the complex or if they display other, phenotype relevant independent functions. The second part provides information about the enzyme activity of the deneddylase DenA of *A. nidulans* and its effect on growth and development of the fungus. The cullin containing ubiquitin ligase regulator CandA is analyzed in the third part of this work. This includes the discovery that CandA is encoded by the two genes *candA-N* and *candA-C* in *Aspergilli*. It directs the question on the role of the CandA proteins for growth, development and secondary metabolism and provides information about the interplay of CandA-N and CandA-C with CSN and the E3 ligase subunit cullin on the molecular level.

2 Materials and Methods

2.1 Growth media and growth conditions

Chemicals used for solutions, buffers and media were obtained from Merck (Darmstadt, D), Roche GmbH (Mannheim, D), Carl Roth GmbH & CoKG (Karlsruhe, D), Invitrogen GmbH (Karlsruhe, D), Fluka (Neu-Ulm, D) or Sigma-Aldrich Chemie GmbH (Steinheim, D).

E. coli strains were propagated in LB (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) or LBLS (1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) medium at 30°C or 37°C modified from Bertani (1951). Expression of genes under the control of the *BAD* promoter in pKOBEG was induced with 0.2% arabinose. For selection 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, 20 µg/ml kanamycin or 25 µg/ml Zeocin™ (Cayla, Toulouse, F) was used.

Saccharomyces cerevisiae strains were grown at 30°C under non-selective conditions in YEPD (2% pepton, 1% yeast extract, 2% glucose) or under selective conditions in SC medium (0.15% Yeast Nitrogen Base without amino acids, 0.5% $(NH_4)_2SO_4$, 0.2 mM myo-inositol, 0.2% amino acid mix containing either 2% glucose or 2% galactose/1% raffinose) supplemented as described (Guthrie and Fink, 1991).

A. nidulans strains were grown at 37°C in or on minimal medium (7 mM KCL, 11.2 mM KH_2PO_4 (pH 5.5), 2 mM $MgSO_4$, trace elements) (Käfer, 1965). As carbon source 1% glucose was used. As nitrogen source 10 mM $NaNO_3$, 70 mM $NaNO_3$ or 10 mM NH_4Cl were added. The medium was supplemented with 4.8 µM pyridoxine-HCl and/or 5 mM uridine as required. For plates 2% agar was added. Selection for strains carrying the dominant marker gene *ble* of *Streptoalloteichus hindustanus* was performed using 10 µg/ml phleomycin (Cayla, Toulouse, F). For selection of strains carrying the dominant marker cassette *ptrA* (Takara Biokom, Junki, PL) 100 ng/ml pyrithiamine was added to the medium. Vegetative mycelia were obtained from submerged liquid cultures inoculated with 10^6 spores/ml and grown on a rotary shaker for 14-30 hours. For induction of development 10^6 spores were spread on agar plates. Asexual development was obtained by incubating the plates in constant white light whereas sexual development was induced under oxygen limiting conditions on tape-sealed plates in the dark (Clutterbuck, 1974).

Strains were grown on plates covered with cellophane film (Merck Chemicals, Nottingham, GB) when cultivated for harvesting. Colony growth was recorded as colony diameter within time. Conidiospore quantification was modified from Bussink and Osmani (1998) as described by Busch *et al.* (2003).

2.2 Strains

2.2.1 *Escherichia coli* strains

For general cloning procedures *E. coli* DH5α [F^-, Φ80dΔ(*lacZ*)$M15^{-1}$, Δ(*lacZYA-argF*) U169, *recA1*, *endA1*, *hsdR17* (r_K^-, m_K^+), *supE44*, λ^-, *thi1*, *gyrA96*, *relA1*] (Woodcock *et al.*, 1989) was used. Recombination in *E. coli* was performed in strain KS272 [F^-, Δ*lacX74*, *galE*, *galK*, *thi*, *rpsL*, Δ*phoA* (*Pvu*II)] carrying the pKOBEG plasmid (Chaveroche *et al.*, 2000).

2.2.2 *Saccharomyces cerevisiae* strains

The strains used and constructed in this study for DenA characterization in *S. cerevisiae* in addition to the yeast strains used for the two-hybrid tests are summarized in Tab. 5. For the strain construction *denA* containing plasmids pME3278 and pME3279 were transformed into *S. cerevisiae yuh1* deletion strain Y06911 (Euroscarf, Frankfurt, D) resulting in strains RH3318 and RH3330. As control the wild-type strain BY4741 (Euroscarf, Frankfurt, D) was used. For testing Den1 deneddylase activity in *S. cerevisiae* plasmid pME3280 was transformed into the yeast wild-type strain BY4741 resulting in strain RH3337, into the Δ*rri1*/Δ*csn5* strain Y03914 (Euroscarf, Frankfurt, D) resulting in strain RH3332 and into the Δ*yuh1* strain Y06911 resulting in strain RH3331. Thereafter plasmids pME3278 and pME3279 were transformed into strain RH3332 resulting in strains RH3333 and RH3334, respectively, and into strain RH3331 resulting in strains RH3335 and RH3336, respectively.

2.2.3 *Aspergillus nidulans* strains

A. nidulans strains including their genotypes used and constructed in this study are summarized in Tab. 6.

2.2.3.1 Construction of *csn* deletion strains of and promoter *lacZ* fusions in *Aspergillus nidulans*

For deletion of *csnB*, the deletion cassette was cut out of plasmid pME2814 with *Hpa*I/*Eco*RV and was transformed into strain AGB152 (Busch *et al.*, 2003). Via selection for uridin prototrophy strain AGB238 was obtained. For complementation of the deletion strain, plasmid pME2815 was transformed into strain AGB238 as an ectopic integration resulting in strain AGB239. For construction of a *csnA*/*csnB* double deletion strain, strain AGB234 (Draht, 2005) was transformed with the *csnB* deletion cassette from plasmid pME2814 resulting in strain AGB250. For construction of strains containing a *csnA* promoter::*lacZ* fusion or a *csnE* promoter::*lacZ* fusion as well as a control strain containing the *lacZ* gene without a promoter, strain WG355 was transformed with linearized plasmids pME2868, pME2817 or pAN923-41B, resulting in arginine prototroph transformants with a single copy integration at the chromosomal *argB* gene locus. AGB246 containing the *lacZ* gene without promoter from plasmid pAN923-41B served as negative control. Strain AGB243 contains the 5´ *csnA::lacZ* fusion and strain AGB248 the 5´ *csnE::lacZ* fusion as single copy as confirmed by PCR and Southern hybridisation analyses.

2.2.3.2 Construction of *denA* deletion and complementation strains in *Aspergillus nidulans*

The *denA* deletion cassette was cut from plasmid pME3275 using the restriction enzymes *Cla*I/*Not*I and integrated into strain AGB152 via homologous recombination. The resulting *denA* deletion strain was named AGB316. Transformation of the *denA* deletion cassette into TNO2A3, carrying the *nkuA* deletion (Nayak *et al.*, 2006), resulted in strain AGB317. Strain AGB316 was complemented by integration of the *denA* genomic fragment and a phleomycin resistance marker cassette of pME3267 resulting in strain AGB318.

Tab. 5: *S. cerevisiae* strains used and constructed for DenA characterization and the yeast two-hybrid tests.

strain	genotype	Reference
BY4741	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;* wild-type	Euroscarf[a]
EGY48-p1840	*MATa, his3, trp1, ura3-52, leu2::pLEU2-LexAop6, URA3::lacZ-LexAop2*	Golemis and Brent (1996)
RH3318	*GAL1(p)::denA, URA3; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJR099w::kanMX4*	This work: pME3278 in Y06911
RH3330	*GAL1(p)::denA::V5, URA3; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJR099w::kanMX4*	This work: pME3279 in Y06911
RH3331	*culD::lexA, HIS3, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJR099w::kanMX4*	This work: pME3280 in Y06911
RH3332	*culD::lexA, HIS3, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDL216c::kanMX4*	This work: pME3280 in Y03914
RH3333	*denA, URA3, culD::lexA, HIS3, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDL216c::kanMX4*	This work: pME3278 in RH3332
RH3334	*denA::V5, URA3, culD::lexA, HIS3, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDL216c::kanMX4*	This work: pME3279 in RH3332
RH3335	*denA, URA3, culD::lexA, HIS3, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJR099w::kanMX4*	This work: pME3278 in RH3331
RH3336	*denA::V5, URA3, culD::lexA, HIS3, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJR099w::kanMX4*	This work: pME3279 in RH3331
RH3337	*culD::lexA, HIS3, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	This work: pME3280 in BY4741
Y03914	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDL216c::kanMX4*	Euroscarf[a]
Y06911	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJR099w::kanMX4*	Euroscarf[a]

[a]European *Saccharomyces cerevisiae* Archive for Functional analysis, Johann Wolfgang Goethe-University, Frankfurt, D.

2.2.3.3 Construction of *Aspergillus nidulans* strains for CandA characterization

pME3306 was restricted with *Nhe*I/*Dra*I to obtain the *candA-N* deletion cassette that was transformed into strain AGB152 resulting in strain AGB264. For complementation of the *candA-N* deletion, strain AGB264 was transformed with the complementation construct of plasmid pME3308 resulting in strain AGB265. For *candA-C* deletion, the corresponding cassette was cut out of plasmid pME3115 with *Not*I and transformed into strain AGB152 resulting in strain AGB262. For complementation of the *candA-C* deletion strain, the complementation cassette of plasmid pME3116 was integrated ectopically into the genome of strain AGB262 resulting in strain AGB263. For construction of the *candA-N*/*candA-C* double deletion strain, AGB264 was transformed with the *candA-C* deletion cassette of plasmid pME3127 resulting in strain AGB268.

Tab. 6: *Aspergillus nidulans* strains used and constructed in this study.

Strain	Genotype	Reference/Construction
A4	*A. nidulans* Glasgow wild-type	FGSC[a] A4
AGB150	*pyrG98; pyroA4; wA3*	constructed by V. Große
AGB152	*pyrG98; pyroA4*	Busch *et al*. (2003)
AGB160	*pyr-4; pyrG98; pyroA4*	Busch *et al*. (2003)
AGB195	Δ*csnD::pyr-4; pyrG98; pyroA4*	Busch *et al*. (2003)
AGB209	Δ*csnE::pyr-4; pyrG98; pyroA4*	Busch *et al*. (2003)
AGB234	Δ*csnA; pyrG98; pyroA4*	Draht (2005)
AGB238	Δ*csnB::pyrG; pyrG98; pyroA4*	This work: pME2814 in AGB152
AGB239	Δ*csnB::pyrG; csnB::ble; pyrG98; pyroA4*	This work: pME2815 in AGB238
AGB243	*biA1; bgaO; argB2, 5´ csnA::lacZ <argB>*	This work: pME2868 in WG355
AGB246	*biA1; bgaO; argB2, lacZ <argB>*	This work: pAN923-41B in WG355
AGB248	*biA1; bgaO; argB2, 5´ csnE::lacZ <argB>*	This work: pME2817 in WG355
AGB250	Δ*csnA;* Δ*csnB::pyrG; pyrG98; pyroA4*	This work: pME2814 in AGB234
AGB262	Δ*candA-C::pyr-4; pyrG98; pyroA4*	This work: pME3115 in AGB152
AGB263	Δ*candA-C::pyr-4; candA-C::ble; pyrG98; pyroA4*	This work: pME3116 in AGB262
AGB264	Δ*candA-N::pyr-4; pyrG98; pyroA4*	This work: pME3306 in AGB152
AGB265	Δ*candA-N::pyr-4; candA-N::ble; pyrG98; pyroA4*	This work: pME3308 in AGB264
AGB266	*candA-C(p)::candA-C::GFP; pyrG98; pyroA4*	This work: pME3120 in AGB262
AGB267	Δ*csnE::pyr-4;* Δ*candA-C::ble; pyrG98; pyroA4*	This work: pME3127 in AGB209
AGB268	Δ*candA-N::pyr-4;* Δ*candA-C::ble; pyrG98; pyroA4*	This work: pME3127 in AGB264
AGB269	*candA-C::GFP; alcA::mRFP1::H2A::pyr-4; pyrG98; pyroA4*	This work: pME3125 in AGB266
AGB316	Δ*denA::pyr-4; pyrG98; pyroA4*	This work: pME3275 in AGB152
AGB317	Δ*denA::pyr-4; pyrG98; pyroA4*	This work: pME3275 in TNO2A3
AGB318	Δ*denA::pyr-4; denA::ble; pyrG98; pyroA4*	This work: pME3267 in AGB316
AGB331	*candA-N::ptrA; candA-C;* Δ*candA-N::pyr-4;* Δ*candA-C::ble; pyrG98; pyroA4*	This work: pME3311 and pME3114 in AGB268
AGB332	*candA-N*(p)::*candA-N::candA-C::candA-C*(t)::*ptrA;* Δ*candA-N::pyr-4;* Δ*candA-C::ble; pyrG98; pyroA4*	This work: pME3310 in AGB268
AGB383	Δ*csnE::pyr-4; pyrG98; pyroA4; wA3*	constructed by S. Busch
TNO2A3	*pyrG98; pyroA4*	Nayak *et al*. (2006)
WG355	*biA1, bgaO, argB2*	van Gorcom *et al*. (1986)

a. Fungal Genetics Stock Center (University of Missouri, Kansas City, MO, USA)

For construction of a strain containing the *candA-C*::*gfp* fusion, strain AGB262 was transformed with the fusion cassette of plasmid pME3120 and counter selection on 5-FOA for homologues integration resulted in strain AGB266. Strain AGB269, containing a nuclear RFP-marker, under the control of an *alcA* inducible promoter, and the *candA-C::gfp* fusion resulted from transformation of plasmid pME3125 into strain AGB266. For a double deletion of *csnE* and *candA-C,* the *candA-C* deletion cassette from plasmid pME3127 was transformed into strain AGB209 (Busch *et al.*, 2003) resulting in strain AGB267. To obtain a strain, carrying a *candA-N::C* fusion construct instead of the single proteins, the *candA-N*/*candA-C* double deletion strain AGB268 was transformed with plasmid pME3310 resulting in strain AGB332. As control, plasmid pME3311, carrying the

dominant *ptrA* marker and the *candA-N* genomic fragment, was co-transformed with the *candA-C* genomic fragment of pME3114 resulting in the complementation strain AGB331. Homologous integration of deletion cassettes and ectopic integration of complementation constructs was confirmed using PCR and Southern hybridisation analyses.

2.3 Genetic manipulation

2.3.1 Transformation

Transformations of *E. coli*, *S. cerevisiae* and *A. nidulans* were performed as described (Eckert *et al.*, 2000; Elble, 1992; Inoue *et al.*, 1990). For transformation of plasmids into *E. coli* strain KS272, competence was achieved by the calcium shock method (Hanahan *et al.*, 1991). Transformation was performed by electroporation, with cells washed extensively in ice cold water and suspended in 10% glycerol. Cells were mixed with the DNA and electroporation was carried out in 0.2 cm electroporation chambers with a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA, USA) at 200 Ω, 25 µF and 2.5 kV. Cells were then diluted with LB medium and incubated 1 hour at 30°C before plated on solid medium.

2.3.2 Primers and plasmids

Primers and plasmids used and constructed in this study are summarized in Tab. 7 and Tab. 8, respectively.

2.3.2.1 Constructs for studies on CsnB in *Aspergillus nidulans*

DNA for a 820 bp *csnB* probe was obtained by PCR on genomic DNA of the wild-type strain A4 with specific primers ES1/ES2. The PCR fragment was cloned into the *Eco*RV site of pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) resulting in plasmid pME3118. By hybridization of a filter representing an *A. nidulans* BAC library (obtained from Clemson University, Clemson, SC, USA) clone B15 of plate1 was identified to contain the *csnB* genomic region. A 7727 bp, *csnB* containing *Aat*II fragment was then cloned into pGEM-Zf (+) resulting in plasmid pME2821. For cloning of a *csnB* deletion blaster cassette the method described by Krappmann and Braus (2003) was used. The *csnB*

5´-flanking region was amplified with primers ES5/ES6 and the 3´-flanking region was amplified with primers ES7/ES8 from genomic DNA. The PCR fragments were cloned into plasmid pME2409 via the *Eco*RV or *Hpa*I site, respectively, resulting in plasmid pME2820. The deletion cassette was excised with *Eco*RV and *Hpa*I from pME2820 and transformed together with pME2821 into *E. coli* strain KS272 carrying the pKOBEG plasmid for recombination (Chaveroche *et al.*, 2000). The deletion construct pME2814 was obtained via *in vivo* recombination. For complementation of the *A. nidulans* Δ*csnB* strain a 6700 bp *Aat*II/*Hpa*I genomic *csnB* fragment of pME2821 was blunt end ligated into the *Xba*I opened plasmid pME1510 (Busch *et al.*, 2003) containing a phleomycin resistance cassette from plasmid pAN8-1 (Punt and van den Hondel, 1992) resulting in plasmid pME2815. To create a 5´ *csnA*::*lacZ* fusion and a 5´ *csnE*::*lacZ* fusion, plasmid pAN923-41B (van Gorcom *et al.*, 1986) was used. The unique *Bgl*II site within the *argB* wild-type gene of this plasmid was filled in with PolIK, creating an insertion mutant *argB* allele (Punt *et al.*, 1990). This mutation allows the selection of arginine prototrophic transformants, generated by recovering the wild-type allele by crossing over with the *argB*2 mutation of WG355. 1000 bp of the 5´-regulatory region of *A. nidulans csnA* and *csnE* were amplified from wild-type DNA with specific primers ES55/ES56 and ES59/ES60 containing *Bam*HI restriction sites. The 1000 bp PCR fragments were fused to *lacZ* in the unique *Bam*HI site of plasmid pAN923-41B resulting in plasmids pME2868 and pME2817, respectively.

2.3.2.2 Constructs for studies on DenA in *Aspergillus nidulans*

For construction of the *denA* deletion cassette, 1000 bp of the *denA* 5´-flanking region were amplified with primers MC1/MC3 and ligated into vector pCR®–Blunt II-TOPO® resulting in plasmid pME3271. Using primers MC4/MC2, the *denA* open reading frame with 1000 bp 3´-flanking region was amplified. The *Not*I cut MC4/MC2 PCR product was ligated via *EcoR*V/*Not*I into plasmid pME3281 (see 2.3.2.3) whereas the *Cla*I/*BstE*II fragment cut out of pME3271 was ligated to the *Cla*I/*BstE*II sites resulting in plasmid pME3267. The *Neurospora crassa pyr4,* obtained from pRG3 (Waring *et al.*, 1989), was amplified using primers MC5/MC6, subcloned into pCR®–Blunt II-TOPO® and cut out of the resulting plasmid pME3273 using the restriction enzymes *Hpa*I/*Eco*RI. The *pyr4*

expression cassette was then ligated to the *Hpa*I/*Mfe*I opened plasmid pME3267, replacing the *denA* open reading frame. The resulting plasmid pME3275 contains the *denA* deletion cassette.

2.3.2.3 Constructs for studies on CandA in *Aspergillus nidulans*

A. nidulans BAC library (obtained from Clemson University, Clemson, SC, USA) was screened for *candA-C* using a gene specific probe, amplified by PCR from genomic wild-type DNA with primers ES61/ES62. A 9 kb *Hpa*I/*Not*I fragment from BAC library clone E19 of plate 9, containing the complete *candA-C* coding region was cloned into pBluescript II SK(+) with a blunted *Xho*I cutting site, resulting in plasmid pME3114. For construction of a deletion cassette for *candA-C*, the *candA-C* open reading frame, except the last four codons, was cut out of plasmid pME3114 via *Xho*I. The gene was substituted with *N. crassa pyr4* obtained from pRG3 (Waring *et al.*, 1989) via blunt ligation resulting in plasmid pME3115. In addition, a *candA-C* deletion construct containing a phleomycin resistance cassette was cloned. The *gpdA* promoter of the resistance cassette of plasmid pAN8-1 (Punt and van den Hondel, 1992) was shortened according to the results of the promoter analysis of Punt *et al.* (1990), resulting in plasmid pME3133. The shortened phleomycin resistance cassette was cut *Bgl*II/*Xba*I out of plasmid pME3133, blunted and ligated either to the *Eco*RV opened or to the *Xho*I opened and blunted pBluescript II SK(+), resulting in plasmids pME3134 and pME3281, respectively. The phleomycin marker cassette was cut *Spe*I/*Xho*I out of plasmid pME3134 and ligated to the *Spe*I/*Xho*I opened plasmid pME3120 resulting in plasmid pME3127 carrying the *candA-C* phleomycin deletion cassette. The *candA-C* complementation construct was provided with the original phleomycin resistance cassette of pAN8-1 in pME1510 (Busch *et al.*, 2003; Punt and van den Hondel, 1992). pME1510 was opened with *Xba*I and blunt end ligated to the *Not*I *candA-C* fragment from plasmid pME3114 resulting in plasmid pME3116.
For deletion of *candA-N* the 5´-flanking region of *candA-N* was amplified with the primers ES191/ES192 and cloned *Bgl*II/*Spe*I into the *Bam*HI/*Spe*I opened pME3281 resulting in plasmid pME3304. The 3´-flanking region of *candA-N* was amplified with the primers ES189/ES190 and cloned *Spe*I/*Sac*II into pME3304 resulting in pME3305. pME3305 was opened *Spe*I and ligated to *pyr-4* of pRG3 resulting in plasmid pME3306. For the

complementation of the *candA-N* deletion strain, the *candA*-N open reading frame together with the 3´-end was amplified with the primers ES195/ES190 and ligated *Spe*I/*Sac*II into pBluescript II SK(+) resulting in plasmid pME3307. The *candA-N* open reading frame with the *candA-N* 3´-flanking region was then cut with *Spe*I/*Xba*I out of the plasmid and was ligated to the *Spe*I/*Xba*I opened plasmid pME3305 resulting in plasmid pME3308.
For the *candA-C*::*gfp2-5* fusion construct, plasmid pME3114 containing a 9000 bp *Hpa*I/*Not*I genomic fragment was cut *Mlu*I/*Cla*I and ligated to the PCR products of ES96/ES131 (*Mlu*I/*Spe*I) and ES132/ES99 (*Spe*I/*Cla*I) amplifying the 3´-end of the *candA-C* gene and the 3´-flanking region of the *candA-C* gene respectively. This way, the stop-codon of the *candA-C* reading frame was replaced by a *Spe*I cutting site in the resulting plasmid pME3117. *gfp2-5* obtained from plasmid pMCB17 (Fernandez-Abalos *et al.*, 1998) was amplified with primers ES155/156 and ligated via *Spe*I to plasmid pME3117 resulting in plasmid pME3120. As a nuclear marker, the mRFP1 (monomeric red fluorescent protein) coding sequence (Campbell *et al.*, 2002) was fused to the histone H2A coding sequence in plasmid pDM8 (Veith *et al.*, 2005). For this, pDM8 was opened *Pac*I/*Asc*I and ligated to histone H2A coding sequence amplified with primers ES165/ES166 from plasmid CFP-H2A (Su *et al.*, 2004) resulting in plasmid pME3125. For the construction of a cassette expressing a CandA-N::C fusion protein, the 5´-promoter region of *candA-N* together with the *candA-N* gene were amplified with primers ES192/ES194 and ligated *Bam*HI/*Spe*I into pBluescript II SK(+) containing a *ptrA* marker in the *Not*I restriction site. This plasmid was named pME3309. It was opened *Spe*I and ligated to the *Spe*I cut PCR fragment of *candA-C* gene and its terminator, amplified with primers ES204/ES205 from plasmid pME3114. The resulting plasmid contains a fusion of *candA-N* to *candA-C* under the control of the *candA-N* promoter and the *candA-C* terminator and was named pME3310. For complementation of the *candA-N*/*candA-C* double deletion strain with *candA-N*, the *ptrA* resistance marker was blunt end ligated into the *Sma*I site of plasmid pME3308 resulting in plasmid pME3311.

Tab. 7: Primers used in this study.

Primer	Sequence	bp
ES1	5′- CAT ATC TAC TGG AGC TAT ACG C -3′	22
ES2	5′- CAA GGC CTG ATT GGT AGA GC -3′	20
ES5	5′- GAT CTA GAT ATC GAA GTT GGC TTG TCA AC -3′	29
ES6	5′- TGT GCG GCA GTC TTG AGT GG -3′	20
ES7	5′- TCT GAG TCG TTC AAA GCA TCG -3′	21
ES8	5′- CTA TGA GTT AAC AGA CGA GCG GAT CGC -3′	27
ES55	5′- TGA GGA TCC AGA GAC CAA ATC CGT CCC -3′	27
ES56	5′- TAG GGA TCC CAT TGC GTC TAT GCT GGA -3′	27
ES59	5′- TGA GGA TCC GGC TTT CTC GTC AAC CAG -3′	27
ES60	5′- TAG GGA TCC CAT GAT GAT TGT CAG GTG -3′	27
ES61	5′- ATG TCT TCC GAC GCA ATG -3′	18
ES62	5′- GAA CGG AAG AAG AGC ATC -3′	18
ES96	5′- TCA TTT GAG AAA CGA TCC AAG G -3′	22
ES99	5′- CTC ATG GAG CAG CTC AAT CG -3′	20
ES110	5′- CTT GAA TTC ATG TCT TCC GAC GCA ATG TC -3′	29
ES111	5′- CAA GAA TTC TTA GAA CTC CGA CTC GAG GT -3′	29
ES117	5′- GTT GTT AAC AAT GCA GCA GAA CTC GAG ATC -3′	30
ES118	5′- CTA GTT AAC CTA AGC TAC ATA TTG GTA TCT G -3′	31
ES119	5′- GTT GGA TCC GTA TGC AGC AGA ACT CGA GAT C -3′	31
ES120	5′- CAT GGA TCC TAA GCT ACA TAT TGG TAT CTG -3′	30
ES131	5′- CCA CTA GTG AAC TCC GAC TCG AGG TTA CTC AG -3′	32
ES132	5′- GGA CTA GTG ACA AGA CTT TGC CAT TGG ATT TTC -3′	33
ES139	5′- AGT CTC GAG ATG AAG AAA GCA GAA ATC CGT C -3′	31
ES155	5′- GGA CTA GTA TGA GTA AAG GAG AAG AAC TTT TC -3′	32
ES156	5′- CTA CTA GTT TAT TTG TAT AGT TCA TCC ATG CC -3′	32
ES165	5′- AGG CGC GCC GGG CAT GAC TGG CGG CAA ATC TG -3′	32
ES166	5′- CTT AAT TAA TTA CAG CTC CTG GCT GCC C -3′	28
ES169	5′- GAC CTC GAG CCT GCA ATG TAA GCG ATT TCA TCG -3′	33
ES186	5′- GAT CTC GAG ATG GGA GAT CGA CAC ACG ATC C -3′	31
ES187	5′- CTA CTC GAG CTA CAT ACC AGT AAT TAG CTT TTC -3′	33
ES189	5′- CCA CTA GTT CTA GCA TTT ATT TAT GGG CTG G -3′	31
ES190	5′- CCA CCG CGG CTC CCT TCA TAT ATG CAT AG -3′	29
ES191	5′- GAA CTA GTC GTG AAC AGA ACC CCG CGC TG -3′	29
ES192	5′- GAG GAT CCG CGA TTG ACA CCG TAG ATG ATA ATC -3′	33
ES194	5′- CAT ACT AGT GGC AAC TGA CGG TAT TGG TGG -3′	30
ES195	5′- GAT ACT AGT ATG GGA GAT CGA CAC ACG ATC C -3′	31
ES204	5′- ACT AGT ATG TCT TCC GAC GCA ATG TCG -3′	27
ES205	5′- ACT AGT CTC ATG GAG CAG CTC AAT CG -3′	26
MC1	5′- GTA ATC GAT GTC ATC GCT GAA AAG GG -3′	26
MC2	5′- CCT GCG GCC GCT CTA CAT GGG TAT GAC TAG AG -3′	32
MC3	5′- GTT GGT CAC CGA TGG TCT AAT CAC GAA CCT C -3′	31
MC4	5′- CAA GGT GAC CAT GCG CGA CGG AGG GCT AGG -3′	30
MC5	5′- GTA GTT AAC TAT GCG GCA TCA GAG CAG -3′	27
MC6	5′- CAA GGT CAC CGC TAA TTA ACT GAG TAG AGA AC -3′	32
MC30	5′- CAA TGC GCG ACG GAG GGC TAG G -3′	22
MC31	5′- TCA CTC AAT ACG CGG CGG ACT C -3′	22
MC32	5′- CTC AAT ACG CGG CGG ACT CC -3′	20

Tab. 8: Plasmids used and constructed in this study.

Plasmid	Description	Reference
pAN8-1	fungal phleomycin resistance cassette; *gpdA*(p)::*ble*::*trpC*(t), *amp*^{R}	Punt and van den Hondel (1992)
pAN923-41B	*lacZ* expression analysis vector; *amp*^{R}	van Gorcom *et al.* (1986)
pBluescript II SK(+)	cloning vector; *amp*^{R}	Stratagene, La Jolla, CA, USA
pCR®-Blunt II-TOPO®	cloning vector for blunt end DNA fragments, *zeo*^{R}, *kan*^{R}	Invitrogen GmbH, Karlsruhe, D
pDM8	*alcA*(p)::*mRFP1*::*apsB1.5* in pMCB17apx-apsB; *pyr4*	Veith *et al.* (2005)
pEG202	2-hybrid bait vector (*amp*^{R}; *ADH*(p)::*lexA*::*ADH*(t)*, HIS3*, 2 µm)	Golemis and Brent, (1996); Gyuris *et al.*, (1993)
pGEM-Zf (+)	cloning vector; *amp*^{R}	Promega, Madison, WI, USA
pJG4-5	2-hybrid prey vector (*amp*^{R}; *GAL1(p)*::*B42*::*ADH(t)*, *TRP1*, 2 µm)	Golemis and Brent, (1996); Gyuris *et al.*, (1993)
pMCB17	*gfp2-5* containing plasmid	Fernandez-Abalos *et al.* (1998)
pME1510	cloning vector containing phleomycin resistance cassette from pAN8-1; *gpdA*(p)::*ble*::*trpC*(t), *amp*^{R}	Busch *et al*. (2003)
pME2355	*csnD* in pEG202	Busch *et al*. (2007)
pME2357	*csnD* in pJG4-5	Busch *et al*. (2007)
pME2409	for construction of *csnB* deletion cassette; *amp*^{R}, *zeo*^{R}	Krappmann and Braus (2003)
pME2501	*csnA* in pJG4-5	Busch *et al*. (2007)
pME2502	*csnA* in pEG202	Busch *et al*. (2007)
pME2814	*csnB* deletion construct; obtained by recombination of pME2820 fragment with pME2821, *csnB*(p)::*pyr4*::*csnB*(t), *amp*^{R}	This work
pME2815	*csnB* complementation; *csnB* genomic fragment in pME1510, *gpdA*(p)::*ble*::*trpC*(t), *amp*^{R}	This work
pME2817	*csnE*(p)*::lacZ* fusion; 1 kb 5´*csnE* via *Bam*HI in pAN923-41B, *amp*^{R}	This work
pME2820	for construction of *csnB* deletion cassette; PCR fragments ES5/ES6 and ES7/ES8 via *Eco*RV and *Hpa*I in plasmid pME2409, *amp*^{R}, *zeo*^{R}	This work
pME2821	*csnB* genomic fragment; 7727 bp *csnB* containing *Aat*II fragment in pGEM-Zf (+), *amp*^{R}	This work
pME2868	*csnA*(p)*::lacZ* fusion;1000 bp 5´ *csnA* via *Bam*HI in pAN923-41B, *amp*^{R}	This work
pME2972	*csnB* in pEG202	Busch *et al*. (2007)
pME2973	*csnC* in pEG202	Busch *et al*. (2007)
pME2974	*csnE* in pEG202	Busch *et al*. (2007)
pME2975	*csnF* in pEG202	Busch *et al*. (2007)
pME2976	*csnG* in pEG202	Busch *et al*. (2007)
pME2977	*csnH* in pEG202	Busch *et al*. (2007)
pME2978	*csnB* in pJG4-5	Busch *et al*. (2007)
pME2979	*csnC* in pJG4-5	Busch *et al*. (2007)
pME2980	*csnE* in pJG4-5	Busch *et al*. (2007)
pME2981	*csnF* in pJG4-5	Busch *et al*. (2007)
pME2982	*csnG* in pJG4-5	Busch *et al*. (2007)
pME2983	*csnH* in pJG4-5	Busch *et al*. (2007)
pME3114	*candA-C* genomic fragment; PCR fragment ES61/ES62 in pBluescript II SK(+), *amp*^{R}	This work

Tab. 8: Plasmids used and constructed in this study, continued.

Plasmid	Description	Reference
pME3115	*candA-C* deletion cassette, *candA-C*(p)::*pyr4*::*candA-C*(t) in pME3114, *amp*^{R}	This work
pME3116	*candA-C* complementation construct; *candA-C*(p)::*candA-C*::*candA-C*(t), *ble* marker cassette in pBluescript II SK(+), *amp*^{R}	This work
pME3117	for *candA-C*::GFP fusion; *candA-C*(p)::*candA-C*::*Spe*I::*candA-C*(t), *amp*^{R}	This work
pME3118	*csnB* probe; 820 bp PCR product ES1/ES2 in pBluescript II SK(+), *amp*^{R}	This work
pME3120	*candA-C*::*gfp* fusion construct; *candA-C*(p)::*candA-C*::*Spe*I::*gfp*::*Spe*I::*candA-C*(t), GFP in pME3117, *amp*^{R}	This work
pME3121	*candA-C* in pEG202	This work
pME3122	*candA-C* in pJG4-5	This work
pME3125	mRFP nuclear marker; *alcA*(p)::*mRFP1*::*H2A* in pDM8, *pyr4, amp*^{R}	This work
pME3126	*culD* in pJG4-5	This work
pME3127	*candA-C* deletion cassette, *candA-C*(p)::*gpdA*(p)::*ble*::*trpC*(t)::*candA-C*(t); *ble* marker from pME3134 in pME3120, *amp*^{R}	This work
pME3133	phleomycin resistance cassette, shortened promoter in pAN8-1; shortened *gpdA*(p)::*ble*::*trpC*(t), *amp*^{R}	This work
pME3134	phleomycin resistance cassette, shortened promoter in *Eco*RV site of pBluescript II SK(+); shortened *gpdA*(p)::*ble*::*trpC*(t), *amp*^{R}	This work
pME3267	*denA*(p)::*denA*::*denA*(t); open reading frame with approx. 1000 bp 5´ and 3´ of the gene, containing a *Bst*EII restriction site 5´of the start codon in pME3281, *amp*^{R}	This work
pME3271	*denA*(p); PCR fragment MC1/MC3 in pCR®–BluntII-TOPO®, *zeo*^{R}, *kan*^{R}	This work
pME3273	*pyr4* marker cassette; in pCR®-BluntII-TOPO®, *zeo*^{R}, *kan*^{R}	This work
pME3275	*denA* deletion construct; *denA*(p)::*pyr4*::*denA*(t) in pME3267, *amp*^{R}	This work
pME3278	*denA*; PCR fragment MC30/MC31 in pYES2.1/*V5-His*-TOPO®	This work
pME3279	*denA*::*V5*; PCR fragment MC30/MC32 in pYES2.1/*V5-His*-TOPO®	This work
pME3280	*culD* in pEG202	This work
pME3281	phleomycin resistance cassette, shortened promoter in blunted *Xho*I site of pBluescript II SK(+); shortened *gpdA*(p)::*ble*::*trpC*(t), *amp*^{R}	This work
pME3301	*candA-N* in pEG202	This work
pME3302	*candA-N* in pJG4-5	This work
pME3303	*culA* in pJG4-5	This work
pME3304	for construction of *candA-N* deletion cassette; *candA-N*(p), PCR fragment ES191/192 in pME3281, *ble* marker cassette, *amp*^{R}	This work
pME3305	for construction of *candA-N* deletion cassette; *candA-N*(p):: *Spe*I::*candA-N*(t), PCR fragment ES189/ES190 in pME3304, *amp*^{R}	This work
pME3306	*candA-N* deletion plasmid; *candA-N*(p)::*pyr4*::*candA-N*(t), *pyr4* in *Spe*I site of pME3305, *amp*^{R}	This work
pME3307	for *candA-N* complementation plasmid; *Spe*I::*candA-N*::*candA-N(t)*, PCR fragment ES195/ES190 in pBluescript II SK(+), *amp*^{R}, *ble* marker cassette	This work
pME3308	*candA-N* complementation plasmid; *candA-N*(p)::*Spe*I::*candA-N*::*candA-N*(t), *Spe*I/*Xba*I fragment from pME3307 in pME3305, *amp*^{R}, *ble* marker cassette	This work
pME3309	for *candA-N*::*C* fusion; *candA-N*(p)::*candA-N*, PCR fragment of ES192/ES194 in pBluescript II SK(+), *ptrA, amp*^{R}	This work
pME3310	*candA-N::C* fusion; *candA-N*(p)::*candA-N*::*candA-C*::*candA::C*(t), *ptrA*, *amp*^{R}	This work

Tab. 8: Plasmids used and constructed in this study, continued.

Plasmid	Description	Reference
pME3311	*candA-N* complementation plasmid; *candA-N*(p)::*Spe*I::*candA-N*::*candA-N*(t), *ptrA* marker in *Sma*I site of pME3308	This work
pPTRII	pPTRII, *ptrA*-vector	Takara Biokom, Junki, PL
pRG3	vector containing *pyr4* expression cassette, amp^R	Waring *et al.* (1989)
pYES2.1/*V5-His*-TOPO®	cloning vector for TA-cloning; *GAL1(p);V5*/*HIS6* Epitop-Tag; *URA3*, amp^R	Invitrogen GmbH, Karlsruhe, D

2.3.2.4 Construction of plasmids for DenA characterization in *Saccharomyces cerevisiae*

A. nidulans denA cDNA was amplified with primers MC30/MC31 from a cDNA library and integrated into vector pYES2.1/*V5-His*-TOPO® (Invitrogen GmbH, Karlsruhe, D) resulting in plasmid pME3278. To yield a *denA::V5* fusion, *denA* cDNA without its stop-codon was amplified with primers MC30/32 and integrated into vector pYES2.1/*V5-His*-TOPO® (Invitrogen GmbH, Karlsruhe, D). The plasmid was named pME3279.

2.3.2.5 Construction of plasmids used in the yeast two-hybrid tests

All cDNAs were amplified from an *A. nidulans* cDNA library (Krappmann *et al.*, 2006). *candA-C* cDNA was amplified using primers ES110/ES111, restricted with *Eco*RI and ligated into vectors pEG202 and pJG4-5 resulting in plasmids pME3121 and pME3122, respectively. *candA-N* cDNA was amplified using primers ES186/ES187 and was ligated into the *Xho*I site of vectors pEG202 and pJG4-5, resulting in plasmids pME3301 and pME3302, respectively. *culA* cDNA was amplified with primers ES139/ES169 and ligated into the *Xho*I site of vector pJG4-5 resulting in plasmid pME3303. *culD* cDNA was amplified using primers ES117/ES118 and cloned *Hpa*I into pJG4-5 resulting in plasmid pME3126. Primers ES119/ES120 were used to amplify *culD* cDNA with *Bam*HI cutting sites and the cDNA was ligated *Bam*HI into pEG202 resulting in plasmid pME3280.

2.3.3 Sequence analysis

BLAST searches were performed at and accession numbers were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). *A. nidulans* proteins were identified from the *A. nidulans* genome sequence (Galagan *et al.*, 2005) at http://www.broad.mit.edu, for sequence alignment Clustal W (http://npsa-pbil.ibcp.fr/) was used. For nuclear localization signal prediction PredictNLS (http://cubic.bioc.columbia.edu/predictNLS/) was applied. Multialignment was done using the software MultAlign (Corpet, 1988) (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). PCI domain analysis was performed using the software at the Sanger Institute (http://www.sanger.ac.uk/Software/Pfam/). DNA was sequenced at the Labor für Genomanalyse in Göttingen. Sequences were analysed with the software Lasergene (DNASTAR, Inc., Madison, WI, USA).

2.3.4 Recombinant DNA methods

Recombinant DNA technologies were performed according to the standard methods (Sambrook *et al.*, 1989). For PCR reactions *Taq* (Fermentas GmbH, St. Leon-Rot, D), *Pfu* (Fermentas GmbH, St. Leon-Rot, D) or *Kod* (Novagen, Nottingham, UK) polymerases were used. Custom oligonucleotides were ordered from Operon Europe (Köln, D). Restriction enzymes and T4 DNA ligase were ordered from Fermentas GmbH (St. Leon-Rot, D). 5´ RACE was performed using the GeneRacer Kit (Invitrogen GmbH, Karlsruhe, D) according to the manual.

2.3.5 DNA isolation and hybridisation

Plasmid DNA from *E. coli* was prepared using Qiagen Plasmid Midi or Mini Kit (Qiagen, Hilden, D) according to the manual. DNA gel extraction was performed using the QIAquick Gel Extraction Kit (Qiagen, Hilden, D). To obtain homogenized cell material of *A. nidulans*, mycelia were harvested by filtration through sterile Miracloth (Merck Chemicals, Nottingham, UK) and grinded in liquid nitrogen using mortar and pestle. Isolation of genomic DNA of *A. nidulans* was performed as described (Lee and Taylor, 1990).

For Southern blot analysis the standard technique was used (Southern, 1975). Non-radioactive labeling of probes and detection was performed with the Gene Images™ Random-Prime DNA labeling kit and the Gene Images™ CDP-Star™ Detection Kit (GE Healthcare Life Sciences, München, D). For detection of chemiluminescent signals Amersham Hyperfilm™ ECL™ was used (GE Healthcare Life Sciences, München, D). Probes used for BAC filter hybridisation were [α-^{32}P]-dATP labeled with the HexaLabel™ DNA Labeling Kit (MBI Fermentas GmbH, St. Leon-Rot, D) and detection was performed using the BioMaxMS film (Kodak Molecular Imaging, New Haven, CT, USA).

2.4 Protein methods

2.4.1 Protein isolation and analysis

For protein isolation from *S. cerevisiae*, strains were grown over night at 30°C in SC-medium with 2% glucose or 2% galactose / 1% raffinose as carbon source. From this preculture, the main culture was inoculated and grown at 30°C until an OD_{595nm} of 1 was reached. The cells were harvested by centrifugation at 3000 rpm for 4 min and resuspended in 2.5 ml breaking buffer (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 20% glycerol, 5 mM EDTA pH 8; containing freshly added 1 µl/ml β-mercaptoethanol and 5 µl/ml 200x PIM (100 mM p-aminobenzamidin-HCL, 100 mM *Na*-p-tosyl-L-lysin-chlormethylketon, 100 mM *Na*-p-tosyl-L-phenylalanin-chlormethylketone, 100 mM o-phenanthrolin and 100 mM phenylmethylsulfonyl-fluoride in DMSO)). The cell suspension was vortexed for 5 min at 4°C and centrifuged for 15 min with 13.000 rpm at 4°C. The protein containing supernatant was stored at -20°C or used immediately for further analyses. For protein isolation from *A. nidulans*, the breaking buffer described for protein isolation from *S. cerevisiae* was used. Strains were grown in liquid medium and harvested by filtration through Miracloth (Merck Chemicals, Nottingham, UK). 500 µl B^+-buffer were added to 300 µl grinded, frozen mycelia and vortexed 4 times for 15 sec. After centrifugation at 4°C for 10 min the supernatant was used directly for further analyses. Protein concentrations were determined as described (Bradford, 1976). For Western hybridization experiments, separated proteins were transferred onto a nitrocellulose membrane by electro blotting. As first antibodies goat-anti-Cdc53 IgG (Santa Cruz Biotechnology, Heidelberg, D), rabbit-

anti-Rub1 IgG (US Biological, Swampscott, MA, USA), mouse-anti-V5 IgG (Invitrogen GmbH, Karlsruhe, D) or mouse anti-GFP (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, F) were used. As secondary antibodies peroxidase-coupled donkey-anti-goat IgG-HRP (Santa Cruz Biotechnology, Heidelberg, D), goat-anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Heidelberg, D), rabbit-anti-mouse IgG-HRP (Zymed, Wien, A) or goat anti-mouse IgG antibody (Invitrogen GmbH, Karlsruhe, D) were used. As marker the PageRuler^{TM} Prestained Protein Ladder (Fermentas GmbH, St. Leon-Rot, D) was used. Detection after incubation was done using the ECL technology (GE Healthcare Life Sciences, München, D).

2.4.2 Yeast two-hybrid analysis

To identify interaction proteins, a yeast two-hybrid test was performed using the yeast two-hybrid system described by (Golemis and Brent, 1996; Gyuris *et al.*, 1993) which was based on plasmids pEG202 (bait) and pJG4-5 (prey). Plasmids were transformed into *S. cerevisiae* strain EGY48-p1840. For the interaction tests overnight cultures were washed in saline. 10 µl of liquid cultures of OD_{546} = 0.01 were dropped on test plates. All strains were tested for growth on a plate containing 2% glucose and 2% leucine as positive control and on a plate containing 2% galactose and no leucine as negative control. SC selective medium containing 2% galactose without leucine was used for the leucine growth test. SC selective medium with 2% galactose and 0.2 g/l leucine was covered with a filter paper for the β-galactosidase activity test. Plates were incubated for 2-5 days at 30°C. For the β-galactosidase activity test, the filter was frozen in liquid nitrogen and placed on a Whatman paper soaked in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) supplemented with 40 µl 1% XGal in DMF. Filters were incubated at 37°C for 1 to 6 h.

2.4.3 Specific β-galactosidase activity assay of *Aspergillus nidulans* proteins

For β-galactosidase assays protein extracts were isolated from the respective *A. nidulans* strains. The assay was carried out at 28°C as described by Miller (1972) using 0.5-5 µl of protein extracts.

2.5 Microscopic analysis

Pictures of *A. nidulans* colonies, hyphae and structures were taken with a Kappa PS30 digital camera (KAPPA opto-electronics GmbH, Gleichen, D) used in combination with an Olympus SZX12 binocular (Olympus, Hamburg, D) or a ZEISS Axiolab (ZEISS AG, Oberkochen, D) light microscope. KAPPA ImageBase software (KAPPA opto-electronics GmbH, Gleichen, D) was used for editing pictures and the calibration of magnifications. Fluorescence microscopy was conducted with a ZEISS Axiovert S100 microscope and photographs were taken with a Xillix Microimager digital camera and the Improvision Openlab 5.01 software (Improvision, Coventry, UK).

2.6 Chemical analysis

Chemical analysis was performed by Mieke Westermann and Stephanie Grond at the Institut für organische und biomolekulare Chemie, Georg-August-Universität Göttingen (Göttingen, D). 1H NMR spectra were recorded on a Varian Inova 600 and Unity 300 spectrometer, respectively, at 600 MHz and 300 MHz at 298 K (VARIAN Inova, Palo Alto, CA, USA). Chemical shifts in CD3OD are reported as δ values (ppm) relative to CH3OH (δ = 3.30) as internal reference unless stated otherwise. 13C NMR spectra were recorded at 150.8 MHz. Chemical shifts in CD3OD are reported as δ values relative to CD3OD (δ = 49.0); the multiplicity of the signals was determined by the HSQC (edited) technique. 2D NMR spectra: H,H-COSY (H,H-Correlated Spectroscopy, gCOSY pulse sequence), HSQC (Heteronuclear Singular Quantum Coherence, gHSQC pulse sequence), HMBC (Heteronuclear Multiple Bond Connectivity, g HMBC pulse sequence). EI-MS: ionizing voltage of 70 eV (Finnigan MAT 95). UV spectra were obtained in methanol on a Varian Cary 3E (VARIAN, Palo Alto, CA, USA). Infrared spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer as KBR pellets (Perkin Elmer, Waltham, MA, USA). Solvents for extraction and chromatography were of technical grade and distilled before use. Thin layer chromatography (TLC) was carried out: type A) on silica gel 60 F_{254} plates/ 0.2 mm (Merck, Darmstadt, D) using $CHCl_3$: MeOH (9:1) as solvent or type B) on RP-18 F_{254S}: plates/0.2 mm (Merck, Darmstadt, D) using MeOH:H_2O (7:3) as solvent. Silica gel 60/0.040–0.063 mm (Machery&Nagel, Düren, D) and Sephadex LH-20 (Pharmacia, D) were used for column chromatography. Flash column chromatography was

performed using silica gel 60/0.025–0.040 mm (Machery& Nagel, Düren, D). MPLC was performed with Knauer Wellchrom Maxi-Star K 1000 pumps (Knauer, Berlin, D) using a Merck LiChroprep RP-18/0.040-0.063 mm column (B, 310-25). HPLC was carried out on JASCO HPLC-systems with PU-2080 Plus or PU-1587 pumps (JASCO, Gross-Umstadt, D), respectively, using column A (Macherey&Nagel, Superspher-100 RP-18 endc., 4 µm, 100 x 2 mm, flow rate: 3.0 ml/min), column B (Macherey&Nagel, Superspher-100 RP-18 endc., 4 µm, 100 x 20 mm, flow rate: 18.0 ml/min or column C (Jasco Kromasil, 100 C-18, 5 µm, 250 x 8 mm, flow rate: 2.5 ml/min). High performance liquid chromatography/mass spectrometry/diode array detection (HPLC-MS-DAD) analysis was carried out using Flux Instruments Rheos 4000, PDA detector (Finnigan Surveyor) and MS-LC-Q detector (Finnigan) with Xcalibur^{TM} 1.3 software (Finnigan).

2.7 Metabolite analysis and isolation

In general, 2 l minimal medium cultures containing 10 mM NH_4Cl or 10 mM $NaNO_3$ as nitrogen source grown in the light or in the dark were used for metabolite analysis and isolation. The culture broth was separated from the mycelium by filtration using a Miracloth filter (Calbiochem, Merck Biosciences Ltd., Nottingham, UK). Metabolite analysis and isolation was performed by Mieke Westermann and Stephanie Grond at the Institut für organische und biomolekulare Chemie, Georg-August-Universität Göttingen (Göttingen, D). The mycelium was extracted with MeOH:acetone (3:2, 3 x 1 l). The culture filtrate was adjusted to pH = 5.0 and extracted with ethyl acetate (3 x 2 l), the solvent was removed by evaporation to yield the crude residue. The crude extracts were dissolved each in 1 ml of MeOH and analyzed by TLC. For HPLC-MS-DAD analysis the crude extracts (5 mg/ml) and pure compounds (1 mg/ml) were dissolved in MeOH. Analytical HPLC was performed to investigate crude extracts, fractions and pure compounds. Preparative purification of compounds proceeded from subjection to silica gel chromatography (50 x 2 cm, cyclohexane:ethyl acetate:methanol (5:10:1) which yielded diorcinol. Subsequent size exclusion chromatography (100 x 2.5 cm, Sephadex LH-20, Acetone) and reverse phase HPLC gave the other compounds. The metabolites orcinol (up to 1.3 mg/l), diorcinol (up to 16 mg/l), cordyol C (up to 2.4 mg/l), violaceol I (up to 0.8 mg/l) and violaceol II (up to 2.0 mg/l) were obtained as pure compounds. Additionally,

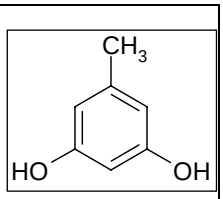
fractions with mixtures of known and presumably unknown metabolites were obtained from all chromatographic steps.

HPLC programs: Solution A: H_2O, solution B: acetonitrile, solution C: methanol. HPLC-columns: A: Jasco Kromasil 100 C-18, 5 µm, 250 x 8 mm; B: Machery-Nagel Superspher-100, RP-18 endc., 4 µm, 100 x 2 mm; C: Machery-Nagel Supersphere-100 RP18 endc., 4 µl, 100 x 20 mm.

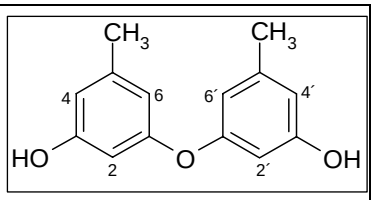
Isolation of orcinol and cordyol: 20% to 100% B in 25 min, 5 min 100% B, 100% B to 20% B in 2 min, 20% B 8 min, column A. Isolation of violaceol I and violaceol II: 20% to 60% B (A, B with 0.05% HCOOH) in 25 min, 60% to 100% B in 2 min, 3 min 100% B, 100% B to 20% B in 5 min, 20% B 5 min, column C. Physico-chemical properties of orcinol, diorcinol, cordyol C, violaceol I and violaceol II are summarized in Tab. 9 - Tab. 13.

For plate diffusion assays, 50 µg of the desired compound was solved in acetone or MeOH and dropped on 0.5 mm thick paper disks with a diameter of 6 mm. These were dried under sterile conditions and placed on agar plates inoculated with the respective test organism *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* or *Candida albicans*.

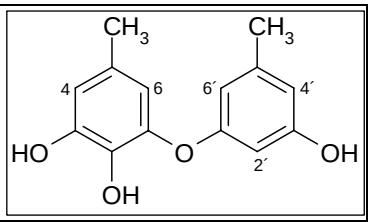
Tab. 9: Orcinol [1,3-Dihydroxy-5-methylbenzol]

	$C_7H_8O_2$ (124.14), colorless solid, turns orange in MeOH solution. 
R_f	0.29 ($CHCl_3$:MeOH = 9:1)
Anisaldehyde staining reagent	yellow-orange
UV	(MeOH): λ_{max} (lg ε) = 207 (sh, 2.65), 280 (0.27) nm (MeOH/ HCl): λ_{max} (lg ε) = 207 (sh, 2.84), 275 (0.20) nm (MeOH/ NaOH): λ_{max} (lg ε) = 215 (sh, 2.20), 286 (0.37) nm
IR (KBr)	$\tilde{\nu}$ = 3446, 2625 (sh), 1157, 1017 cm^{-1}
EI-MS	m/z = 124.1 $[M]^+$, 123.0 $[M-H]^+$
^{1}H-NMR (CD_3OD, 600 MHz)	δ = 2.16 (s, 3H, 7-H_3), 6.04 (t, *J* = 2.0 Hz, 1H, 2-H), 6.10 (dd, *J* = 2.0, 1.0 Hz, 2H, 4-H, 6-H) ppm
^{13}C-NMR (CD_3OD, 125.7 MHz)	δ = 21.59 (q, C-7), 100.66 (d, C-2), 108.49 (d, C-4, C-6), 141.11 (s, C-5), 159.32 (s, C-1, C-3) ppm

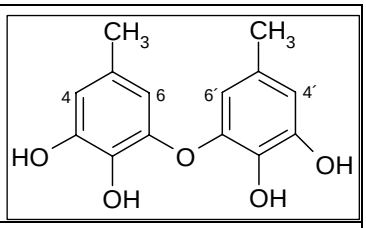
Tab. 10: Diorcinol [3,3´-Dihydroxy-5,5´-dimethyldiphenylether]

	$C_{14}H_{14}O_3$ (230.27), colorless solid, turns orange in MeOH solution. 
R_f	0.42 ($CHCl_3$:MeOH = 9:1)
Anisaldehyde staining reagent	orange-red
UV	(MeOH): λ_{max} (lg ε) = 212 (5.61), 276 (0.63) nm (MeOH/ HCl): λ_{max} (lg ε) = 211 (5.88), 275 (0.49) nm (MeOH/ NaOH): λ_{max} (lg ε) = 220 (5.57), 291 (1.09) nm
IR (KBr)	$\tilde{\nu}$ = 3424, 1601, 1464 (sh), 1324, 1156 (sh), 1038, 837 cm^{-1}
EI-MS	m/z = 230.1 $[M]^+$
optical rotation value	$[\alpha]_D^{20}$ = -2° (c = 1, MeOH)
^{1}H-NMR (CD_3OD, 600 MHz)	δ = 2.20 (s, 6H, 7-H_3, 7´-CH_3), 6.18 (t, *J* = 2.0 Hz, 2H, 2-H, 2´-H), 6.25 (dd, *J* = 2.0, 1.0 Hz, 2H, 6-H, 6´-H), 6.34 (dd, *J* = 2.0, 1.0 Hz, 2H, 4-H, 4´-H) ppm
^{13}C-NMR (CD_3OD, 125.7 MHz)	δ = 21.54 (q, C-7, C-7´), 104.23 (d, C-2, C-2´), 111.76 (d, C-6, C-6´), 111.97 (d, C-4, C-4´), 141.62 (s, C-5, C-5´) 159.54 (s, C-3, C-3´), 159.63 (s, C-1, C-1´) ppm

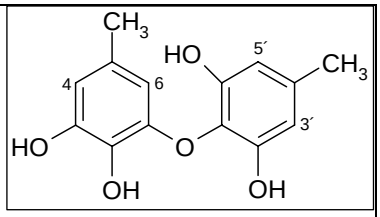
Tab. 11: Cordyol C [2,3,3´-Trihydroxy-5,5´-dimethyldiphenylether]

	$C_{14}H_{14}O_4$ (246.27) colorless solid, turns deep-red in MeOH solution. 
R_f	0.27 ($CHCl_3$:MeOH = 9:1)
Anisaldehyde staining reagent	orange
UV	(MeOH): λ_{max} (lg ε) = 205 (2.74), 278 (0.38), 462 (0.08) nm (MeOH/ HCl): λ_{max} (lg ε) = 204 (2.59), 280 (0.22), 465 (-0.02) nm (MeOH/ NaOH): λ_{max} (lg ε) = 219 (1.29), 282 (0.37), 489 (0.13) nm
IR (KBr)	$\tilde{\nu}$ = 3446, 1636 (sh), 1384, 1136 (sh) cm^{-1}
EI-MS	m/z = 246.2 $[M]^+$
optical rotation value	$[\alpha]_D^{20}$ = +4° (c = 0.50, MeOH)
^{1}H-NMR (CD_3OD, 600 MHz)	δ = 2.14 (s, 3H, 7-H_3), 2.20 (s, 3H, 7´-CH_3), 6.15 (t, *J* = 2 Hz, 1H, 2´-H), 6.20 (d, *J* = 2 Hz, 1H, 4-H), 6.24 (br. s, 1H, 6-H), 6.29 (br. s, 1H, 4´-H), 6.44 (d, *J* = 2 Hz, 1H, 6´-H) ppm
^{13}C-NMR (CD_3OD, 125.7 MHz)	δ = 20.92 (q, C-7), 21.59 (q, C-7`), 102.70 (d, C-2´), 110.28 (d, C-6), 111.17 (d, C-6´), 113.04 (d, C-4´), 113.43 (d, C-4), 130.01 (s, C-5), 135.98 (s, C-2), 141.32 (s, C-5´), 145.29 (s, C-1), 147.70 (s, C-3), 159.38 (s, C-1´), 160.56 (s, C-3´) ppm

Tab. 12: Violaceol I [2,2´,3,3´-Tetrahydroxy-5,5´-dimethyldiphenylether, Ethericin A, Aspermutarubrol]

	colorless solid, turns yellow in MeOH solution. $C_{14}H_{14}O_5$ (262.26) 
R_f	0.16 ($CHCl_3$:MeOH = 9:1)
Anisaldehyde staining reagent	brownish-orange
UV	(MeOH): λ_{max} (lg ε) = 216 (7.28) nm (MeOH/ HCl): λ_{max} (lg ε) = 214 (7.90), 277 (0.74) nm (MeOH/ NaOH): λ_{max} (lg ε) = 220 (4.46), 290 (0.90) nm
IR (KBr)	$\tilde{\nu}$ = 3445, 1631, 1384 (sh), 1017 cm^{-1}
EI-MS	m/z = 262.1 $[M]^+$, 124.1 $[M-138]^+$, 280.2 $[M+NH_4]^+$
optical rotation value	$[\alpha]_D^{20}$ = -3.75° (c = 0.80, MeOH)
^{1}H-NMR (CD_3OD, 300 MHz)	δ = 2.12 (s, 6H, 7- H_3, 7´-H_3), 6.14 (s, 2H, 6-H, 6´-H), 6.39 (s, 2H, 4-H, 4´-H) ppm
^{13}C-NMR (CD_3OD, 125.7 MHz)	δ = 21.02 (q, C-7, C-7´), 111.60 (d, C-6, C-6´), 112.40 (d, C-4, C-4´), 129.89 (s, C-5, C-5´), 134.92 (s, C-2, C-2´), 146.54 (s, C-1, C-1´), 147.48 (s, C-3, C-3´) ppm

Tab. 13: Violaceol II [2,2´,3,6´-Tetrahydroxy-4´,5-dimethyldiphenylether]

	$C_{14}H_{14}O_5$ (262.26) colorless solid, turns yellow in MeOH solution. 
R_f	0.19 ($CHCl_3$:MeOH = 9:1)
Anisaldehyde staining reagent	orange-brown
UV	(MeOH): λ_{max} (lg ε) = 205 (1.65), 347 (0.13) nm (MeOH/ HCl): λ_{max} (lg ε) = 206 (1.72), 283 (0.03), 347 (-0.09) nm (MeOH/ NaOH): λ_{max} (lg ε) = 221 (0.66), 332 (0.02), 347 (0.02) nm
IR (KBr)	$\tilde{\nu}$ = 3447, 1636, 1384 (sh) cm^{-1}
EI-MS	m/z = 262.1 $[M]^+$, 124.0 $[M-138]^+$
optical rotation value	$[\alpha]_D^{20}$ = -6° (c = 0.33, MeOH)
^{1}H-NMR (CD_3OD, 300 MHz)	δ = 2.05 (s, 3H, 7-H_3), 2.20 (s, 3H, 4´-CH_3), 5.89 (s, 1H, 4-H), 6.26 (s, 2H, 3`-H, 5´-H), 6.30 (s, 1H, 6-H) ppm
^{13}C-NMR (CD_3OD, 125.7 MHz)	δ = 21.21 (q, C-7´), 21.40 (q, C-7), 107.83 (d, C-6), 109.64 (d, C-3´, C-5´), 111.21 (d, C-4), 129.77 (s, C-1´), 130.06 (s, C-5), 132.78 (s, C-4´), 136.64 (s, C-1), 147.10 (s, C-2), 148.25 (s, C-3), 151.58 (s, C-2´,C-6´) ppm

3 Results

3.1 Isolation and characterization of the fungal COP9 signalosome subunit CsnB

3.1.1 *Aspergillus nidulans csnB* encodes a PCI domain subunit of the COP9 signalosome

To investigate if the second largest subunit of the COP9 signalosome is conserved in *A. nidulans*, the genome database was searched for a protein with high similarity to human Csn2. The *A. nidulans* gene AN4783.3 was identified as the gene, coding for a protein with high amino acid identity to subunit 2 of CSN of higher eukaryotes (Fig. 15). According to the *Aspergillus* nomenclature the protein was named CsnB.

The *csnB* cDNA was amplified from an *A. nidulans* cDNA library (Krappmann and Braus, 2003), cloned and sequenced. The open reading frame of 1683 bp is disrupted by three introns of 61 bp, 52 bp and 49 bp at positions +44, +264 and +377 relative to the start codon resulting in a 1521 bp long mRNA. The mRNA codes for a 507 aa protein with a calculated mass of 58,1 kDa. The protein contains a conserved PCI domain (Hofmann and Bucher, 1998) from amino acid 314 to 417 (Fig. 14).

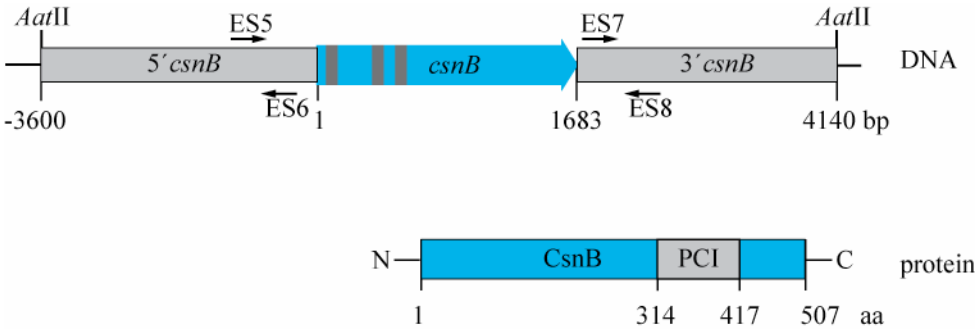


Fig. 14: Scheme of *csnB* gene and protein of *Aspergillus nidulans*.

csnB gene and CsnB protein are depicted in blue, introns of the gene and the PCI domain in the protein are indicated in grey. Restriction sites (*Aat*II) and primers used for cloning are indicated. aa, amino acid; bp, base pairs.

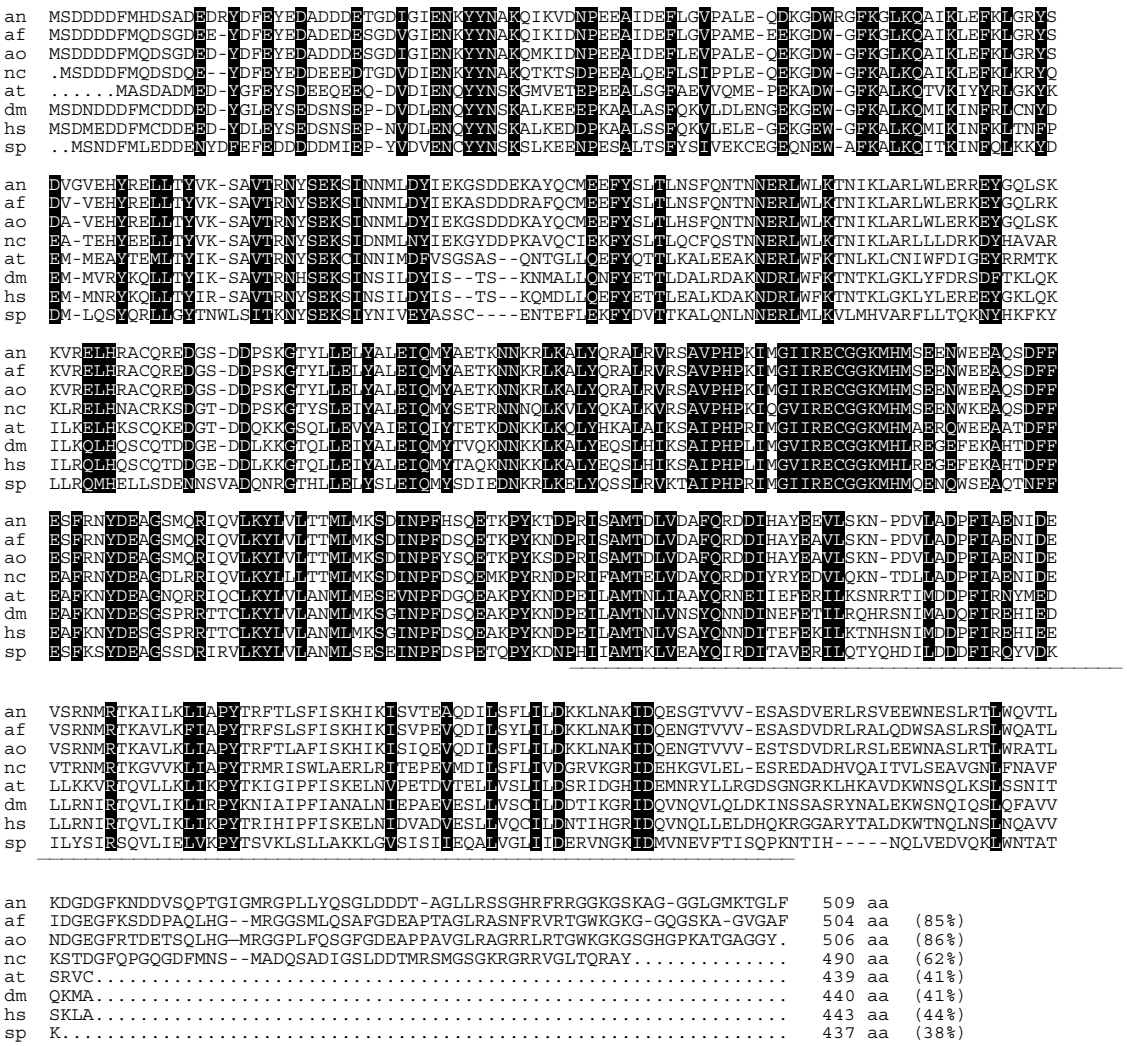


Fig. 15: *A. nidulans* CsnB shows high amino acid identities to Csn2 sequences of other eukaryotes.

Multialignment of the amino acid sequence of *Aspergillus nidulans* CsnB (an, AN4783.3) to the corresponding Csn2 sequences of *Aspergillus fumigatus* (af, EAL92898), *Aspergillus oryzae* (ao, BAE63528), *Neurospora crassa* (nc, XM324772), *Arabidopsis thaliana* (at, AF395058), *Drosophila melanogaster* (dm, AF129079), *Homo sapiens* (hs, AF084260) and *Schizosaccharomyces pombe* (sp, AF314168) is shown. Residues with consensus value ≥ 90% are indicated by black boxes. The conserved PCI domain is underlined. Identities of the respective protein sequences to *A. nidulans* CsnB are given in parentheses.

3.1.2 *Aspergillus nidulans* strains deleted for *csnB* and *csnA*/*csnB* are blocked in sexual development and produce red colored hyphae

Deletion of subunits *csnA*, *csnD* or *csnE* leads to a typical *csn* deletion phenotype, characterized by a block in sexual development and the production of a red color (Busch *et al.*, 2003; Draht, 2005). To investigate the role of subunit CsnB in growth and development and to find out whether deletion of this subunit also leads to the typical *csn* deletion phenotype, a *csnB* deletion strain and a *csnA*/*csnB* double deletion strain were constructed. The resulting strains were characterized and compared to the phenotype of the other available *csn* deletion strains.

Both, the *csnB* and *csnA*/*csnB* deletion strains show defects identical to the already characterized *csn* deletion strains. The strains grow wild-type like in vegetative cultures (Fig. 16 A, B). When grown on an air medium interface, the deletion strains produce beside wild-type like hyphae also shortened, knobby cells. In many of these hyphae the accumulation of a red color can be observed. While this color accumulates mainly in the hyphae, a part of it is also released into the medium (Fig. 16 C, D).

In addition to that, the Δ*csnB* and Δ*csnA*/Δ*csnB* mutants are blocked in sexual development. Grown on an agar plate under sexual development inducing conditions, the deletion strains produce primordia in a nest, including Hülle cells. But at this point, development stops and the primordium does not become a mature cleistothecium (Fig. 16 E, F). These results suggest a similar role of CsnB and the already investigated CSN subunits in the regulation of growth and development of *A. nidulans*.

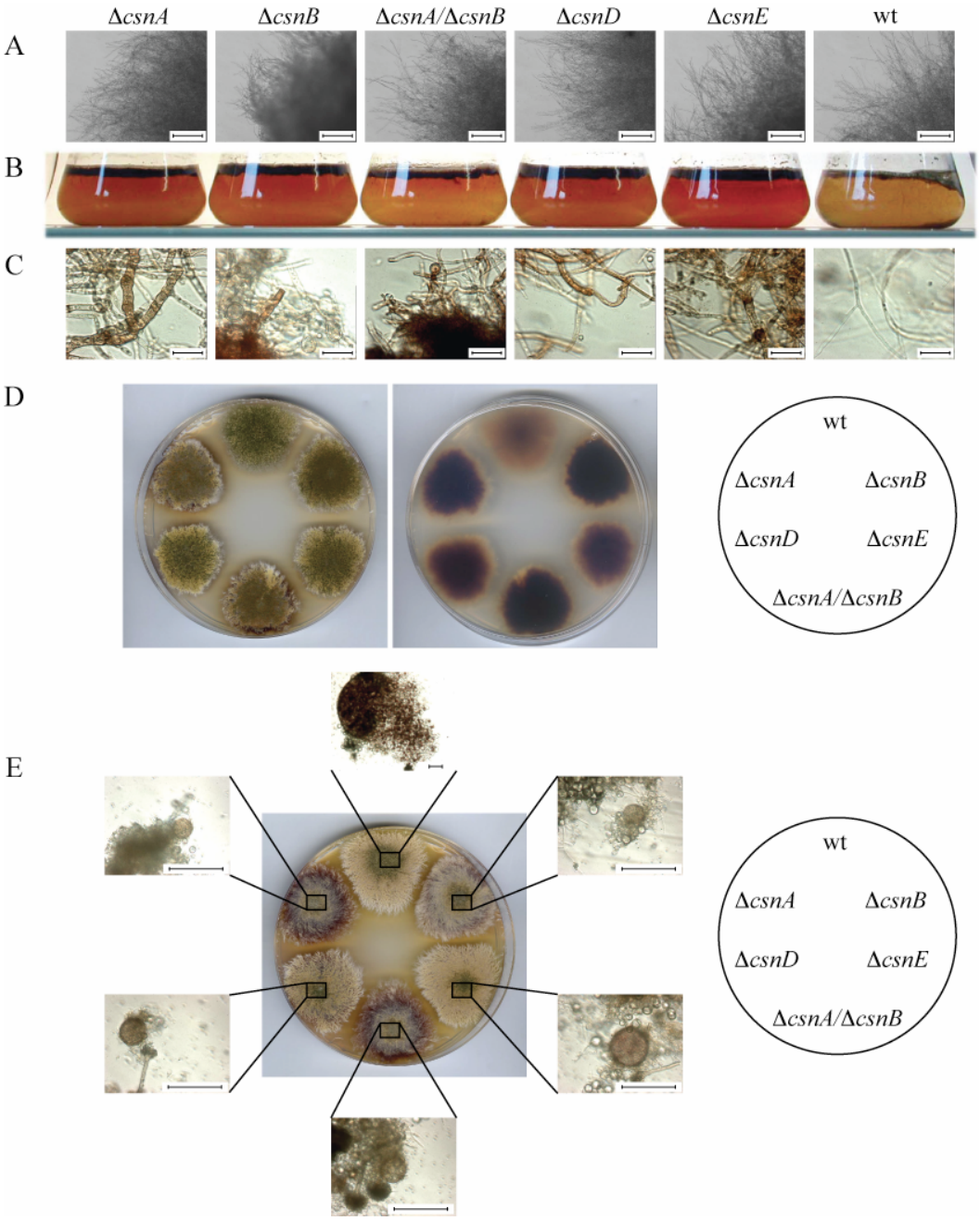


Fig. 16: CSN subunit deletion strains of *A. nidulans* show pleiotropic defects.

Strains AGB234 (Δ*csnA*), AGB238 (Δ*csnB*), AGB195 (Δ*csnD*), AGB209 (Δ*csnE*) and AGB250 (Δ*csnA*/Δ*csnB*) were compared to the wild-type AGB160 (wt) grown in or on minimal medium containing nitrate as a nitrogen source. A. Mutant strains grown for 24 h in vegetative, liquid cultures grow wild-type like. Scale bars: 200 µm. B. Mutant strains grown for 7 days at 37°C on the surface of liquid medium in flasks in the light produce a red color that accumulates mainly in the mycelium but is also released into the medium that becomes reddish. C. The microscopic picture shows that this mycelium consists of hyphae containing shortened, knobby cells. The red color accumulates in some cells and hyphae. The wild-type cells are longer and do not produce a red dye. Scale bars: 10 µm D. Picture of an agar plate from the front (left) and the back side (right). Mutant strains are reddish compared to the wild-type when grown on an agar plate for 5 days at 37°C in the light. E. Strains were grown for 5 days at 37°C under sexual development inducing conditions (center). Mature cleistothecia with ascospores are produced by the wild-type while all mutant strains develop only primordia and Hülle cells (small pictures). Scale bars: 50 µm.

3.1.3 CsnB interacts with CulD in the yeast two-hybrid test

To perform its deneddylase function, the COP9 signalosome interacts with cullin containing ubiquitin ligases. In human cells, binding of Csn2 to Cul1 is crucial for cullin deneddylation (Yang *et al.*, 2002). To investigate whether subunits of the fungal COP9 signalosome are able to interact with cullins, the yeast two-hybrid system was used. The fungal cullin CulD was tested in this experiment as a representative for the three cullins of *A. nidulans*. *culD* and *csn* cDNAs were fused to the activation domains (AD) and DNA binding domains (DBD) of the yeast two-hybrid plasmids pEG202 (bait) and pJG4-5 (prey), respectively (Gyuris *et al.*, 1993). Interactions were tested by leucine prototrophy and *β*–galactosidase activity. In both test systems CulD and CsnB show a strong interaction (Fig. 17). In the less sensitive *β*–galactosidase activity test a positive signal for the interaction of CulD and CsnD was obtained when CulD was cloned as prey. Because this result could neither be reproduced in the more sensitive leucine prototrophy growth test nor when CulD was cloned as bait, it might be an unspecific interaction that was not further investigated. The interaction of CsnB and CulD indicates that in *A. nidulans* the binding of the COP9 signalosome to the cullins is mediated by CsnB in analogy to mammals.

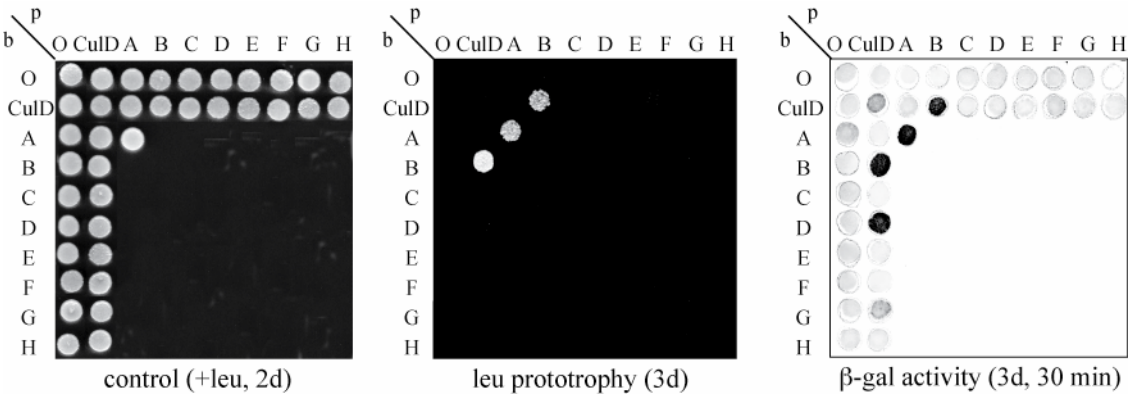


Fig. 17: *A. nidulans* CsnB and CulD interact in the yeast two-hybrid test.

The eight CSN subunits (A-H) were tested for interaction with CulD in the yeast two-hybrid test. All cDNAs were cloned into the prey (p) and bait (b) vector. Empty vectors (0) were used as negative controls, a yeast strain containing *csnA* (A) in the bait- and prey vector was used as positive control. Of each strain 10 µl liquid culture of OD_{546} = 0.01 were dropped on the plates. All strains were tested for viability (left panel). Interaction was tested as leucine prototrophy (middle panel) and *β*-galactosidase activity (right panel) after 3 days of growth.

3.1.4 Transcription of *csnA* and *csnE* is unaffected by DNA damaging agents

The COP9 signalosome influences protein levels of several E3 ligase target proteins. By changing the activity of a Pcu4/Cul4 containing ubiquitin ligase, CSN indirectly affects the abundance of ribonucleotide reductase (RNR) and thereby the ability for DNA repair in response to DNA damage in *S. pombe* (Liu *et al.*, 2003). This important function of the CSN suggests that unidentified upstream factors might also regulate the availability of the complex in response to DNA damage. And in fact, Lima *et al.* (2005) showed by quantitative RT-PCR, that mRNA levels of *csnD* and *csnE* increase upon exposure of *A. nidulans* to the DNA damaging agents CPT, MMS, BLEO and 4-NQO. To investigate if the higher amount of mRNA present in the samples is due to a stabilization of mRNA or increased transcription a *lacZ* reporter gene system was used (van Gorcom *et al.*, 1986). *A. nidulans* strains expressing a *lacZ* reporter gene under the control of 1000 bp 5´ *csnA* (AGB243) and 1000 bp 5´ *csnE* promoter region (AGB248) were constructed. Strains were grown in liquid medium and *lacZ* activity was measured upon treatment with different DNA damaging agents (Fig. 18). No significant change of *lacZ* activity was observed. This effect could have been caused by a compensation of transcriptional induction by promoter dependent inhibition of translation. Alternatively, the increased amount of *csnE* mRNA detected via RT-PCR is due to increased mRNA stability while the expression levels of *csnA* and *csnE* are independent of the DNA damaging agents CPT, MMS, BLEO and 4-NQO under the conditions tested.

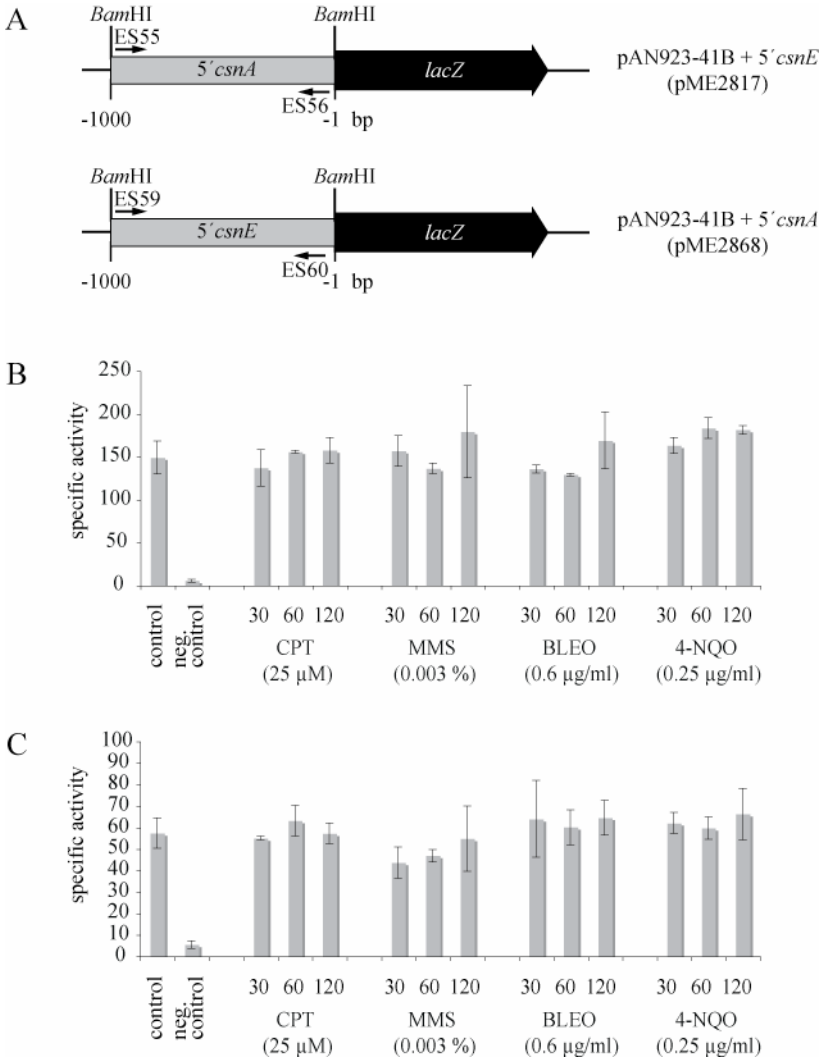


Fig. 18: 5´ *csnA* and 5´ *csnE* dependent *lacZ* expression is unaffected by the application of DNA damaging agents.

A. Scheme of 5´ *csnA* and 5´ *csnE* promoter regions fused to a *lacZ* reporter gene. *Bam*HI restriction sites and primers used for cloning are indicated. B. Specific β-galactosidase activity of the 5´ *csnA*::*lacZ* strain (AGB243) and C. the 5´ *csnE*::*lacZ* strain (AGB248) in response to the indicated amounts of the DNA damaging agents camptothecin (CPT), methyl methane sulfonate (MMS), bleomycin (BLEO) and 4-nitroquinoline oxide (4-NQO) after 30, 60 and 120 min, respectively. Each value represents the mean of two independent measurements with standard deviations not exceeding 20%. The control shows the mean specific β-galactosidase activity of the strain grown without any drug. The strain AGB246 containing pAN923-41B does not show significant β-galactosidase activity (neg. control).

3.2 Isolation and characterization of the deneddylase DenA

3.2.1 *Aspergillus nidulans denA* encodes a putative cysteine protease

The deneddylase Den1 (human deneddylase 1) belongs to the family of cysteine proteases (Gan-Erdene *et al.*, 2003; Mendoza *et al.*, 2003; Wu *et al.*, 2003). Blast search in the *A. nidulans* database (Broad Institute, Cambridge, MA, USA) revealed the putative protein AN10456.3 with 26% identity to mammalian Den1. According to the *A. nidulans* nomenclature, this protein was named DenA. The gene *denA* is located on chromosome 2. Amplification and sequencing of *denA* cDNA from an *A. nidulans* cDNA library (Krappmann and Braus, 2003) confirmed a 1137 bp open reading frame including six exons and five introns (Fig. 19). The resulting deduced 258 bp long protein of approximately 29 kDa contains the typical histidine (H), aspartate (D) and cysteine (C) catalytic triad along with a glutamine (Q) residue typical for cysteine proteases (Wilkinson and Hochstrasser, 1998) (Fig. 20). In addition to that, *A. nidulans* DenA contains a seven amino acids loop, typical for Den1 protease family members.

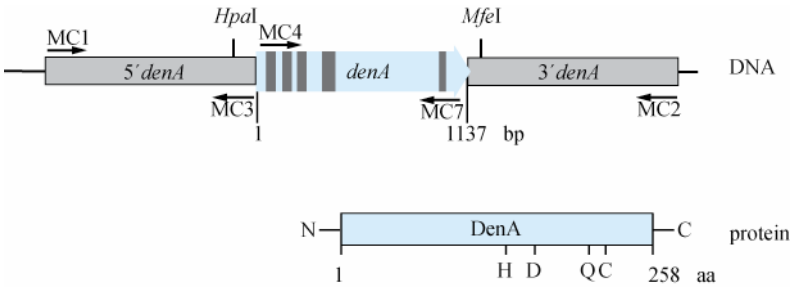


Fig. 19: Scheme of *denA* gene (AN10456.3) and protein.

Gray boxes depict introns in the *denA* gene; primers, restriction sites used for cloning and the conserved amino acids histidine (H), aspartate (D), cysteine (C) and glutamine (Q) are indicated; *denA* gene and DenA protein, light blue; bp, base pair; aa, amino acid.

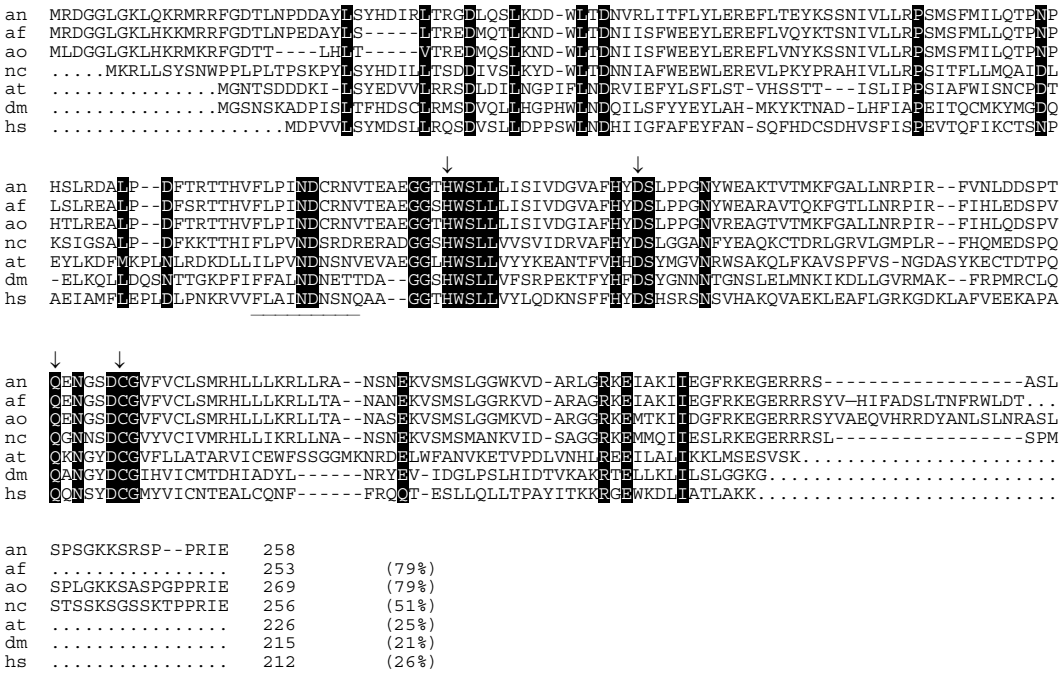


Fig. 20: The deduced protein sequence of *A. nidulans* DenA shows high amino acid identities to Den1 sequences of other eukaryotes.

Arrows indicate amino acids participating in the formation of the cysteine protease active center and the active cysteine. The seven amino acid loop typical for Den1 protease family members is underlined. Residues with high consensus are indicated by black boxes. Identities of the respective protein sequence to *A. nidulans* DenA are given in parentheses. *Aspergillus nidulans* (an, AN10456.3), *Aspergillus fumigatus* (af, XM_743956), *Aspergillus oryzae* (ao, AB223957), *Neurospora crassa* (nc, XM_959294), *Arabidopsis thaliana* (at, NM_125412), *Drosophila melanogaster* (dm, AY119635), *Homo sapiens* (hs, BC031411).

3.2.2 DenA is required for correct development of *Aspergillus nidulans*

To investigate the influence of DenA on fungal growth and morphology, the *denA* gene was deleted. To test whether the *denA* gene is essential for growth, we first transformed the *denA* deletion construct into TNO2A3, a strain deleted for *nkuA* (Nayak *et al.*, 2006). The *nkuA* deletion leads to a highly reduced rate of heterologous integration in these strains resulting in an increased rate of transformants containing a homologous integrated deletion construct. The high rate of correct deletion strains makes the *nkuA* deletion strains a fast and convenient tool to test whether knockout of a gene is lethal. Transformation of the *denA* deletion cassette into AGB152 and TNO2A3 both resulted in *denA* deletion strains named AGB316 and AGB317 respectively. Homologous integration of the deletion cassette as single copy was confirmed for both strains by PCR and Southern analyses (data not shown). Because possible synergistic effects of *nkuA* and *denA* deletions in a TNO2A3

strain cannot be excluded the *denA* deletion strain based on strain AGB152 was used for further analyses. Spore production of the *denA* deletion strain was quantified and compared to spore production of the *csnE* deletion strain and the wild-type strain AGB160 (Fig. 21).

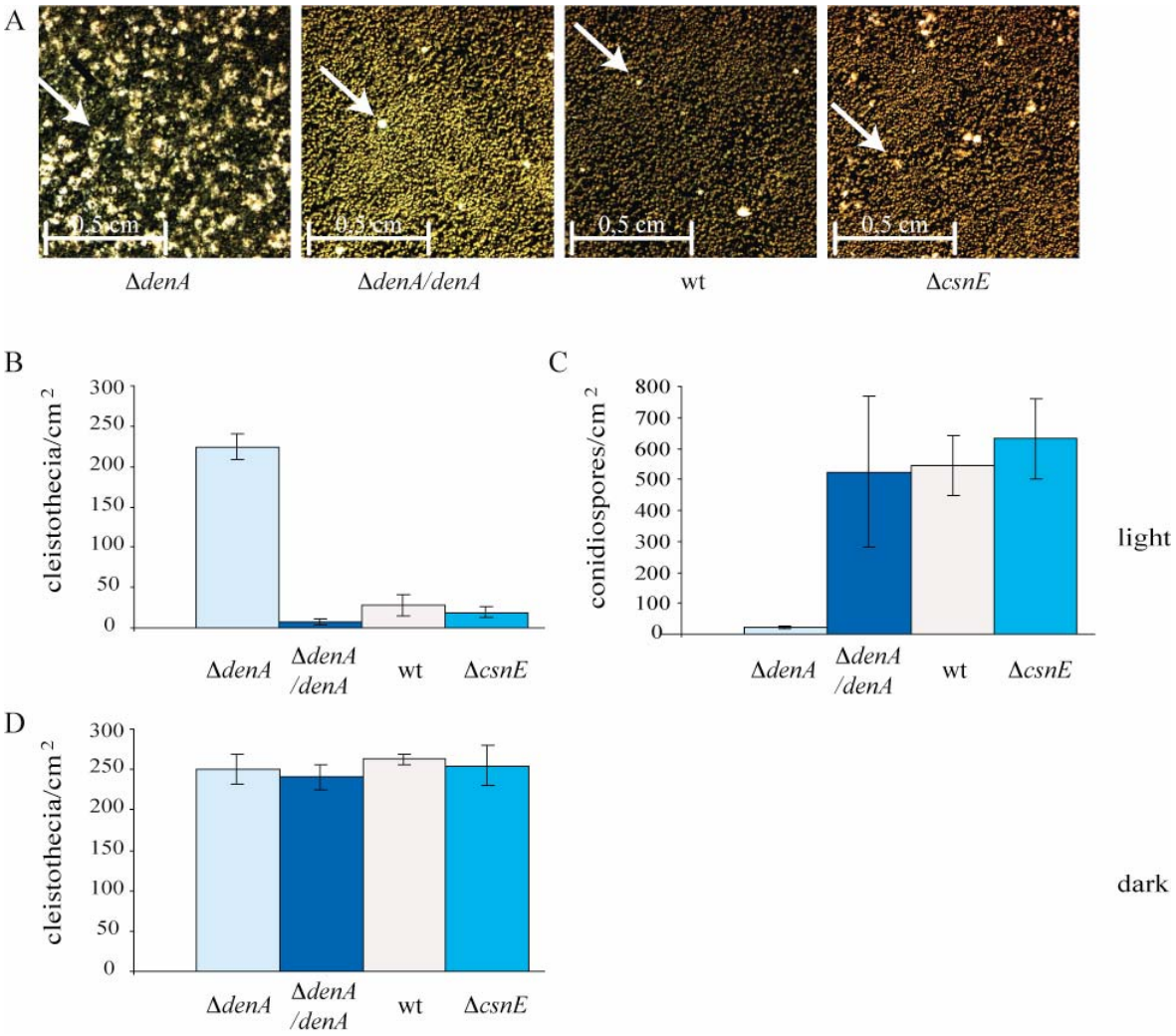


Fig. 21: Sexual development of the *A. nidulans denA* deletion strain is constitutively induced when grown on an air-medium interface.

Strains Δ*denA* (AGB316), Δ*denA*/*denA* (AGB318), wt (AGB160) and Δ*csnE* (AGB209) were grown on minimal medium agar plates supplemented with pyridoxin-HCl and uridine. A. Structures produced by the fungi after growth for 7 days at 30°C under conditions inducing asexual sporulation; sexual structures are indicated by arrows. B. Number of produced cleistothecia/cm^2 and C. conidiospores/cm^2 for strains incubated in the light for 2 days at 30°C. D. Number of cleistothecia/cm^2 produced by the strains incubated in the dark for 7 days at 30°C. Bars represent the standard deviation (n=3).

Strains were grown on minimal medium supplemented with pyridoxin-HCl and uridine at 30°C either in the light for two days (Fig. 21A) or on taped plates resulting in an increased CO_2 partial pressure in the dark for seven days. The number of conidiospores and cleistothecia was calculated per cm^2. The *denA* deletion strain produces similar amounts of cleistothecia and shows wild-type like growth under sexual development inducing conditions (Fig. 21D). Interestingly, in the light the *denA* strain produces about 25 times less conidia than the wild-type and the *csnE* deletion strain (Fig. 21C). Simultaneously, the amount of cleistothecia is increased about twelve-fold compared to the wild-type and the number of primordia produced by the *csnE* deletion strain (Fig. 21B) suggesting that light perception of the fungus is impaired. All deletion phenotypes could be restored by integration of a wild-type genomic copy of *denA* into the deletion strain. These results show that DenA is an important factor for the correct light dependent regulation of asexual and sexual spore production.

3.2.3 *Aspergillus nidulans* DenA is unable to complement a Δ*yuh1* mutant of *Saccharomyces cerevisiae*

In *S. cerevisiae* the DenA corresponding but presumably not homolog protein Yuh1 is a Nedd8 processing enzyme needed to sustain a pool of processed Nedd8/Rub1 (Linghu *et al.*, 2002). To investigate the ability of DenA to process the Nedd8 homolog Rub1 in yeast DenA was expressed under the control of a galactose inducible promoter with and without a V5 tag in the *S. cerevisiae yuh1* deletion strain Y06911. It was wondered whether expression of DenA would lead to a pool of processed Nedd8/Rub1 that is ready to be attached to cullins. This should result in neddylated/rubbylated cullin and the complementation of the Yuh1 deletion phenotype. Galactose-dependent expression of DenA::V5 was confirmed using a V5 antibody in Western analysis (Fig. 22A).

The western blot shows that yeast cullin Cdc53 is not neddylated/rubbylated in the *yuh1* deletion strain while most of the Cdc53 is neddylated/rubbylated in a wild-type yeast strain (Fig. 22B). DenA expression in the *yuh1* deletion strain does not lead to a significant increase of neddylated/rubbylated Cdc53 (Fig. 22C), suggesting that DenA activity is insufficient to process the yeast homolog of Nedd8.

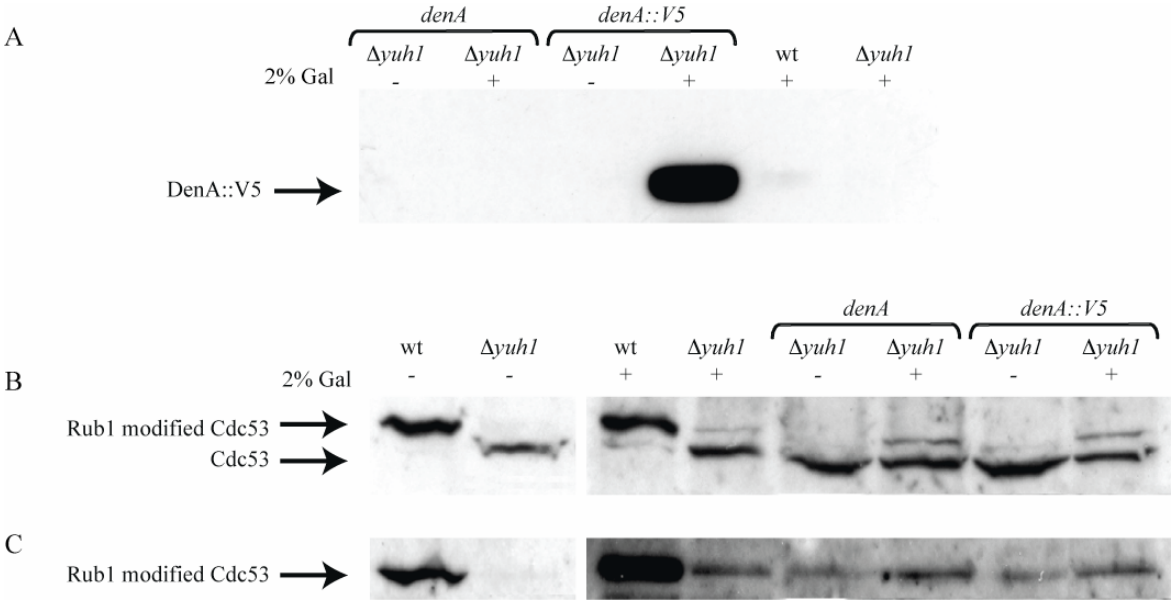


Fig. 22: *A. nidulans* DenA expression in a *S. cerevisiae yuh1* deletion strain is insufficient to restore neddylation/rubbylation of Cdc53.

A. Western analysis using the V5 antibody, B. Cdc53 antibody and C. Nedd8/Rub1 antibody. Protein extracts of the *yuh1* deletion strain containing *GAL1(p)::denA* on plasmid pME3278 (strain RH3318) or *GAL1(p)::denA::V5* on plasmid pME3279 (strain RH3330) were analyzed. Strains were grown either on galactose (+), inducing *denA*/*denA::V5* expression or glucose (-), repressing *denA*/*denA::V5* expression. Protein extracts of the wild-type strain (wt, BY4741) and the *yuh1* deletion strain (Δ*yuh1*, Y06911) were used as controls.

3.2.4 The *Aspergillus nidulans* deneddylase DenA is sufficient to detach yeast Nedd8/Rub1 from *A. nidulans* CulD in *Saccharomyces cerevisiae*

To test the ability of DenA to deneddylate/derubbylate the *A. nidulans* cullin CulD *in vivo*, the heterologous system *S. cerevisiae* was used. *culD* was cloned into the yeast two-hybrid plasmid pME3280 and was constitutively expressed as a lexA::CulD fusion protein in a *S. cerevisiae* wild-type, Δ*csn5* and Δ*yuh1* strain. To exclude that the intrinsic deneddylase/derubbylase activity of yeast Csn5 has an influence on the CulD neddylation/rubbylation status, we also used the *rri1*/*csn5* deletion strain for this experiment. Western analysis with protein extracts of these strains revealed that CulD is neddylated/rubbylated in the wild-type strain as well as the Δ*csn5* strain while no neddylation/rubbylation occurred in the Δ*yuh1* strain (Fig. 23). This shows that the yeast enzymes for Nedd8/Rub1 modification are able to modify CulD while yeast Rri1/Csn5

seems not to be able to deneddylate/derubbylate CulD. The neddylated/rubbylated CulD cannot be detected in a strain co-expressing DenA and CulD while the neddylation/rubbylation status of yeast Cdc53 is not significantly altered. This shows that the deneddylase DenA of *A. nidulans* has deneddylase/derubbylase function *in vivo*.

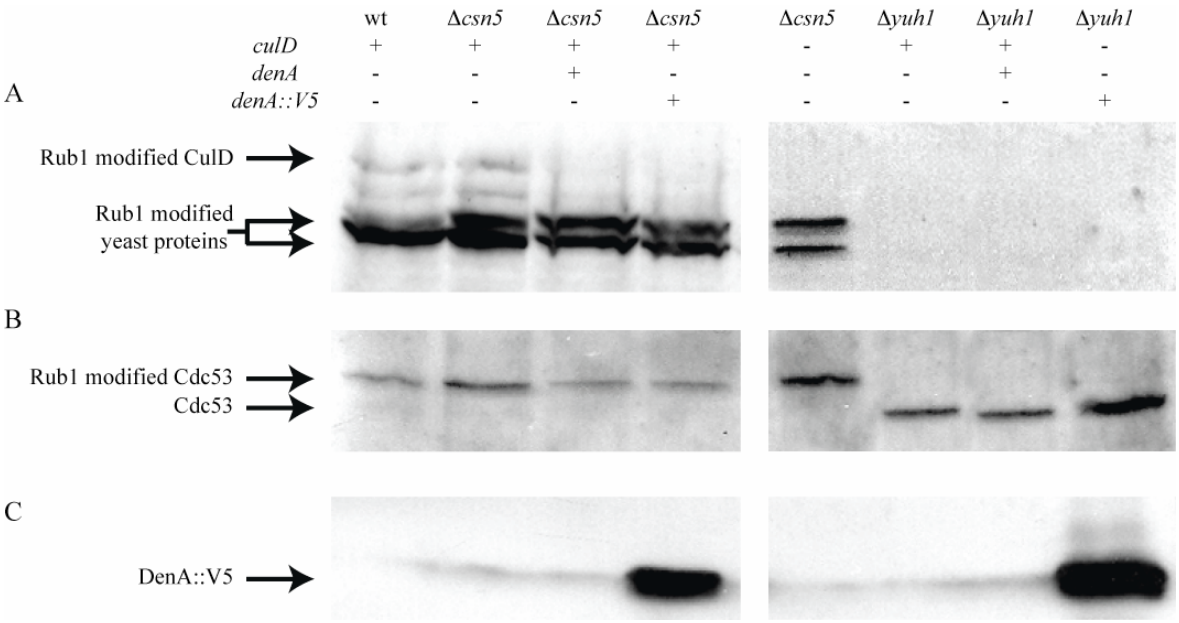


Fig. 23: *A. nidulans* DenA deneddylates/derubbylates CulD in *S. cerevisiae*.

Protein extracts of wild-type (wt, BY4741), Δ*csn5* (Y03914) or Δ*yuh1* (Y06911) yeast strains with (+) or without (-) plasmids for constitutive expression of *culD* or galactose induced expression of *denA* or *denA::V5* were analyzed. All strains were grown in medium containing 2% galactose as carbon source, inducing *denA* and *denA::V5* expression. A. For Western analysis the Rub1 antibody was used detecting neddylated/rubbylated CulD-lexA, neddylated/rubbylated Cdc53 and another protein visible in the wt and Δ*csn5* strains that is putatively the yeast cullin Cul3. The second, lower band visible in the strains expressing *culD::lexA* but not *denA* is putatively a degradation product of the CulD::lexA fusion protein. B. The Cdc53 antibody detects Cdc53 and the neddylated/rubbylated Cdc53. C. DenA::V5 expression was confirmed by Western analysis with a V5 antibody.

3.3 Isolation and characterization of the ubiquitin ligase regulator CandA of *Aspergillus nidulans* that is split into two genes

3.3.1 Cand1 is encoded by the separated genes *candA-N* and *candA-C* in *Aspergillus nidulans*

In animals and plants, the HEAT repeat protein Cand1/AtCand1 is assumed to be a key player in the regulation of the assembly and disassembly of E3 ubiquitin ligases in the ubiquitin/proteasome protein degradation pathway. Inspection of the genome of the filamentous fungus *Aspergillus nidulans in silico* revealed that the corresponding fungal homolog is divided into two genes. We named these two genes according to the encoded protein part of highest similarity in comparison to higher eukaryotes *candA-N* (AN10306.3) and *candA-C* (AN2458.3), respectively (Fig. 24). The two genes are both located on Chr VII. in relative proximity to each other separated by four open reading frames for conserved hypothetical proteins.

To identify other organisms harboring a split CandA/Cand1 protein, the genomes of several other organisms possessing a conserved CSN-cullin-system including 13 fungi representing Ascomycota and Basidiomycota and Cand1 sequences of *Arabidopsis thaliana*, *Drosophila melanogaster* and *Homo sapiens*, respectively, were searched (Fig. 25).

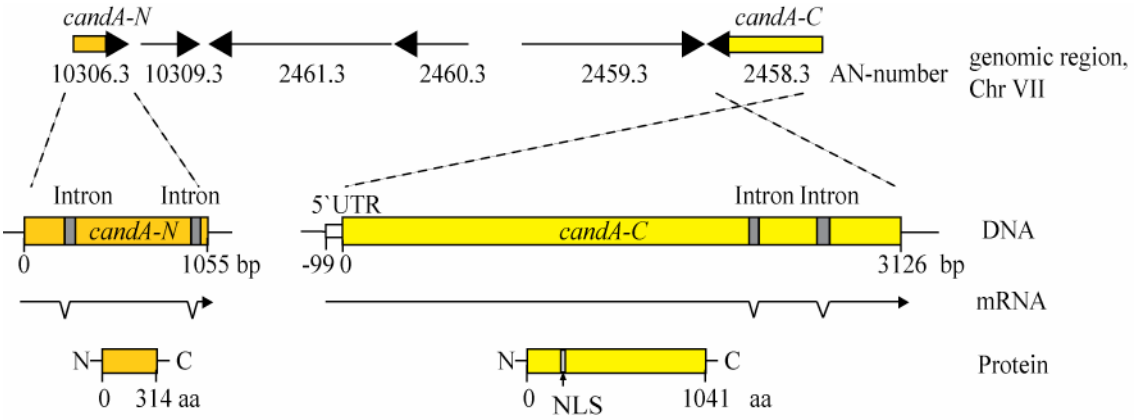


Fig. 24: Scheme of *candA-N* and *candA-C* coding regions.

Genes are located on chromosome VII; DNA, mRNA and protein sequences of CandA-N (orange) and CandA-C (yellow) are shown. Introns, *candA-C* 5´-untranslated region (5´UTR) and putative NLS (RKRRR) at position 197 of CandA-C are indicated.

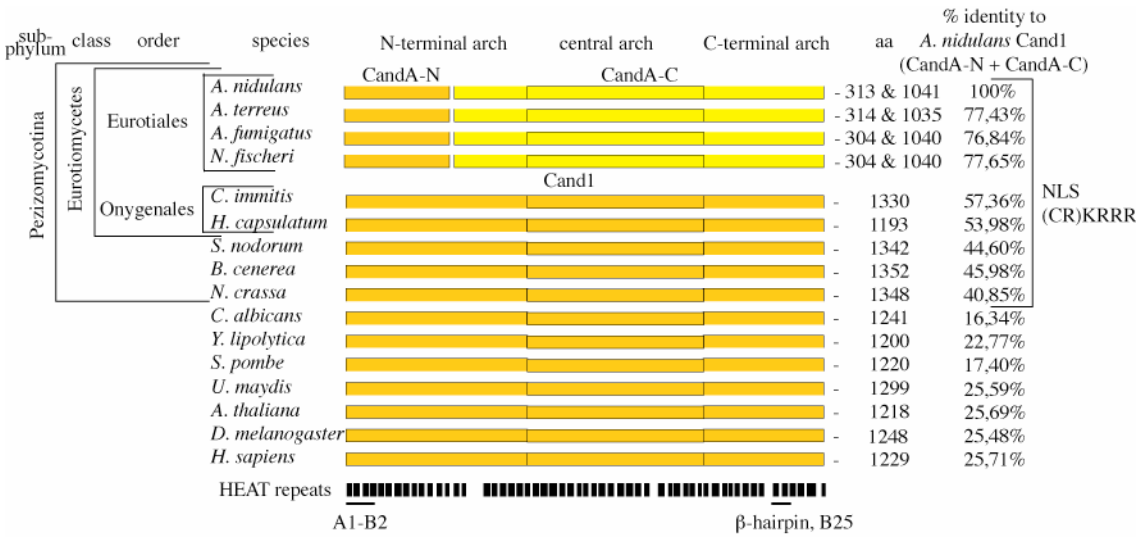


Fig. 25: CandA-N and CandA-C alignment of *Aspergilli* to Cand1 of other eukaryotes.

Numbers represent the exact protein length. Fungi containing a putative nuclear localization signal (NLS) in Cand1/CandA are indicated. CandA-N and CandA-C protein sequences were treated as one CandA sequence and % identity of *A. nidulans* CandA to every other Cand1 sequence was determined by ClustalW. A1-B2 indicate the two HEAT repeats involved in Cul1 Nedd8 binding site blocking, β-hairpin, B25 indicate the region involved in Skp1 binding site blocking on Cul1 (Goldenberg *et al.*, 2004). Cand1 sequences were obtained from NCBI or the Broad Institute (Cambridge, MA, USA). *Homo sapiens* (NM_018448); *Drosophila melanogaster* (NM_135545); *Arabidopsis thaliana* (NM_126312); *Aspergillus nidulans* CandA-N (AN10306.3); *Aspergillus nidulans* CandA-C (AN2458.3, XM_654970); *Aspergillus terreus* CandA-N (XM_001211664.1); *Aspergillus terreus* CandA-C (ATEG_02478.1, XM_001211656); *Aspergillus fumigatus* CandA-N (Afu6g10440, XM_745839.1); *Aspergillus fumigatus* CandA-C (XM_745833); *Aspergillus oryzae* CandA-N (AO090023000238); *Aspergillus oryzae* CandA-C (AO090023000228); *Neosartorya fischeri* CandA-N (XM_001258169); *Neosartorya fischeri* CandA-C (XM_001258163.1); *Histoplasma capsulatum* (HCAG_02472.1); *Stagonospora nodorum* (SNOG_10271.1); *Botrytis cinerea* (BC1G_11706.1); *Neurospora crassa* (XM_955575); *Candida albicans* (XM_714735); *Yarrowia lipolytica* (XM_503036); *Schizosaccharomyces pombe* (NM_001018716); *Cryptococcus neoformans* (XM_567799); *Ustilago maydis* (XM_754531). aa, amino acid.

Most species investigated possess only a single *cand1* homolog encoding a protein of approximately 1300 amino acids. Only the genomes of *Aspergilli* and *Neosartorya* of the fungal order Eurotiales comprise two *cand1* genes coding for an N-terminal Cand1 of approximately 300 aa and a C-terminal Cand1 of approximately 1000 aa, respectively. The % identity of *A. nidulans* CandA to other Cand1 sequences ranges from 77% for Cand1 of other *Aspergilli* to 25% for human Cand1. While *cand1* has not been found in *S. cerevisiae*, the identity of *A. nidulans* CandA to Cand1 of other yeasts is only 16% to 23%. Strikingly, there is a very high conservation of both HEAT repeat A1/B1 and the β-hairpin protrusion. A1/B1 is located in the N-terminal part of the Cand1 protein and blocks the binding site for the ubiquitin-like modifier Nedd8 on Cul1 in mammalian cells. The protrusion domain is located in the C-terminal part of Cand1 and partially occupies the

adaptor-binding site on Cul1 (Goldenberg *et al.*, 2004). For further analyses of the two *candA* genes of *A. nidulans*, the DNAs and cDNAs of *candA-N* and *candA-C* were cloned (Fig. 24). Sequencing and comparison of both genomic and cDNA nucleotide sequences of *candA-C* confirmed two introns at the 3´-end of *candA-C*. The mRNA codes for a deduced protein sequence of 1041 aa with a molecular mass of 113,5 kDa. To verify the CandA-C protein N-terminus, we determined the 5´-untranslated region of the transcript by 5´ RACE. The 5´-end of the *candA-C* transcript is located 99 bp upstream of the start codon corresponding to the first AUG of the mRNA. DNA and cDNA sequencing of *candA-N* showed that the 314 aa, 33,64 kDa CandA-N is encoded by a 1055 bp long reading frame interrupted by two introns. This finding further shows that the CandA gene of *A. nidulans* is split into two genes resulting in two separate proteins.

3.3.2 The *candA* genes are required for fungal development

To analyze the function of the CandA proteins in *A. nidulans*, deletion strains of *candA-N* (AGB264), *candA-C* (AGB262) and a double deletion strain *candA-N*/*candA-C* (AGB268) were constructed. The *candA* single deletion mutants of *candA-N* and *candA-C* as well as the double mutant *candA-N*/*candA-C* show wild-type like growth in vegetative cultures and radial colony expansion from a point spore inoculum on an agar plate does not differ significantly from the control strain (data not shown). Grown on an air-medium interface that induces development, the *candA* deletion strains appear dark red (Fig. 26A). The pigments are not evenly spread in the mycelium but are concentrated in distinct parts of hyphae as well as asexual conidiophores (Fig. 26B). The production of a red color is reminiscent to the phenotype described for deletion strains of the COP9 signalosome, another regulator of cullin containing ubiquitin ligases (see 3.1) (Busch *et al.*, 2003).
Asexual spore formation of wild-type and *candA* deletion strains were compared for the fungi grown on an agar plate and illuminated in the presence of sufficient oxygen supply to support asexual conidia formation. Asexual spore quantification of all three *candA* deletion strains showed a significantly decreased number of asexual spores compared to the wild-type strain (Fig. 27A-C).

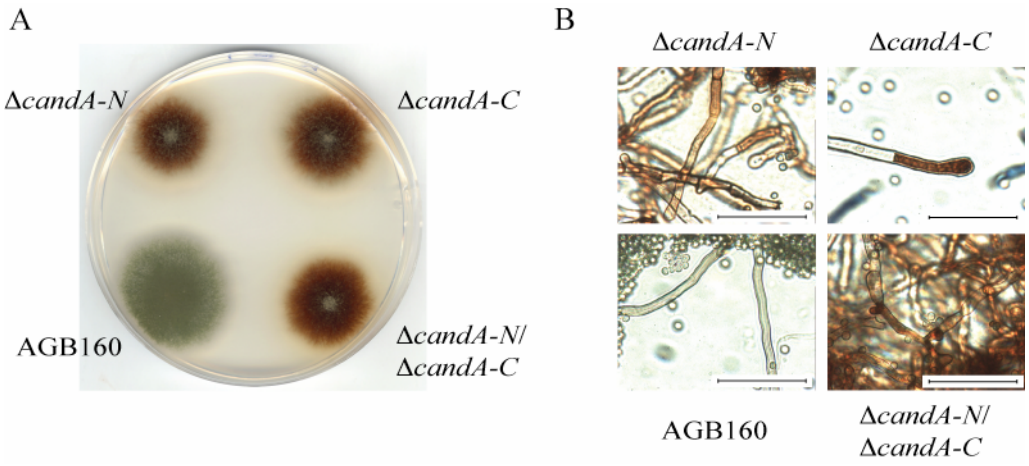


Fig. 26: *A. nidualns candA-N* and *candA-C* deletion strains show a red color phenotype.

A. Both deletion strains, Δ*candA-N* (AGB264) and Δ*candA-C* (AGB262) as well as the double deletion strain Δ*candA-N*/Δ*candA-C* (AGB268) show a red color phenotype grown on an air-medium interface. Strains were grown for four days at 37°C. B. The red color accumulates in some, but not all cells. Scale bars: 20 µm

Growing the *candA* deletion strains under conditions inducing the sexual cycle leads to the induction of sexual development but it is blocked at early nest stage (Fig. 27D, E). Hyphae aggregate to a small white or yellow structure including Hülle cells, but they do not develop a nest with primordia. However, hyphae of the *candA-N* and *candA-C* deletion strains are able to fuse and develop mature cleistothecia (Fig. 27F). The mutant phenotypes were complemented by ectopic integration of the corresponding wild-type *candA-N* and *candA-C* fragments into the deletion strains, resulting in strains AGB265 and AGB263, respectively (data not shown). The three *candA* deletion strains *candA-N*, *candA-C* and *candA-N*/*candA-C* have identical phenotypes according to all attributes tested supporting an involvement of CandA-N and CandA-C in similar functions within the cell and a possible cooperation of the proteins on the molecular level.

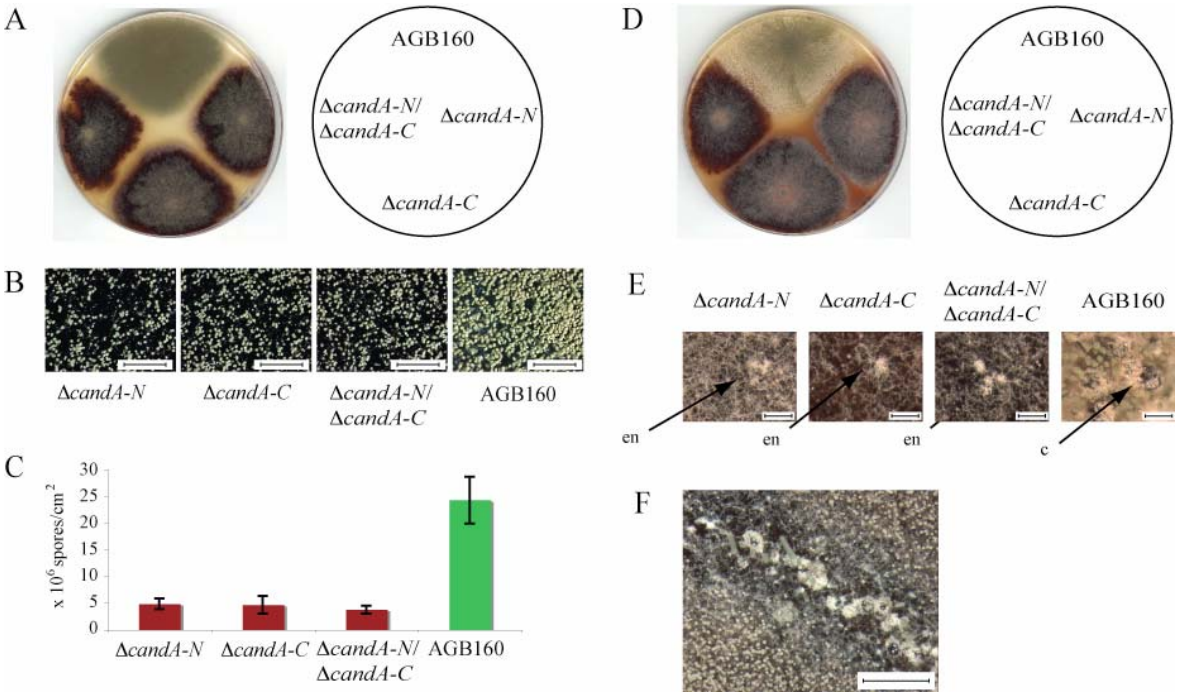


Fig. 27: Δ*candA-N* (AGB264) and Δ*candA-C* (AGB262) mutant strains show strong developmental defects.

A. *A. nidulans* deletion strains grown on an agar plate at 37°C for 6 days B. Amount of spores produced by the deletion strains is highly reduced compared to the wild-type. Scale bars: 600 µm C. Deletion strains produce significantly less asexual spores per area compared to the wild-type (AGB160); standard deviation (n=3) is indicated by black bars D. Strains grown for 6 days on a sealed agar plate in the dark. E. Development of *candA* deletion strains stops at the stage of early nests while the wild-type produces mature cleistothecia. Scale bars: 200 µm F. Hyphae of *candA-N* and *candA-C* deletion strains can fuse and the generated heterokaryon develops mature cleistothecia. en, early nest; c, cleistothecium; scale bars: 600 µm.

3.3.3 A CandA-N::C fusion protein can replace the independent proteins CandA-N and CandA-C

To test whether the split of CandA is necessary for CandA-N and CandA-C function a *candA-N::C* fusion under the control of the native *candA-N* promoter and *candA-C* terminator was constructed (Fig. 28). The fusion was integrated ectopically into the *candA-N*/*candA-C* double deletion strain (AGB268). As a control, the genomic, wild-type regions of *candA-N* and *candA-C* were integrated into the double deletion strain. The resulting *candA-N::C* fusion strain (AGB332) and the complementation strain *candA-N*, *candA-C* (AGB331) both show a wild-type like phenotype and the ability to form germinable asexual and sexual spores is restored. The fact that the fusion construct complements the double deletion phenotype reveals that both the split CandA proteins and the CandA fusion

protein are functionally efficient. This indicates that the Cand1 protein from mammals and the fungal CandA-N and CandA-C proteins may affect ubiquitin ligase activity via the same molecular mechanism.

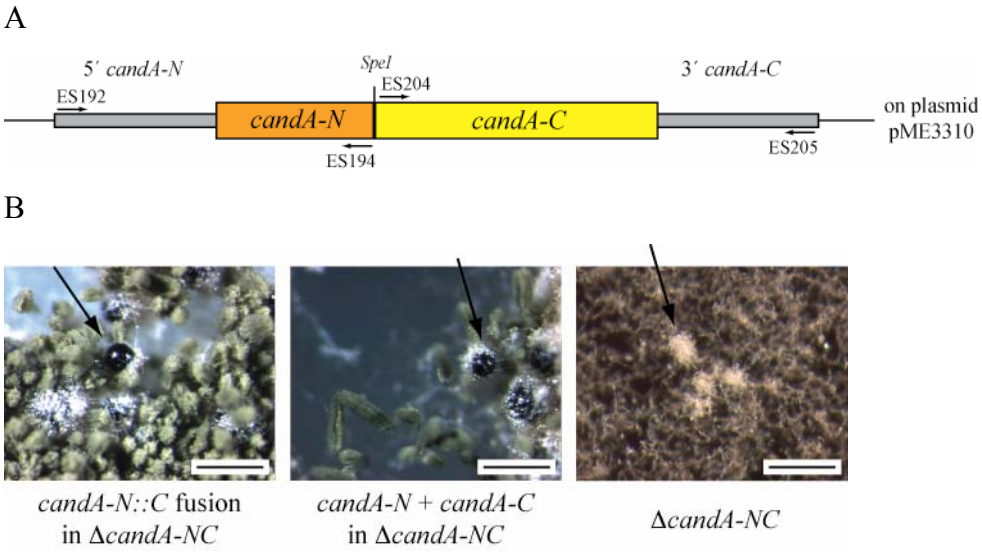


Fig. 28: *candA-N::C* fusion construct complements the *candA* double deletion phenotype of *A. nidulans*

A. Scheme of the *candA-N::C* fusion construct of plasmid pME3310. Primers and restriction sites used for cloning are indicated. The stop-codon of *candA-N* was replaced by a *Spe*I restriction site (*Spe*I) via PCR. 5′-flanking region of *candA-C* (5′ *candA-N*); 3′-flanking region of *candA-C* (3′ *candA-C*). B. Integration of the *candA-N::C* fusion (AGB332, left panel) or the single genes *candA-N* and *candA-C* (AGB331, middle panel) into the Δ*candA-N*/Δ*candA-C* deletion strain (AGB268, right panel) results in complementation of the deletion phenotype. Arrows indicate the mature ascospores-containing cleistothecia of the complementation strains and the early nest structures of the double deletion strain. Scale bars: 200 µm

3.3.4 Deletion of *candA-N* and *candA-C* as well as *csnE* leads to production of phenylethers

To investigate which substances are produced by the *candA-N*, *candA-C*, and *csnE* deletion strains but not the wild-type, the mutants and the wild-type strain AGB160 were grown on the surface of liquid medium for ten days at 37°C. The culture filtrate was extracted with ethylacetate and the organic phase was subsequently concentrated in vacuum. The resulting crude extract was analyzed by thin-layer chromatography (Fig. 29) and high performance liquid chromatography/mass spectrometry/diode array detection (HPLC-MS-DAD). Metabolites were isolated by column-chromatography, gel chromatography and HPLC to yield the pure compounds in micro-scale. The structural elucidation was performed using spectroscopic methods and databases. Five substances were identified

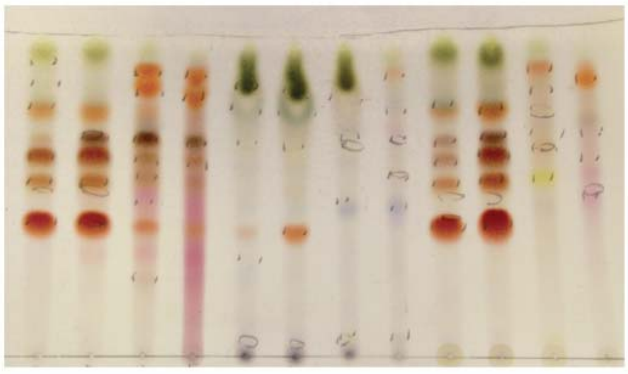
namely orcinol, violaceol II, violaceol I, cordyol C and diorcinol that were isolated from the culture filtrate of *candA-N*, *candA-C* and *csnE* deletion strains, but not from the wild-type control. The formation of these substances was found to be light-independent.

Higher amounts of the five substances were formed by the fungi when the strains were grown on ammonium as a nitrogen source, while the intensity of the red color is lower compared to the growth on nitrate as a nitrogen source (Fig. 29).

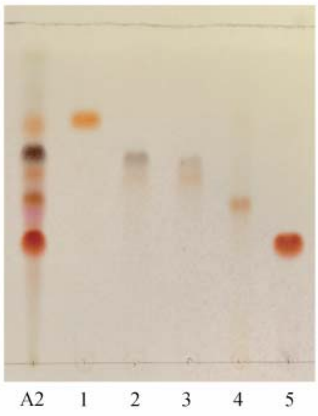
Diorcinol (3,3´-dihydroxy-5,5´-dimethyldiphenylether) is a symmetrical diphenylether first isolated from *Aspergillus rigulosus* (Ballantine *et al.*, 1968) and subsequently found in *Hypocrea citrina* (Nair and Carey, 1979), *Aspergillus nidulans* (Butnick *et al.*, 1984a), *Emericella falconensis* (Itabashi *et al.*, 1993) and *Cordyceps sp.* (Bunyapaiboonsri *et al.*, 2007) as well as in the lichen *Graphis* (Takenaka *et al.*, 2003). Orcinol (1,3-dihydroxy-5-methylbenzol) is apparently the monomer of the diphenylether diorcinol. This substance was first described for *Aspergillus fumigatus* (Pettersson, 1964). In addition it was found in the fungus *Gliocladium roseum* (Pettersson, 1965), the bacterium *Pseudomonas putida* (Chapman and Ribbons, 1976) and the lichen *Lasallia pustulata* (Mosbach and Schultz, 1971). Cordyol C (2,3,3´-trihydroxy-5,5´-dimethyldiphenylether) was first described as a substance isolated from the insect pathogenic fungus *Cordyceps* sp. (Bunyapaiboonsri *et al.*, 2007). Violaceol II (2,2´,3,6´-tetrahydroxy-4´,5-dimethyldiphenylether) has been isolated from *Aspergillus violacea* (Yamazaki and Maebayashi, 1982), the lichen *Graphis rikuzensis* (Takenaka *et al.*, 2003) and the fungus *Cordyceps sp.* (Bunyapaiboonsri *et al.*, 2007). Violaceol I (2,2´,3,3´-tetrahydroxy-5,5´-dimethyldiphenylether) also known as ethericin A (König *et al.*, 1978) or aspermutarubrol (Taniguchi *et al.*, 1978) represents the symmetrical isomer of violaceol II. These violaceols isomerize in methanol forming an isomeric mixture. Violaceol I is known from *Aspergillus funiculosus* (König *et al.*, 1978), *Aspergillus sydowi* (Taniguchi *et al.*, 1978), *Aspergillus violacea* (Yamazaki and Maebayashi, 1982), *Graphis rikuzensis* (Takenaka *et al.*, 2003) and *Cordyceps sp.* (Bunyapaiboonsri *et al.*, 2007). All substances are colorless powders. When dissolved in methanol the substances are presumably oxidized resulting in yellow, orange or red coloration. Although the biological functions of the substances are still unclear, some secondary metabolites are known to exhibit antibiotic activity. All substances were tested for biological activity in an agar diffusion test. From each substance, 50 µl of a 2 mg/ml solution was used to test its activity against *Bacillus subtilis*, *E. coli*, *Staphylococcus*

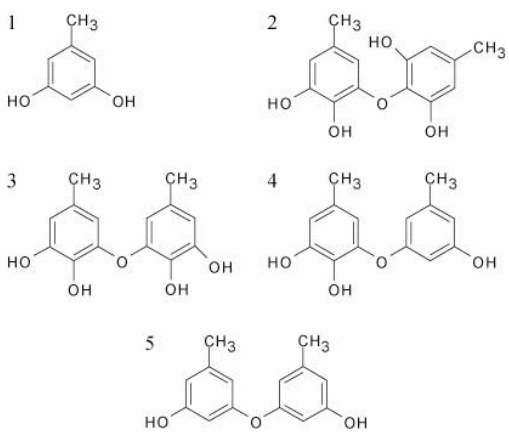
aureus and *Candida albicans*. Under these conditions, none of the substances showed biological activity against the tested organisms (data not shown).

A



B





C

	name	molecular formula	molecular weight (g/mol)	characteristics
1	Orcinol	$C_7H_8O_2$	124	colorless powder, orange in methanol
2	Violaceol II	$C_{14}H_{14}O_5$	262	colorless powder, yellow in methanol
3	Violaceol I	$C_{14}H_{14}O_5$	262	colorless powder, yellow in methanol
4	Cordyol C	$C_{14}H_{14}O_4$	246	colorless powder, deep red in methanol
5	Diorcinol	$C_{14}H_{14}O_3$	230	colorless oil, orange in methanol

Fig. 29: *candA* deletion strains produce substances that are also found in the *csnE* deletion strain.

A./B. Thin-layer chromatography MeOH:H_2O 7:3 developed with anisaldehyde. A. Comparison of secondary metabolite production of Δ*candA-C* (A), wild-type (wt) and Δ*csnE* (E) strains under different culture conditions, NH_4-medium, dark (1); NH_4-medium, light (2); NO_3-medium, dark (3); NO_3-medium, light (4). B. Comparison of the metabolite pattern of Δ*candA-C* grown in NH_4-medium in the light (A2) to the isolated and identified substances (lane 1-5) (left panel) and their chemical structures (right panel) 1: Orcinol; 2: Violaceol II; 3: Violaceol I; 4: Cordyol C; 5: Diorcinol. These substances were isolated from the culture filtrate of Δ*candA-N* (AGB264), Δ*candA-C* (AGB262) and Δ*csnE* (AGB209) strains. C. Summary of the substances and their characteristics. Chemical analysis was performed by M. Westermann and S. Grond.

3.3.5 Production of phenylethers in the *csnE* deletion strain is *wA3* independent

The exact way of orcinol, violaceol II, violaceol I, cordyol C and diorcinol biosynthesis and its regulation in the fungi is not clear but it is likely that all substances derive from orsellinic acid via decarboxylation. Polyketide synthase PKSI of *Colletotrichum lagenarium* has been shown to produce orsellinic acid when expressed heterologously in *A. oryzae* (Fujii *et al.*, 1999). The putative homolog of the PKSI of *A. nidulans* is the polyketide synthase WA (AN8209.3). To test whether the PKS has an effect on polyphenylether and red color formation in the mutant strains, the *wA3* mutation was introduced into the Δ*csnE* strain (AGB383). The analysis of the culture filtrate revealed that the strain containing the *wA3* mutation in combination with the *csnE* deletion produced a similar amount of red color and phenylethers than the *candA* and *csnE* deletion strains (Fig. 30). This indicates that the PKS WA is not involved in the synthesis of the red pigment and orcinol-like substances. However, it is not known which part of the WA is affected by the *wA3* mutation. The finding of these five substances in both, *candA* and *csnE* deletion mutants indicates that CandA-N, CandA-C and the COP9 signalosome, both effectors of cullin containing ubiquitin ligases, influence secondary metabolism in a similar manner.

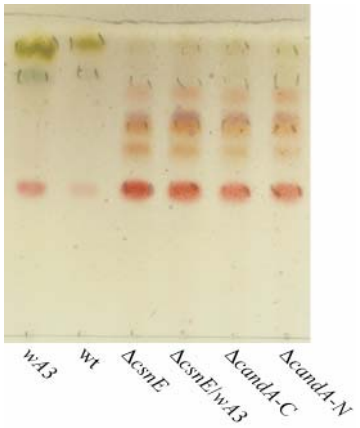


Fig. 30: The *wA3* mutation has no effect on the Δ*csnE* dependent formation of secondary metabolites.

Comparison of secondary metabolites from the culture filtrate of *wA3*, wild-type (wt), Δ*csnE*, Δ*csnE/wA3,* Δ*candA-C* and Δ*candA-N* strains. Strains were grown for 10 days at 37°C on liquid minimal medium containing 10 mM NH_4Cl as a nitrogen source. Thin-layer chromatography MeOH:H_2O 7:3 developed with anisaldehyde. Chemical analysis was performed by S. Grond and M. Westermann.

3.3.6 A *candA-C*/*csnE* double deletion strain shows a combination of the *candA-C* and *csnE* deletion phenotypes

To observe if there is a genetic interaction of CandA and CSN, we constructed a *candA-C*/*csnE* double deletion strain (AGB267). Interestingly, this strain has a defect in radial growth expansion like the *csnE* deletion mutant and the strain shows a dark red color as well as the severe developmental defects described for the *candA* deletion strain (Fig. 31).

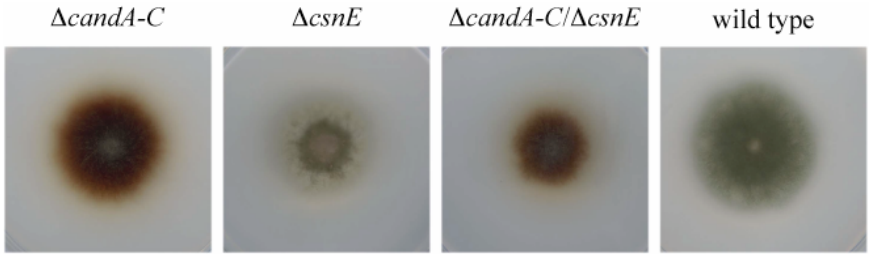


Fig. 31: Phenotype of *A. nidulans candA-C* and *csnE* deletion strains in comparison to the double deletion and the wild-type.

Comparison of *A. nidulans* Δ*candA-C*/Δ*csnE* strain (AGB267) to the single deletion strains Δ*candA-C* (AGB262) and Δ*csnE* (AGB209) and the parental wild-type strain (AGB152). The double deletion strain shows the severe developmental defects of the *candA* deletion strains in combination with the radial growth defect of the *csnE* deletion strain.

3.3.7 The nuclear localized CandA-C is expressed in vegetative mycelia and degraded upon induction of fruit body formation

To examine the localization of CandA-C in the fungal cell, a CandA-C::GFP fusion strain was constructed by replacing the *candA-C* deletion cassette of the *candA-C* deletion strain (AGB262) by a homologously integrated *candA–C*::*GFP* fusion (Fig. 32A). The resulting strain (AGB266) is phenotypically indistinguishable from the wild-type showing that the fusion protein complements the deletion phenotype. A RFP::Histon2A fusion was used as nuclear marker. Strains were grown for 14 h as vegetative cultures and analyzed by fluorescence microscopy. The co-localization of CandA-C::GFP with the nuclear RFP signal supports that the CandA-C protein is transported to the nucleus and accumulates there (Fig. 32B), suggesting a conserved function for CandA in the nucleus. The strong developmental phenotype of the *candA* deletions prompted us to investigate at which time points CandA-C is fulfilling its nuclear function.

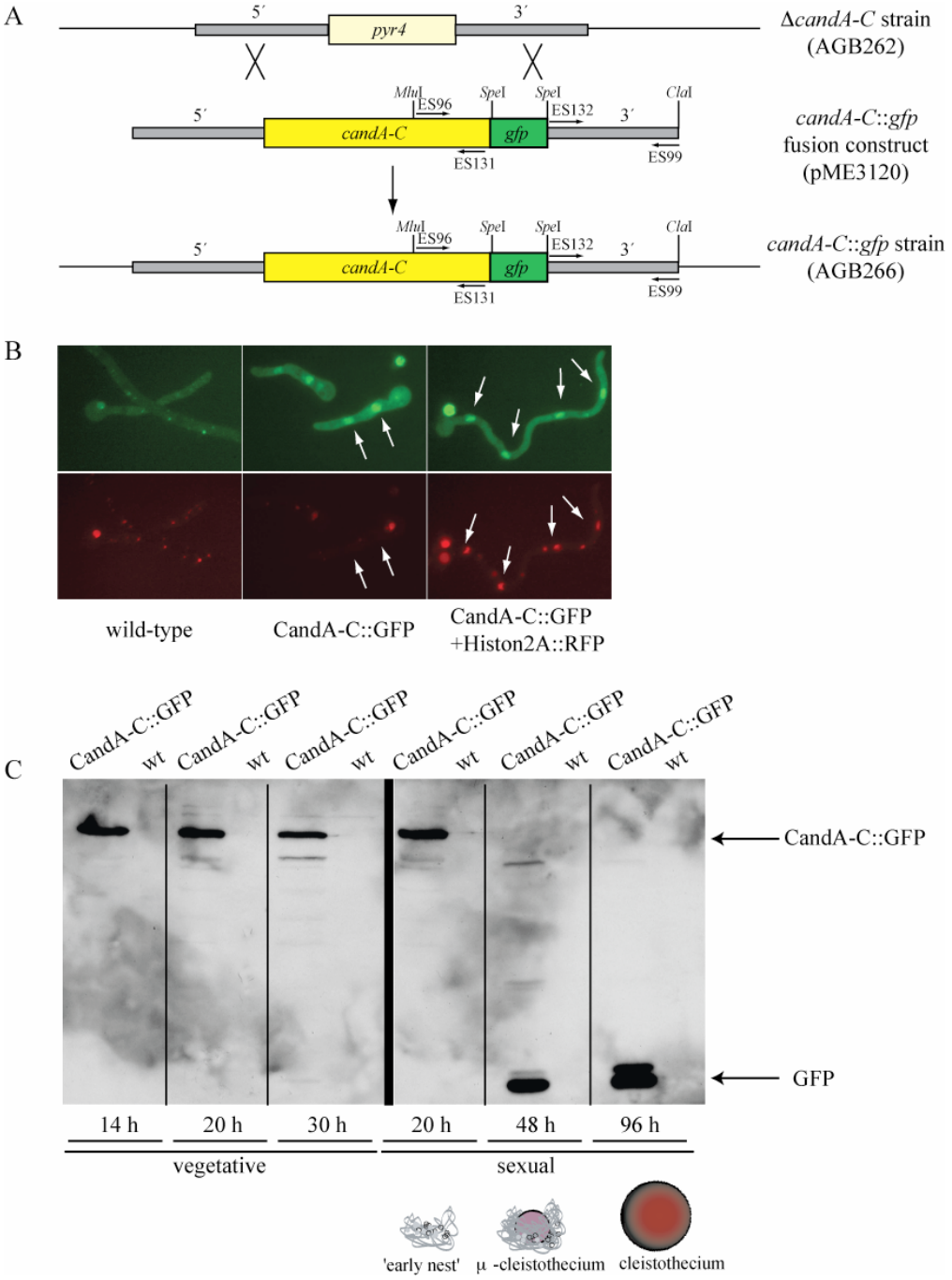


Fig. 32: CandA-C of *A. nidulans* localizes to the nucleus, is expressed during vegetative growth and degraded after induction of sexual development.

A. Scheme of CandA-C::GFP fusion strain construction. The fusion construct was integrated via homologous recombination into the original *candA-C* locus. Primers and restriction enzymes used for cloning are indicated; 5´, 5´-flanking region. B. Localization study of CandA-C::GFP fusion protein. Strains AGB266 (CandA-C::GFP) and AGB269 (CandA-C::GFP + Histone2A::RFP), containing the nuclear marker, were grown for 14 h and analyzed by fluorescence microscopy. The GFP signal colocalizes with the RFP signal in the nucleus as indicated by arrows. C. Western analysis using a GFP-antibody, with protein extracts of the CandA-C::GFP fusion strain AGB266 and the wild-type strain A4 used as negative control. Strains were grown vegetatively and under sexual development inducing conditions for the indicated time. The stages of sexual development at the tested time points are indicated. Expected sizes of the fusion protein CandA-C::GFP of 140,4 kDa and GFP of 26,9 kDa are indicated by arrows.

Expression of CandA-C during vegetative growth and sexual development was studied in the CandA-C::GFP fusion strain (AGB266).

Using a GFP antibody, we were able to detect the CandA-C::GFP fusion protein in Western experiments (Fig. 32C). Strains were grown as vegetative cultures for 14, 20 and 30 h and under conditions inducing sexual differentiation for 20, 48 and 96 h. In all samples of vegetative growth a single band, representing CandA-C::GFP protein was detectable indicating that CandA–C protein amounts were stable during vegetative growth. Under sexual development inducing conditions, CandA-C is only detectable after 20 h while after 48 h and 96 h of growth CandA-C had been degraded and only a single band representing the stable GFP-tag was detected. These experiments suggest that CandA-C fulfills its function in the nucleus during vegetative growth and at the beginning of sexual development while the protein is not needed in the later stages of spore production.

3.3.8 CandA-C and CandA-N interact in the yeast two-hybrid system but only CandA-C interacts with cullins

To test whether the two CandA proteins have the conserved function to regulate cullin containing ubiquitin ligases, the ability of CandA-N to bind to CandA-C and interaction of these proteins with CulA and CulD of *A. nidulans* (Busch *et al.*, 2007) was tested in the yeast two-hybrid system. cDNAs of *candA-N, candA-C*, *culA* and *culD* were cloned into the yeast two-hybrid vectors and transformed into the yeast two-hybrid strain in all possible combinations. Empty vectors were used as negative controls. All strains were tested for interaction in the leucine growth and β-galactosidase activity test (Fig. 33).

Both read out systems showed interaction of CandA-N with CandA-C. This suggests that CandA-N and CandA-C might also bind *in vivo* and could act as a heterodimer. An interaction of CandA-C with CulA and CulD was observed in both the β-galactosidase activity test and in the more sensitive growth test with *candA-C* cloned as bait. It is supposed that protein interaction of CandA-C with CulA or CulD is impeded because of sterical hindrance when *candA-C* is cloned as prey. Interestingly, CandA-N did not interact with CulA or CulD. This suggests that CandA-C has to mediate the binding of the only 200 aa long protein CandA-N into a putative complex consisting of CandA-C, CandA-N and a cullin. In analogy to mammals, CandA-C does neither interact with CsnB nor with

any other CSN subunit in a yeast two-hybrid test (data not shown). It was shown that the bigger part of the split CandA of *A. nidulans* is able to bind CandA-N. In addition it was shown that CandA-C is able to bind cullins in the absence of CandA-N while CandA-N does not have this ability. This suggests that CandA-C is required to allow a stable binding of CandA-N to cullins.

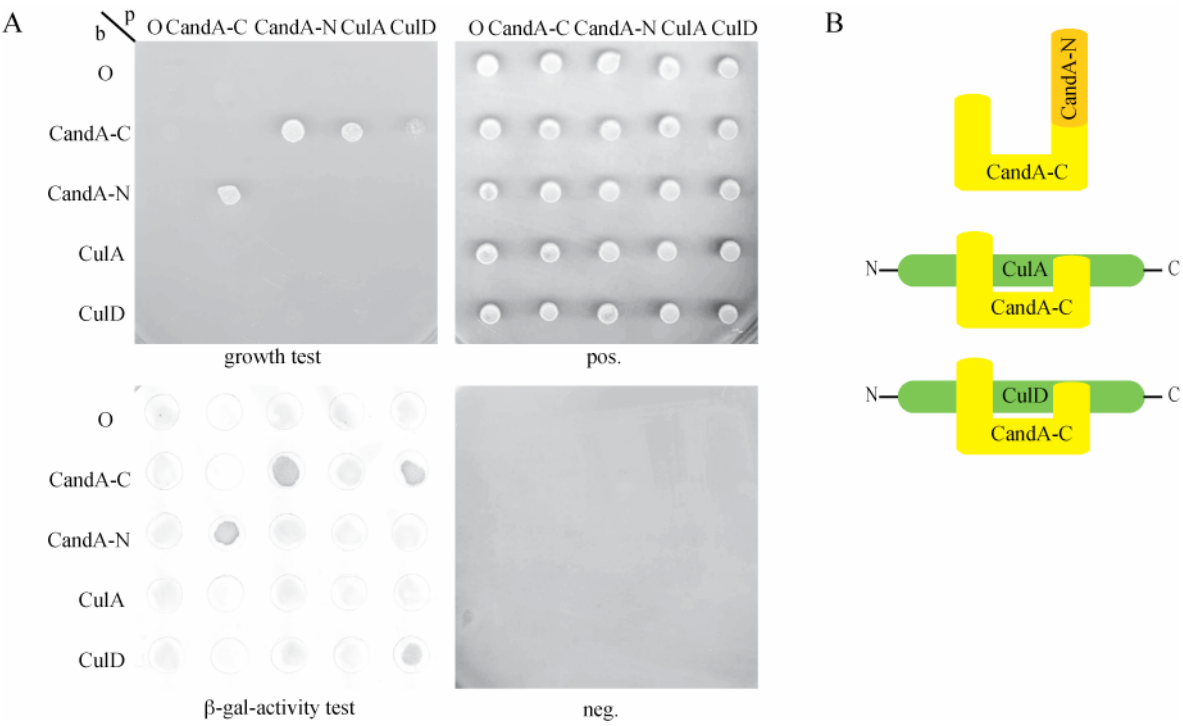


Fig. 33: Interaction study of *A. nidulans* CandA proteins, CulA and CulD, using a yeast two-hybrid system.

A. Yeasts containing combinations of *candA-C*, *candA-N*, *culA* and *culD* cDNAs, cloned into the bait and prey vector, and/or empty bait (b) and prey (p) control vectors (O) were viable (pos) and did not grow on the negative control (neg) after three days. Interaction was tested in a leucine growth test after 3 days of growth and as specific β-galactosidase activity (β-Gal), of colonies grown for two days. Of each strain 10 µl liquid culture of OD_{546} = 0.01 were dropped on the plates. B. Scheme of the interactions found.

4 Discussion

4.1 High sequence conservation of ubiquitin ligase interacting proteins in *Aspergillus nidulans*

The system of ubiquitin dependent protein degradation is an essential regulatory mechanism important for a multitude of cellular processes. The components of this system are highly conserved in mammals and higher eukaryotes whereas *S. cerevisiae* only contains some parts of the system (Draht *et al.*, 2007). Recently, genome sequencing of *A. nidulans*, *A. oryzae* and *A. fumigatus* provided the tool for detailed analysis of these fungal genomes (Galagan *et al.*, 2005).

The proteins isolated in this study, CsnB, DenA, CandA-N and CandA-C, all show high amino acid sequence identity to the corresponding proteins of higher eukaryotes. The fungal CsnB represents the homolog of the second best conserved subunit of the COP9 signalosome, Csn2, while the cysteine protease of the Ulp1-endopeptidase family DenA is the homolog of mammalian Den1, the second deneddylase identified in mammals. The fungal homolog of the mammalian cullin binding protein Cand1 is highly conserved but split into two independent proteins (see 4.2). Not conserved are the sequences of the ubiquitin ligase regulators CsnB, DenA and CandA in *S. cerevisiae* so that the yeast is a less suitable model organism to study aspects of protein degradation connected to these proteins.

The findings of this study suggest that the three regulators CsnB, DenA and CandA of the ubiquitin dependent protein degradation system of *A. nidulans* display important functions in the fungus. While CsnB and CandA are necessary for complete sexual development, DenA is needed for the correct balance between asexual and sexual development. So *A. nidulans* is a convenient model organism to study the functions of the proteins in an easy to handle, fast growing organism.

4.2 The separation of Cand1/CandA into two genes is a specific feature of the *Aspergilli*

Two independent genes, *candA-N* and *candA-C* encode CandA in the genome of *A. nidulans*. The *candA-N* and *candA-C* open reading frames oppose each other on the same chromosome, separated by four open reading frames. One possible explanation is that by a rearrangement of the DNA, the CandA encoding open reading frame was split apart.

Examination of the so far known genome sequences revealed that the highly conserved, split CandA can only be found in the fungi of the order Eurotiales. The two CandA genes *candA-N* and *candA-C* are highly conserved in these species. This suggests that independently of the split, a high sequence conservation of CandA-N and CandA-C was maintained. In contrast, the gene arrangement of the DNA between the two CandA genes is not conserved among the species harboring a split CandA although some similarities can be found. The appearance of the split gene in this single order suggests that the rearrangement happened as a single occasion in an ancestor of this order while the development of the non-conserved regions between the genes can supposedly be explained by lineage-specific recombination (Kay Hofmann, personal communication). Nevertheless, to obtain further insights into the formation of these regions, more detailed bioinformatical analysis will be required.

That the splitting at any position in the protein leads to two proteins that co-exist in the cell and are still functional together is not a likely scenario. It is all the more surprising that in *Aspergilli*, both parts of the gene ended up with a functional start codon, a promoter and a terminator allowing transcription of the genes, which was confirmed by cDNA amplification in this study. In this study it was also shown that the split of *candA* resulted in genes encoding for two independent proteins that nevertheless fulfil the conserved function of non-split Cand1/CandA proteins. Further research on the split CandA of *Aspergilli* might give interesting insights into the function of the originally fused Cand1/CandA.

4.3 Functional conservation of the ubiquitin ligase regulators in *Aspergillus nidulans*

4.3.1 The deneddylases CSN and DenA are functionally conserved

In this work an interaction of *A. nidulans* CulD with CsnB in analogy to Csn2 from human cells is shown and recently, our lab proved the existence of a CsnB containing, eight subunit COP9 signalosome in *A. nidulans* (Busch *et al.*, 2007). In mammals, the mediation of CSN binding to the SCF complex by Csn2 is crucial for CSN deneddylase function (Yang *et al.*, 2002). This binding-function in *A. nidulans* supports that CsnB mediates the binding of the COP9 signalosome to cullin containing ubiquitin ligases in a conserved way homologous to mammals (Fig. 34). The human protein Den1 has been described as a deneddylase that processes hyperneddylated cullins, deconjugates Nedd8 from cullins and processes the C-terminus of Nedd8, thereby opening the access to the diglycine motif needed for Nedd8 ligation to substrates (Gan-Erdene *et al.*, 2003; Mendoza *et al.*, 2003; Wu *et al.*, 2003). To test the putative conserved processing and deneddylase enzyme activities of *A. nidulans* DenA, we used *S. cerevisiae* as a heterologous system. In *S. cerevisiae*, Den1 is not conserved but the yeast encodes for a Nedd8/Rub1 processing enzyme, Yuh1.

This protein is needed for Rub1 C-terminus processing and *yuh1* mutants display a reduced level of neddylated/rubbylated cullin (Linghu *et al.*, 2002). Heterologous DenA expression under the control of an inducible promoter in a wild-type strain and a *yuh1* deletion strain leaves neddylation/rubbylation unaffected. The result could mean that DenA is not a Nedd8/Rub1 processing enzyme and/or that DenA is not able to process Rub1 of *S. cerevisiae*. While *A. nidulans* Nedd8 has a five amino acid C-terminal extension, characteristic for higher eukaryotes, *S. cerevisiae* Rub1 has only one amino acid.

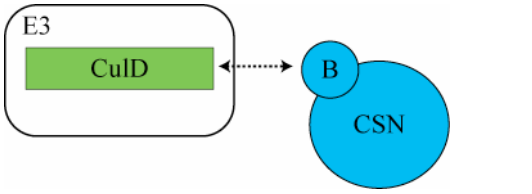


Fig. 34: Interaction of *A. nidulans* COP9 signalosome with CulD.

CsnB mediates the binding of the COP9 signalosome (CSN) to cullin containing ubiquitin ligases (E3). Dashed arrow indicates the interaction found in the yeast two-hybrid experiment.

This might lead to different sterical requirements of the processing enzymes in these organisms. Surprisingly, it seems as if some neddylated/rubbylated Cul1/Cdc53 accumulates in the *yuh1* deletion strain when it is grown on the alternative sugar galactose instead of the preferred glucose as a carbon source. The reasons for this effect remain to be elucidated.

DenA was also tested for its ability to cut the Nedd8 homolog of yeast, Rub1, from *A. nidulans* CulD in *S. cerevisiae*, a process called deneddylation/derubbylation. First, expression of *A. nidulans* CulD in yeast was verified, resulting in neddylation/rubbylation of CulD by the yeasts enzyme cascade. The result that *A. nidulans* CulD indeed is neddylated/rubbylated in yeasts is in agreement with the report that also cullin from *A. thaliana* is neddylated/rubbylated in *S. cerevisiae* (Feng *et al.*, 2004). Upon DenA expression in a CulD containing yeast strain, the signal of the neddylated/rubbylated CulD disappears, proving *A. nidulans* DenA to be a deneddylating/derubbylating enzyme *in vivo*. However, although the signals for the endogenous yeast cullins were strong and the signals of neddylated/rubbylated CulD were clear, not a lot neddylated/rubbylated CulD was detected, indicating that only a limited amount of CulD can be neddylated/rubbylated in yeast under the tested conditions. This low level might be caused by an unexpectedly low CulD expression or impairment caused by the heterologous system. The finding that DenA is able to deneddylate/derubbylate CulD in the heterologous host *S. cerevisiae* strongly suggests that the enzyme DenA also has deneddylating activity in *A. nidulan*s.

DenA was not able to deneddylate/derubbylate the intrinsic yeast cullin Cdc53 although the neddylation/rubbylation site of cullins with the consensus sequence IVR(V/I)MK are highly conserved from yeast to human. To exclude deneddylase/derubbylase activity of intrinsic Csn5 on CulD, we used a yeast strain deleted for *csn5*.

But interestingly, in comparison to the CulD expressing *csn5* deletion strain, the CulD expressing wild-type strain contains as much neddylated/rubbylated CulD as the *csn5* deletion strain. These observations lead to the conclusion that although cullins and Nedd8/Rub1 are conserved in *S. cerevisiae*, *A. nidulans* CulD, yeast Cdc53 and Csn5 are not closely related enough to allow correct enzyme activity in the heterologous system. These experiments demonstrated that the deneddylase function of DenA is conserved in *A. nidulans* and that it shows high substrate specificity.

4.3.2 The CandA proteins are functionally conserved

A. nidulans CandA is split into two proteins, CandA-N and CandA-C. The three *candA* deletion strains revealed that deletion of either or both *candA* genes leads to the same phenotype, suggesting that both proteins are needed for correct protein function. If the two proteins CandA-N and CandA-C represent a split, functional homolog of Cand1 of higher eukaryotes, an artificial fusion of CandA-N and CandA-C should be able to complement all defects of the deletions. And in fact, this study demonstrates that a CandA-N::C fusion is able to complement the deletion phenotype of the *candA-N*/*candA-C* double deletion strain, showing that both the split CandA proteins and the CandA fusion protein are functionally efficient and that independency of the two CandA proteins is not necessary for CandA function.

It was shown in this study that CandA-C is able to bind to cullins independent of CandA-N, while in contrast CandA-N is not able to bind to cullins. The high sequence conservation of *A. nidulans* CandA-N and CandA-C to mammalian Cand1 suggests that also the structures of these proteins are conserved. The mammalian 1229 amino acid Cand1 protein is characterized by its ability to bind to unneddylated cullins, which has been shown in cells in different studies *in vitro* and *in vivo* (Feng *et al.*, 2004; Liu *et al.*, 2002; Min *et al.*, 2003; Zheng *et al.*, 2002a). The crystal structure of Cand1 in complex with Cul1 and Rbx1 (Fig. 6) (Goldenberg *et al.*, 2004) shows that the six HEAT repeats (containing two helices each) similar to the 313 amino acid long *A. nidulans* CandA-N at the N-terminal end of Cand1 display only 21 interaction sites with Cul1 whereas the 1041 amino acid long CandA-C has 57 interaction sites with Cul1. Assuming a similar way of Cand1-Cul1 interaction in *A. nidulans* and mammals, CandA-N probably does not have enough interaction points with the *A. nidulans* Cul1 homolog CulA for a stable interaction. In contrast, CandA-C is long enough and displays enough interaction points with cullins to expect a stable binding. As shown in this study, CandA-N and CandA-C are able to interact. So CandA-C could mediate the binding of CandA-N to cullins to form a stable complex consisting of CandA-N, CandA-C and a cullin.

If the binding of CandA-C and CandA-N to cullin is successive, if the proteins bind before they get contact with the unneddylated Cul1 or if it is a combination of both possibilities cannot be determined yet (Fig. 35). However, simultaneous binding of the two proteins as a fusion is sufficient for functionality favoring a one step mechanism for CandA function.

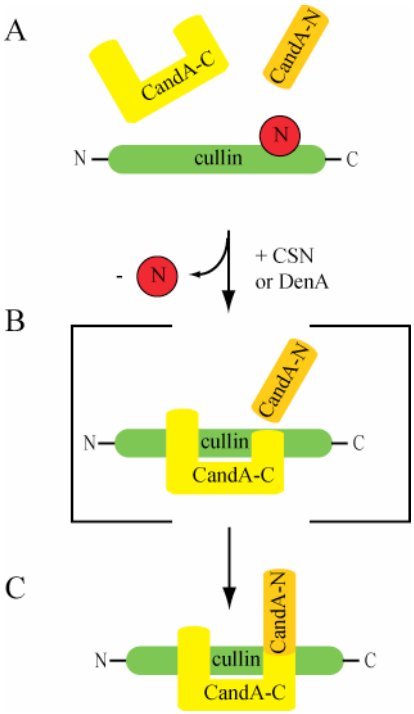


Fig. 35: Proposed sequence of events for binding of CandA-N and CandA-C to cullins in *A. nidulans*.

A. Neither CandA-C nor CandA-N are able to bind to neddylated cullins. B. After deneddylation of the cullin by the COP9 signalosome or DenA, CandA-C and Cand-N bind in subsequent steps or C. form a heterodimer before binding to cullins. N, Nedd8/RubA; N-, N-terminus; C-, C-terminus.

The split CandA of *A. nidulans* is an interesting case of a rearrangement event in an evolutionary successful organism without any obvious evolutionary advantages for the organism. It will be a future challenge to investigate if the split is in fact evolutionarily advantageous or if the split displays simply no disadvantages for the organism.

CandA-C *in vivo* localization study in *A. nidulans* revealed that the protein localizes to the nucleus. This is in agreement to studies in mammalian cells, where Cand1 was detected in the nucleus in immunofluorescence and Co-IP studies (Oshikawa *et al.*, 2003; Yogosawa *et al.*, 1996). A search for nuclear localization signals (NLS) for CandA-N and CandA-C revealed a putative NLS signal at position 197 (RKRRR) of CandA-C. The NLS sequence is conserved in the Cand1 homologs of all Pezizomycotina investigated, including not split Cand1/CandA sequences, except for *Stagonospora nodorum* containing another putative NLS, CKRRR. To function as a heterodimer CandA-N and CandA-C have to co-localize in the nucleus. In contrast to CandA-C, CandA-N does not contain a predictable NLS, which

might be the result of the split, where the C-terminal part of the protein, harboring the NLS was rearranged. In this case, it would be necessary that a yet unknown factor or CandA-C binds to CandA-N and that the proteins are co-transported into the nucleus (Fig. 36A, B). Alternatively CandA-N might contain a functional non-canonical NLS that has not yet been identified with the available bioinformatical tools leading to transportation into the nucleus (Fig. 36C). It will be a challenge for future research to investigate the localization pattern of CandA-N and the mechanism of how CandA-N gets transported to the nucleus.

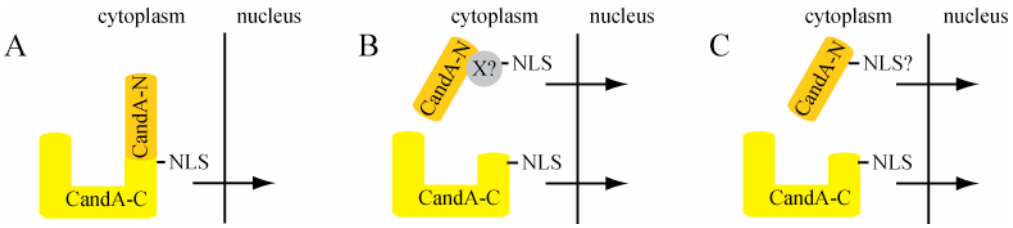


Fig. 36: Alternative models of CandA-N transport into the nucleus.

A. The NLS containing CandA-C might bind to CandA-N in the cytoplasm and the two proteins are transported as a heterodimer into the nucleus. B. CandA-N is transported into the nucleus with the help of a unknown factor or C. CandA-N is transported independently of CandA-C into the nucleus due to a non-canonical NLS. NLS, nuclear localization signal; X?, unknown factor; NLS?, non-canonical, not yet identified NLS.

4.4 CSN, DenA and CandA in development

As shown in this study, the three proteins CsnB (as a part of the CSN), DenA and CandA display a conserved function in *A. nidulans*, most likely via their regulatory function in protein degradation. The analysis of deletion mutants of *csnB, csnA*/*csnB*, *denA* and *candA* revealed that all these factors play a role in development of *A. nidulans* but also display significant differences. The *csnB* and *csnA*/*csnB* deletion strains show the same phenotype as the *csnA*, *csnD* and *csnE* deletion strains with regard to the developmental defects and the red color formation. In the following section they will be referred to as *csn* deletion strains. The three *candA* mutant strains, *candA-N*, *candA-C* and *candA-N*/*candA-C*, also show identical phenotypes and will be referred to as *candA* deletion strains during the discussion of the deletion phenotypes. All investigated deletion strains show no obvious phenotype in liquid culture but display severe defects in development. The *denA* and

candA deletions both produce significantly less asexual spores than the wild-type while the *csn* deletion strains do not show a significant reduction of asexual reproduction at 30°C. Sexual development is affected in all mutant strains. *denA* and *csn* deletion strains both show light independent induction of sexual development. But while deletion of *denA* leads to the production of mature cleistothecia, the *csn* deletion strains are blocked in development at the stage of primordia. In contrast, light regulation is not disturbed in *candA* deletion strains but sexual development is already blocked at an early nest stage (Fig. 37). Both deneddylases connect sexual development to light signaling while CandA does not, suggesting that the deneddylases and CandA affect different regulatory pathways.

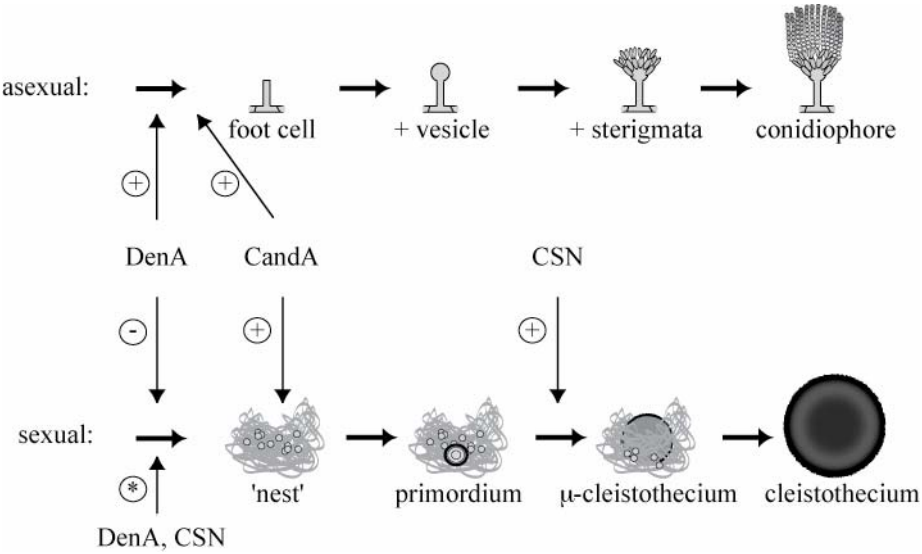


Fig. 37: Effect of DenA, CandA and CSN on development of *A. nidulans*.

Asexual and sexual development of *A. nidulans* is depicted schematically. The effect of DenA, CandA and CSN on development is indicated. Arrows indicate which way of development the proteins influence positively (+) or negatively (-). Light dependent repression is indicated by a star (*).

4.4.1 CandA-C is important for the onset of development

Monitoring of the CandA-C protein during development revealed that the protein is expressed during vegetative growth and is only present at the beginning of the sexual induction, before the protein is degraded during the onset of development (see 3.3.7). The CandA-C protein was detected via a GFP-tag which was not detectable in the vegetative stages of *A. nidulans* development, although GFP is known to be very stable protein. This allows the conclusion that there is no CandA-C protein turnover at this stage of growth. At the beginning of the sexual induction, CandA-C is still present. However after 48 h of development, CandA-C disappears and weak signals of different sizes become visible on the blot while the GFP signal is very prominent. This pattern suggests that CandA-C is degraded. Interestingly, deletion of *candA* indicates that it is not essential for vegetative growth but has a positive influence on the onset of development. This results suggest that CandA-C is a factor, needed for the onset of the developmental program, probably by regulating degradation of several cellular factors. Vegetative hyphae are phenotypically not affected by the *candA* deletion although CandA-C is expressed during vegetative growth and Northern analysis indicates that also *candA-N* mRNA is abundant in the cells at this stage (data not shown). So it is likely that CandA has a yet unclear function during vegetative growth, although it does not have an impact on the phenotype at this stage of growth. Investigating its functions during vegetative growth and identifying target proteins influenced by CandA will be a challenge for future research.

4.4.2 The two deneddylases DenA and CSN influence development differently

In this work DenA, a new deneddylase of *A. nidulans*, was identified. This enzyme is, beside the COP9 signalosome, the second deneddylase characterized in the fungus. A *denA* deletion strain showed that the protein is necessary to repress sexual development in the light. Also the COP9 signalosome affects the production of sexual structures in a light dependent manner; induction of sexual development is light independent in the *csnE* deletion strain. But while deletion of *denA* leads to a light independent high amount of mature cleistothecia containing fertile ascospores, sexual development is blocked at the stage of primordia in the *csn* deletion mutants. Furthermore, the number of asexual spores

produced by the *denA* deletion strain is highly reduced compared to the wild-type while *csn* deletion mutants show a less severe, temperature dependent reduction of asexual spore production. The comparison of the *csn* and *denA* deletion phenotypes indicates that the two deneddylases DenA and CSN have different functions.

Putatively, DenA as well as CSN display their regulatory role by deneddylating cullins of the cullin containing ubiquitin ligases. It will be a challenge to identify the rules underlying the deneddylation of specific ubiquitin ligases by one and/or the other deneddylase at a specific time point.

Target proteins degraded in a CSN or DenA dependent manner have not been identified for *A. nidulans* yet. However, a promising candidate would be the protein VeA (Kim *et al.*, 2002; Stinnett *et al.*, 2007). Although the *veA* transcript level is not influenced by *csnD* deletion (Busch *et al.*, 2003), it might be possible that a deneddylase regulates VeA stability indirectly on the protein level. Notably, the *denA* deletion strain phenotype reminds strongly of the overproduction phenotype of *veA*, a strain described to produce high amounts of cleistothecia while conidia production is decreased. It could be possible that VeA protein level is increased in a *denA* deletion strain due to the missing cullin-deneddylating activity of DenA, which is caused by malfunctioning of the ubiquitin ligase responsible for VeA degradation. The deneddylases play different roles in regulating development; therefore it is likely that deletion of both deneddylases would lead to mutants displaying an stronger developmental defect. The attempt to construct a *csnE*/*denA* double deletion strain by crossing the two single deletion strains was not successful suggesting that a *csnE*/*denA* double deletion in *A. nidulans* is synthetic lethal.

4.4.3 CSN and CandA function in the development of complex eukaryotic multicellular organisms is conserved

Deletion and dysfunctional mutants of CSN subunits have been studied extensively, while the *denA* deletion mutant of *A. nidulans* presented in this study is the first deletion mutant of a Den1 homolog available. The *csn* mutants of higher eukaryotes are characterized by severe developmental phenotypes and post-embryonic lethality (reviewed by Schwechheimer, 2004). As shown in this study, the *A. nidulans csnB* deletion strain as well as the *csnA*/*csnB* deletion strain shows a developmental defect in sexual reproduction,

similar to the already described *csn* deletion strains *csnA*, *csnD* and *csnE*. Nonfunctional mutants of Cand1 in *A. thaliana* have been described (Cheng *et al.*, 2004; Chuang *et al.*, 2004; Feng *et al.*, 2004) and interestingly, both *A. nidulans* and *A. thaliana* need CandA for correct development. *A. nidulans* CandA deletion strains produce significantly less conidia than the wild-type and the sexual spore production is highly impaired. In analogy to that, Cand1 mutants of *A. thaliana* show a phenotype indicating that the vegetative to reproductive growth transition of the primary shoot apical meristem is affected (Feng *et al.*, 2004) assigning Cand1 as an important regulator of development in *Aspergillus* as well as in plants. It can be concluded that the developmental defects caused by CSN or CandA malfunction support a high conservation of their functions from fungi to higher eukaryotes. Interestingly, the CandA phenotypes in plants and fungi remind of CSN deletion strains. Both CandA and CSN are important for auxin response in *A. thaliana* (Cheng *et al.*, 2004; Chuang *et al.*, 2004; Feng *et al.*, 2004; Serino *et al.*, 2003) and for the regulation of development and secondary metabolism in *A. nidulans*. An *A. nidulans candA*/*csnE* double deletion strain revealed that the double deletion has a stronger defect with regard to growth radius, intensity of the red color and conidia formation than the single deletion strains. So the data from *A. nidulans* suggest that CSN and CandA regulate different targets. However, the identical secondary metabolites identified in *csn* and *candA* deletion strains as well as the auxin response phenotype of *csn* and *candA* mutants of *A. thaliana* indicate that CSN and CandA regulate at least some ubiquitin ligases in a similar way.

The intensity of the developmental defects of *csn* or *candA* deletions differs in *A. nidulans* and *A. thaliana*. According to *A. nidulans* development, the effect of *candA* deletion is even stronger than the effect of *csn* deletion. While *candA* deletion leads to a block of cleistothecia formation already at the very beginning of development, the *csn* deletion strains are able to form primordia before development is blocked. In contrast, a *csn* deletion in *A. thaliana* leads to a constitutive photomorphogenic phenotype and lethality at seedling stage (Serino and Deng, 2003), while a loss of function mutant of *candA* only displays a partial constitutive photomorphogenic phenotype (Chuang *et al.*, 2004; Feng *et al.*, 2004) and is still able to propagate sexually, although on a low level. CSN and CandA are assumed to be required for species and developmental stage specific degradation of proteins. Therefore it is likely that the organism specific characteristics of the mutation strains originate from the differences in the developmental processes of fungi and plants.

4.5 Expression of CSN subunits is independent of DNA damaging agents

In fission yeast, *csn* subunit deletion leads to DNA damage and delayed cell cycle progression. This suggests that CSN could be involved in the correct regulation of DNA repair and it cannot be excluded that the CSN subunits are regulated on the transcriptional level upon DNA damage. In this study we tested if the DNA damaging agents CPT, MMS, BLEO and 4-NQO have an influence on transcription of a *lacZ* reporter gene under the control of the 1 kb promoter regions of *csnA* or *csnE*. Under the conditions tested, no change of *lacZ* enzyme activity was observed; hence transcription from the promoters tested is unaffected by these DNA damaging agents. Nevertheless, mRNA levels of *csnD* and *csnE* increase upon treatment with the DNA damaging agents as determined by quantitative RT-PCR (Lima *et al.*, 2005). These findings suggest, that the increased mRNA levels are due to increased mRNA stability, mediated by DNA damage. However, the connection between the sensing of DNA damaging agents or DNA damage and *csn* mRNA stabilization is still unclear and needs to be elucidated in future studies.

4.6 CSN and CandA in secondary metabolism

The most obvious phenotype of all existing *csn* deletion strains and the *candA* deletion strains, but neither the *den1* deletion nor the wild-type strain, is the production of substances leading to a red color. Deletion strains of *csn* and *candA* produce these red pigments in the hyphae after 2-3 days of growth on an air/medium interface while it cannot be observed for the *A. nidulans* wild-type strains. Interestingly, the coloring of the *candA* deletion strains is even more intensive than for the CSN deletion strains (Fig. 16 and Fig. 26). The substances causing the red color mainly locate to the hyphae and are only partially released into the growth medium. Because this phenotype hints to an alteration in secondary metabolism, culture filtrate of the *candA-N, candA-C* and *csnE* deletion strains was analyzed for substances of the secondary metabolism not produced by the wild-type control. Although the filtrate did not show an intensive red color, five putatively polyketide-derived substances specifically produced by the deletion strains were identified, namely orcinol, violaceol II, violaceol I, cordyol C and diorcinol.

Interestingly, diorcinol production has been previously described for strains defective in sexual development, including *acoB202* (strain WIM-145) and *acoC193* (Butnick *et al.*, 1984a; Butnick *et al.*, 1984b) and it is noticeable that both mutations affect CSN subunits; *acoB* is located on chromosome II-R and has been shown to be *csnG* (Lewis and Champe, 1995) while *acoC* is locate on chromosome VII and is identical to *csnD* (Tsitsigiannis *et al.*, 2004). So pigments resulting in a red color and diorcinol production have been identified and described in *csnD, csnE, csnG* and *candA* mutation/deletion strains. This suggests that deletion of each subunits of the COP9 signalosome as well as deletion of *candA* influences phenyl ether production in similar ways.

4.6.1 Bioactivity of the isolated substances

In the literature several reports about antimicrobial activity of polyketide-derived substances can be found. Substituted diphenylethers have been described as antibacterial (Glombitza, 1977) or antifungal (McGahren *et al.*, 1970). Violaceol I has been found to exhibit antimicrobial activity against gram-positive bacteria when 50 µg/ml was used against *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus lysodeikticus*. When used at a concentration of 200 µg/ml the substance also inhibits growth of *Escherichia coli*. Growth of the violaceol I producing fungus *Aspergillus sydowi* is already inhibited at a concentration of 12.5 µg/ml (Taniguchi *et al.*, 1978). Like violaceol I, also violaceol II exhibits weak antimicrobial activity (Yamazaki and Maebayashi, 1982). In addition, violaceols exhibit anti-malarial activity against *Plasmodium falciparum* K1 and show moderate cytotoxic activities. Cordyol C exhibit anti-herpes simplex virus type 1 activity in a concentration of 1.3 µg/ml and is moderately toxic against cancer cells (Bunyapaiboonsri *et al.*, 2007). Diorcinol has antibiotic activity against *Staphylococcus aureus* (Nair and Carey, 1979) and is weakly effective against *Mycobacterium tuberculosis* in a concentration of 50 µg/ml (Bunyapaiboonsri *et al.*, 2007). In our study none of the isolated substances showed antibacterial or antifungal activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. However, we tested orcinol, violaceol II, violaceol I, cordyol C and diorcinol in an agar diffusion test using 50 µl of a 2 mg/ml solution of each substance. These conditions differ from the concentrations and test methods used in the studies described in the literature, which

makes comparison of the data difficult. It is likely that a higher concentration of the tested substances might be necessary for an antimicrobial effect.

4.6.2 Possible ways of orcinol formation in *csn* and *candA* deletion strains

Orcinol derives from the tetraketide orsellinic acid via decarboxylation (Fig. 38) as described for the fungus *Gliocladium roseum* (Pettersson, 1965). Also the isolated substituted diphenylethers violaceol II, violaceol I, cordyol C and diorcinol are putatively derived from orsellinic acid. All of them are composed of two orcinol-like precursors that can derive from orsellinic acid by oxidative phenole coupling (Turner, 1971). It is possible that the diphenylethers are formed by enzymes in the organism or that the monomers are chemically coupled in the growth medium. So it is likely that all substances isolated from the culture filtrate of the deletion mutants derive from orsellinic acid.

To obtain a deeper insight into the production of orsellinic acid it would be interesting to know which polyketide synthase is responsible for the production of the precursor of orcinol, violaceol II, violaceol I, cordyol C and diorcinol. Orsellinic acid has been connected to DHN-melanin production. When expressed heterologously in *Aspergillus oryzae* the PKSI of *Colletotrichum lagenarium* has been found to produce the pentaketide 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) (Fig. 12) (Fujii *et al.*, 1999).

As a byproduct orsellinic acid was identified. This would suggest that in the *A. nidulans* mutant strains the decarboxylated orsellinic acid derives from a dysfunction of the PKS as a byproduct of DHN-melanin synthesis (Fig. 38A) while the dark red substance is possibly a polymerized polyketide. The putative homologs of *C. lagenarium* PKSI are the PKS WA in *A. nidulans* and PKSP in *A. fumigatus*, both producing naphthopyrone (Fujii *et al.*, 2000; Fujii *et al.*, 2001). Deletion of these PKSs leads to the formation of white conidia in both species (Langfelder *et al.*, 1998; Mayorga and Timberlake, 1990). The heptaketide naphthopyrone is subsequently converted to 1,3,6,8-tetrahydroxynaphthalene by the enzyme AYG1 as shown for *A. fumigatus* (Tsai *et al.*, 2001). Putative homologs of AYG1 can also be found in the genomes of *A. nidulans* and *A. oryzae* (data not shown).

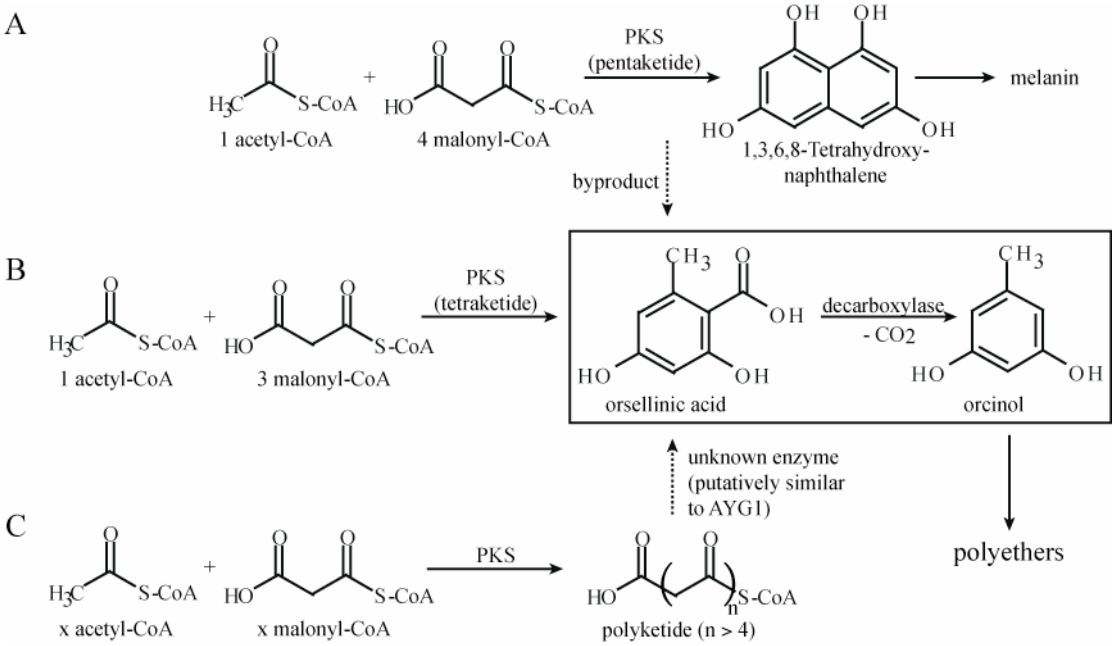


Fig. 38: Three hypothetical ways of orsellinic acid production in *A. nidulans csn* and *candA* mutants.

A. Orsellinic acid derives as byproduct of DHN-melanin biosynthesis. B. Orsellinic acid is synthesized by a yet unknown PKS producing tetraketides that is activated in the mutants. C. A yet unknown enzyme converts polyketides of unknown length into the tetraketide. Orsellinic acid is then decarboxylated to orcinol.

Strains harboring a mutation in the *wA* gene (*wA3*) resulting in white conidia (Pontecorvo *et al.*, 1953) have been widely used in *A. nidulans* genetics. Interestingly the red color and orcinol formation in a *csnE* deletion strain is not affected by the *wA3* mutation indicating that in *A. nidulans* the PKS WA probably does not produce orsellinic acid as a byproduct in the mutant strains. Nevertheless, we cannot exclude that the *wA3* mutation is located in a region of the PKS that eliminates spore color formation but maintains a putative orsellinic acid production via WA. However, in this case, the truncated WA is not constitutively producing tetraketide orsellinic acid congeners, but CSN deletions are specifically switching on phenylether biosynthesis. There is no such an example of polyketide synthase regulation known.

It has been already shown that the second enzyme known to be important for spore color production, YA, is not involved in the formation of the red color in *csn* deletion strain. *yA* encodes for a phenoloxidase essential for development of the green spore pigment from a yellow precursor. The *csnD* and *csnG* defective, red hyphal and diorcinol synthesizing strains WIM-145 and WIM146 carry the defective *yA2* allele as a marker (Butnick *et al.*,

1984a; Butnick *et al.*, 1984b) demonstrating that diorcinol- and red color formation are *p*-diphenol-oxidase independent. The asexual spore color, red color formation and diorcinol production of the investigated *A. nidulans* strains are summarized in Tab. 14.

The coloration of the *csn* and *candA* deletion mutant colonies reminds of the ascospore color of *A. nidulans*. The color appears usually at the end of spore maturation of the wild-type and the substance causing the red color is known as ascoquinone A (Brown and Salvo, 1994) (Fig. 13A). It is putatively derived from an oktaketide synthesized by a polyketide synthase. It is still not clear if ascoquinone biosynthesis is connected to the red pigment and orcinol-phenylethers found in the deletion strains. Hitherto, we were not able to isolate ascoquinone A from the culture filtrate. While the light conditions did not have an influence on the production of the isolated substances, the nitrogen source was shown to be important. The mutants produced a more intense red color when grown on nitrate than on ammonium as nitrogen source. Interestingly, the amount of the identified secondary metabolites produced by the mutants was higher when grown on ammonium. However, the true chemical structure of the deep red pigment which is not extractable by common chemical methods from the hyphae (Westermann, 2007) remains elusive.

Tab. 14: Asexual spore color, red color formation and diorcinol production of different *A. nidulans* strains.

strain	effected gene(s)	red color formation	diorcinol-production	spore color	reference
AGB160	-	-	-	green	FGSC[a] A4
AGB264	Δ*candA-N*	+++	+	green	this work
AGB262	Δ*candA-C*	+++	+	green	this work
AGB234	Δ*csnA*	+	n.d.	green	Draht (2005)
AGB238	Δ*csnB*	+	n.d.	green	this work
AGB195	Δ*csnD*	+	n.d.	green	Busch *et al*. (2003)
AGB209	Δ*csnE*	+	+	green	Busch *et al*. (2003)
AGB383	Δ*csnE*, *wA3*	+	+	white	constructed by S. Busch
WIM-146	*acoB202* (*csnG*), *ya2*	+	+	yellow	Butnick et al., 1984a; Butnick et al., 1984b
WIM-145	*acoC193* (*csnD*), *ya2*	+	+	yellow	
AGB150	*wA3*	-	-	white	constructed by V. Große
DVAR1	Δ*veA*	+	n.d.	yellow	Kim *et al.* (2002) Busch *et al*. (2003)

n.d., not determined

4.6.3 The *Aspergillus* genome encodes several polyketide synthases

It is possible that the tetraketide orsellinic acid is produced by another not yet described tetraketide producing PKS (Fig. 38B). The *A. nidulans* genome encodes approximately 28 putative polyketide synthases containing a β-ketoacyl synthase (N- and C-terminal domain) and an acyltransferase domain (Tab. 15). In addition, Nahlik (2007) identified a putative citrinin PKS (AN7903.3, manually annotated) upregulated in the *csnE* deletion strain on the transcriptional level during early vegetative growth. So it is conceivable that deletion of *csn* or *candA* leads to an altered expression or activity of an orsellinic acid producing polyketide synthase. Furthermore, a combination of a PKS and an enzyme similar to the AYG1 of *A. fumigatus* could lead to the production of tetraketides (Fig. 38C) by a mechanism similar to the polyketide shortening recently described by Brachmann *et al.* (2007).

Alternatively, the red color production of *Aspergillus* deletion and mutant strains has been assumed to be the result of an unspecific, general stress reaction. But interestingly, deletion of both CSN and CandA, but not DenA leads to the formation of the red color. Our results hint to a role of CSN and CandA as central regulators at a possible interface between stress signaling and production of secondary metabolites. Due to their function in ubiquitin ligase regulation it is likely that CSN and CandA influence the stability of several regulatory proteins. A protein which might be possibly affected in the *csn* and *candA* deletion strains is LaeA, a transcriptional regulator of multiple secondary metabolite clusters (Bok *et al.*, 2006).

The identification of the yet unknown pathway of orcinol-family substance production in *A. nidulans* and its triggers will be a fascinating challenge for future research.

Tab. 15: Putative polyketide synthases of *A. nidulans* (*Aspergillus* comparative database, Broad Institute, Cambridge, MA, USA).

putative polyketide synthase (PKS)	annotated protein(s) containing β-ketoacyl synthase and acyltransferase domain	annotated protein containing thioesterase	chromosome
hypothetical protein similar to PKS	AN0150.3	-	VIII
conserved hypothetical protein (expression controlled by LaeA, (Bok *et al.*, 2006))	AN0523.3	-	VIII
conserved hypothetical protein	AN1034.3	-	VIII
conserved hypothetical protein	AN1036.3	-	VIII
conserved hypothetical proteins	AN1784.3	AN1783.3	VII
hypothetical protein	AN2032.3	-	VII
conserved hypothetical protein	AN2035.3	-	VII
hypothetical protein similar to PKS	AN2547.3	-	VII
conserved hypothetical protein	AN3230.3	-	VI
conserved hypothetical protein	AN3386.3	-	VI
conserved hypothetical protein	AN10430.3	-	II
conserved hypothetical protein	AN3612.3	-	II
conserved hypothetical protein	AN6000.3	-	I
conserved hypothetical protein	AN6431.3	-	I
conserved hypothetical protein	AN6448.3	-	I
conserved hypothetical protein	AN6791.3	-	I
conserved hypothetical protein	AN7071.3	-	IV
conserved hypothetical proteins	AN7815.3/AN7814.3	-	IV
sterigmatocystin biosynthesis PKS StcA (Brown *et al*., 1996) metabolic product: norsolorinic acid	AN7825.3	AN7825.3	IV
conserved hypothetical proteins	AN7837.3/AN7838.3	-	IV
hypothetical protein similar to PKS, putative citrinin PKS	AN7903.3 (manually annotated)	-	II
conserved hypothetical protein	AN7909.3	AN7909.3	II
conidial yellow pigment biosynthesis PKS WA (Mayorga and Timberlake, 1990) metabolic product: naphthopyrone	AN8209.3	AN8209.3	II
conserved hypothetical protein	AN8383.3	-	Unmapped scaffolds
conserved hypothetical protein	AN8412.3	-	Unmapped scaffolds
conserved hypothetical protein	AN8910.3	-	VII
conserved hypothetical protein	AN9005.3	-	VII
conserved hypothetical protein	AN11191.3	-	IV
fatty acid synthase subunit alpha reductase/ hypothetical protein similar to fatty acid synthase	AN9407.3/AN9408.3	-	VIII

4.7 Outlook

In this study, the three different ubiquitin ligase regulators CsnB as a component of the COP9 signalosome, DenA and CandA of *A. nidulans* were investigated. The deneddylases CSN and DenA probably regulate protein degradation by controlling the neddylation/deneddylation of the cullin subunit of cullin containing ubiquitin ligases, while CandA is supposed to control the assembly/disassembly of the complex (Fig. 39).

CsnB is a component of the eight subunit fungal COP9 signalosome. The yeast two-hybrid data presented in this study suggest that the function of CsnB to mediated interaction of CSN with cullins is conserved. Future *in vivo* studies will be useful to confirm the yeast two-hybrid data and to find out more about the temporal and spatial distribution of CsnB-cullin binding. This might also give deeper insights into the role of CSN in ubiquitin ligase regulation during development. Although it is likely that also the deneddylase DenA binds to cullins, it has not been proven yet and will be a task for future research.

The COP9 signalosome and DenA are both deneddylases, able to deneddylate cullins. Interestingly, the phenotypes of the respective *csn* or *denA* deletion mutants show different defects in development, indicating that the enzymes control different targets. This different impact on fungal development is conceivably caused by activity alterations of the deneddylases dependent on the specific cullin, CulA, CulC or CulD and the developmental stage of the fungus. The *csn* and *denA* deletion mutants constructed in this study could be a useful tool to find out which deneddylase is responsible for which regulations. It should be possible to study the neddylation status of the different cullins on protein level at different developmental stages, dependent on the presence or absence of the respective deneddylase. It would also be interesting to see if overproduction of DenA could at least partially rescue the phenotype of the *csn* deletion strains and vice versa.

Cand1/CandA cullin-binding function is highly conserved in *A. nidulans*, although CandA is split into CandA-N and CandA-C. If the two proteins work together, CandA-N has to be transported to the nucleus where CandA-C is localized. Because there is no obvious nuclear localization signal in the *candA-N* sequence, it is not clear how the CandA-N protein gets transported into the nucleus. To investigate whether the transport of CandA-N depends on CandA-C, localization studies of CandA-N in a wild-type and a *candA-C* deletion strain should prove effective.

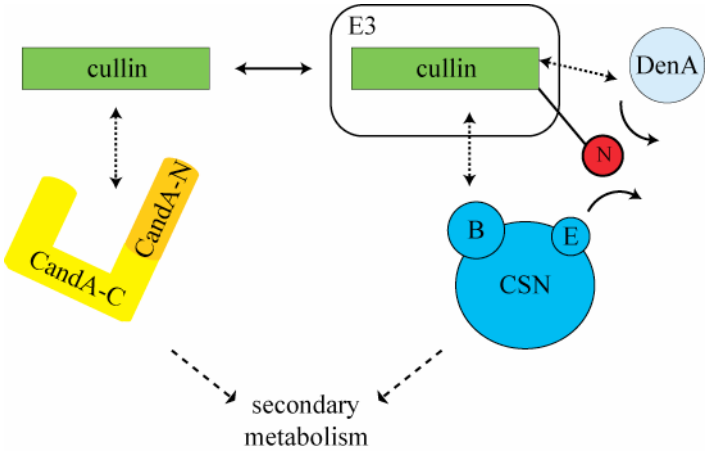


Fig. 39: Model of cullin containing ubiquitin ligase regulation in *A. nidulans*.

The three regulators COP9 signalosome (CSN), DenA and CandA influence the activity of cullin containing ubiquitin ligases (E3). While CSN and DenA control neddylation/deneddylation of the cullin, CandA influences the assembly/disassembly of the complex. CSN binds to cullin via subunit CsnB and deneddylates the cullin due to its intrinsic deneddylase activity located in subunit CsnE. DenA also deneddylates cullin and putatively binds for this purpose to the ubiquitin ligase. CandA binding to cullin prevents the assembly of the complex. Both CandA as well as CSN are connected to secondary metabolism. Double-headed arrows: interactions, round arrows: deneddylase activity, arrows, putative influence.

It has been shown *in vitro* in mammalian cells that Cand1 binding to Cul1 is in equilibrium with the binding of the adaptor protein Skp1 in complex with an F-box protein (Bornstein *et al.*, 2006). Molecular analysis of protein extracts from *A. nidulans* could be very useful to test whether the level of cullins associated with their adaptor proteins changes upon the presence or absence of CandA.

Deletion of both *csnE* and *candA* leads to the formation of red colored hyphae. The substances forming the red pigmentation are assumed to be melanin. Revealing the structure and function of these putatively highly polymerized substances would bring some light into the structures of melanins. This study shows that orcinol and four other orsellinic acid related substances are specifically produced by the deletion mutants. This might indicate that an orsellinic biosynthesis pathway is connected to a general stress response in *A. nidulans* caused by the deletions. On the other hand, it is also possible that the orcinol production is a more direct, specific effect of ubiquitin ligase misregulation due to the loss of CsnE and CandA. Using deletion mutants of polyketide synthases and putative

components of the regulatory ways leading to the production of orcinol in the mutant strains will give insights into the biosynthetic pathways of these substances. In addition, these experiments should also reveal in what way orcinol- and red color formation are linked.

The results of this study reveal the multiple effects of ubiquitin ligase regulators on development of a filamentous fungus. To obtain more knowledge about the mechanisms underlying ubiquitin ligase regulation in *A. nidulans*, it will be essential to identify and characterize the upstream factors regulating CSN, CandA and DenA as well as the downstream factors regulated by CSN, CandA and DenA. *A. nidulans* is the first fungus described harboring a complete, eight-subunit COP9 signalosome in addition to the highly conserved factors CandA and DenA that are not encoded by the yeast *S. cerevisiae*. In contrast to higher eukaryotes, none of these factors is essential. This makes the fungus *A. nidulans*, an amenable, well-investigated organism, a highly suitable, convincing tool for studying and understanding the mechanisms of ubiquitin dependent protein degradation.

5 References

Abe, K., Gomi, K., Hasegawa, F., and Machida, M. (2006) Impact of *Aspergillus oryzae* genomics on industrial production of metabolites. *Mycopathologia* **162**: 143-153.

Abida, W.M., Nikolaev, A., Zhao, W., Zhang, W., and Gu, W. (2007) FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity. *J Biol Chem* **282**: 1797-1804.

Adams, T.H., Wieser, J.K., and Yu, J.H. (1998) Asexual sporulation in *Aspergillus nidulans*. *Microbiol Mol Biol Rev* **62**: 35-54.

Amati, B., and Vlach, J. (1999) Kip1 meets SKP2: new links in cell-cycle control. *Nat Cell Biol* **1**: E91-93.

Aoki, T., Okada, N., Ishida, M., Yogosawa, S., Makino, Y., and Tamura, T.A. (1999) TIP120B: a novel TIP120-family protein that is expressed specifically in muscle tissues. *Biochem Biophys Res Commun* **261**: 911-916.

Aramayo, R., and Timberlake, W.E. (1993) The *Aspergillus nidulans yA* gene is regulated by *abaA*. *EMBO J* **12**: 2039-2048.

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

Axelrod, D.E., Gealt, M., and Pastushok, M. (1973) Gene control of developmental competence in *Aspergillus nidulans*. *Dev Biol* **34**: 9-15.

Baboshina, O.V., and Haas, A.L. (1996) Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5. *J Biol Chem* **271**: 2823-2831.

Bae, M.K., Ahn, M.Y., Jeong, J.W., Bae, M.H., Lee, Y.M., Bae, S.K., Park, J.W., Kim, K.R., and Kim, K.W. (2002) Jab1 interacts directly with HIF-1alpha and regulates its stability. *J Biol Chem* **277**: 9-12.

Ballantine, J.A., Hassall, C.H., and Jones, B.D. (1968) The biosynthesis of phenols - XVII. Some phenolic metabolites of mutant strains of *Aspergillus regulosus*. *Phytochemistry* **7**: 1529-1534.

Bayram, Ö. (2007) Blue light-dependent development of the filamentous fungus *Aspergillus nidulans*. Dissertation, *Georg-August-Universität Göttingen*.

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

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

Bell, A.A., and Wheeler, M.H. (1986) Biosynthesis and functions of fungal melanins. *Ann Rev Phytopathol* **24**: 411-451.

Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**: 293-300.

Birse, C.E., and Clutterbuck, A.J. (1990) N-acetyl-6-hydroxytryptophan oxidase, a developmentally controlled phenol oxidase from *Aspergillus nidulans*. *J Gen Microbiol* **136**: 1725-1730.

Blumenstein, A., Vienken, K., Tasler, R., Purschwitz, J., Veith, D., Frankenberg-Dinkel, N., and Fischer, R. (2005) The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. *Curr Biol* **15**: 1833-1838.

Bochtler, M., Ditzel, L., Groll, M., Hartmann, C., and Huber, R. (1999) The proteasome. *Annu Rev Biophys Biomol Struct* **28**: 295-317.

Bok, J.W., Hoffmeister, D., Maggio-Hall, L.A., Murillo, R., Glasner, J.D., and Keller, N.P. (2006) Genomic mining for *Aspergillus* natural products. *Chem Biol* **13**: 31-37.

Bömeke, K., Pries, R., Korte, V., Scholz, E., Herzog, B., Schulze, F., and Braus, G.H. (2006) Yeast Gcn4p stabilization is initiated by the dissociation of the nuclear Pho85p/Pcl5p complex. *Mol Biol Cell* **17**: 2952-2962.

Bondar, T., Ponomarev, A., and Raychaudhuri, P. (2004) Ddb1 is required for the proteolysis of the *Schizosaccharomyces pombe* replication inhibitor Spd1 during S phase and after DNA damage. *J Biol Chem* **279**: 9937-9943.

Bornstein, G., Ganoth, D., and Hershko, A. (2006) Regulation of neddylation and deneddylation of cullin1 in SCF^{Skp2} ubiquitin ligase by F-box protein and substrate. *Proc Natl Acad Sci U S A* **103**: 11515-11520.

Brachmann, A.O., Joyce, S.A., Jenke-Kodama, H., Schwar, G., Clarke, D.J., and Bode, H.B. (2007) A type II polyketide synthase is responsible for anthraquinone biosynthesis in *Photorhabdus luminescens*. *Chembiochem* **8**: 1721-1728.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Braus, G.H., Krappmann, S., and Eckert, S.E. (2002) Sexual development in ascomycetes: fruit body formation of *Aspergillus nidulans*. In *Molecular biology of fungal development*. Osiewacz, H.D. (ed). New York, Basel: Marcel Dekker, Inc., pp. 215-244.

Brown, D.W., and Salvo, J.J. (1994) Isolation and Characterization of Sexual Spore Pigments from *Aspergillus nidulans*. *Appl Environ Microbiol* **60**: 979-983.

Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H., and Leonard, T.J. (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proc Natl Acad Sci U S A* **93**: 1418-1422.

Bunyapaiboonsri, T., Yoiprommarat, S., Intereya, K., and Kocharin, K. (2007) New diphenyl ethers from the insect pathogenic fungus *Cordyceps* sp. BCC 1861. *Chem Pharm Bull (Tokyo)* **55**: 304-307.

Busby, T.M., Miller, K.Y., and Miller, B.L. (1996) Suppression and enhancement of the *Aspergillus nidulans medusa* mutation by altered dosage of the *bristle* and *stunted* genes. *Genetics* **143**: 155-163.

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

Busch, S., Schwier, E.U., Nahlik, K., Bayram, O., Helmstaedt, K., Draht, O.W., Krappmann, S., Valerius, O., Lipscomb, W.N., and Braus, G.H. (2007) An eight-subunit COP9 signalosome with an intact JAMM motif is required for fungal fruit body formation. *Proc Natl Acad Sci U S A* **104**: 8089-8094.

Bussink, H.J., and Osmani, S.A. (1998) A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*. *EMBO J* **17**: 3990-4003.

Butnick, N.Z., Yager, L.N., Hermann, T.E., Kurtz, M.B., and Champe, S.P. (1984a) Mutants of *Aspergillus nidulans* blocked at an early stage of sporulation secrete an unusual metabolite. *J Bacteriol* **160**: 533-540.

Butnick, N.Z., Yager, L.N., Kurtz, M.B., and Champe, S.P. (1984b) Genetic analysis of mutants of *Aspergillus nidulans* blocked at an early stage of sporulation. *J Bacteriol* **160**: 541-545.

Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., Zacharias, D.A., and Tsien, R.Y. (2002) A monomeric red fluorescent protein. *Proc Natl Acad Sci U S A* **99**: 7877-7882.

Castle, L.A., and Meinke, D.W. (1994) A FUSCA gene of *Arabidopsis* encodes a novel protein essential for plant development. *Plant Cell* **6**: 25-41.

Chamovitz, D.A., and Deng, X.W. (1995) The novel components of the *Arabidopsis* light signaling pathway may define a group of general developmental regulators shared by both animal and plant kingdoms. *Cell* **82**: 353-354.

Chamovitz, D.A., Wei, N., Osterlund, M.T., von Arnim, A.G., Staub, J.M., Matsui, M., and Deng, X.W. (1996) The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. *Cell* **86**: 115-121.

Champe, S.P., Nagle, D.L., and Yager, L.N. (1994) Sexual sporulation. *Prog Ind Microbiol* **29**: 429-454.

Chapman, P.J., and Ribbons, D.W. (1976) Metabolism of resorcinylic compounds by bacteria: orcinol pathway in *Pseudomonas putida*. *J Bacteriol* **125**: 975-984.

Chaveroche, M.K., Ghigo, J.M., and d'Enfert, C. (2000) A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res* **28**: E97.

Cheng, Y., Dai, X., and Zhao, Y. (2004) AtCAND1, a HEAT-repeat protein that participates in auxin signaling in *Arabidopsis*. *Plant Physiol* **135**: 1020-1026.

Chory, J., Peto, C., Feinbaum, R., Pratt, L., and Ausubel, F. (1989) *Arabidopsis thaliana* mutant that develops as a light-grown plant in the absence of light. *Cell* **58**: 991-999.

Chory, J., Nagpal, P., and Peto, C.A. (1991) Phenotypic and Genetic Analysis of det2, a New Mutant That Affects Light-Regulated Seedling Development in *Arabidopsis*. *Plant Cell* **3**: 445-459.

Chuang, H.W., Zhang, W., and Gray, W.M. (2004) Arabidopsis ETA2, an apparent ortholog of the human cullin-interacting protein CAND1, is required for auxin responses mediated by the SCF(TIR1) ubiquitin ligase. *Plant Cell* **16**: 1883-1897.

Ciechanover, A., Heller, H., Elias, S., Haas, A.L., and Hershko, A. (1980) ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proc Natl Acad Sci U S A* **77**: 1365-1368.

Ciechanover, A., and Ben-Saadon, R. (2004) N-terminal ubiquitination: more protein substrates join in. *Trends Cell Biol* **14**: 103-106.

Claret, F.X., Hibi, M., Dhut, S., Toda, T., and Karin, M. (1996) A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. *Nature* **383**: 453-457.

Clutterbuck, A.J. (1969) A mutational analysis of conidial development in *Aspergillus nidulans*. *Genetics* **63**: 317-327.

Clutterbuck, A.J. (1974) *Aspergillus nidulans*. In *Handbook of Genetics*. King, R.C. (ed). Plenum, New York, pp. 447-510.

Clutterbuck, A.J. (1990) The genetics of conidiophore pigmentation in *Aspergillus nidulans*. *J Gen Microbiol* **136**: 1731-1738.

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

Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**: 10881-10890.

de Cássia R. Goncalves, R., and Pombeiro-Sponchiado, S.R. (2005) Antioxidant activity of the melanin pigment extracted from *Aspergillus nidulans*. *Biol Pharm Bull* **28**: 1129-1131.

De Lucca, A.J. (2007) Harmful fungi in both agriculture and medicine. *Rev Iberoam Micol* **24**: 3-13.

DeMartino, G.N., and Gillette, T.G. (2007) Proteasomes: machines for all reasons. *Cell* **129**: 659-662.

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

Dittmar, G.A., Wilkinson, C.R., Jedrzejewski, P.T., and Finley, D. (2002) Role of a ubiquitin-like modification in polarized morphogenesis. *Science* **295**: 2442-2446.

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

Dou, X., Wu, D., An, W., Davies, J., Hashmi, S.B., Ukil, L., and Osmani, S.A. (2003) The PHOA and PHOB cyclin-dependent kinases perform an essential function in *Aspergillus nidulans*. *Genetics* **165**: 1105-1115.

Draht, O. (2005) CsnA Dependent Development and Regulation of Amino Acid Biosynthesis of the Filamentous Ascomycete *Aspergillus nidulans*. Dissertation, *Georg-August-Universität Göttingen*.

Draht, O.W., Busch, S., Hofmann, K., Braus-Stromeyer, S., Helmstaedt, K., Goldman, G.H., and Braus, G.H. (2007) Amino acid supply of *Aspergillus*. In *Basic Biology of the Aspergilli*. Osmani, S. and Goldman, G.H. (eds): in press.

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

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

Elble, R. (1992) A simple and efficient procedure for transformation of yeasts. *Biotechniques* **13**: 18-20.

Feng, S., Shen, Y., Sullivan, J.A., Rubio, V., Xiong, Y., Sun, T.P., and Deng, X.W. (2004) *Arabidopsis* CAND1, an unmodified CUL1-interacting protein, is involved in multiple developmental pathways controlled by ubiquitin/proteasome-mediated protein degradation. *Plant Cell* **16**: 1870-1882.

Fernandez-Abalos, J.M., Fox, H., Pitt, C., Wells, B., and Doonan, J.H. (1998) Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans*. *Mol Microbiol* **27**: 121-130.

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

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

Fujii, I., Mori, Y., Watanabe, A., Kubo, Y., Tsuji, G., and Ebizuka, Y. (1999) Heterologous expression and product identification of *Colletotrichum lagenarium* polyketide synthase encoded by the PKS1 gene involved in melanin biosynthesis. *Biosci Biotechnol Biochem* **63**: 1445-1452.

Fujii, I., Mori, Y., Watanabe, A., Kubo, Y., Tsuji, G., and Ebizuka, Y. (2000) Enzymatic synthesis of 1,3,6,8-tetrahydroxynaphthalene solely from malonyl coenzyme A by a fungal iterative type I polyketide synthase PKS1. *Biochemistry* **39**: 8853-8858.

Fujii, I., Watanabe, A., Sankawa, U., and Ebizuka, Y. (2001) Identification of Claisen cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*. *Chem Biol* **8**: 189-197.

Fujii, I., Yasuoka, Y., Tsai, H.F., Chang, Y.C., Kwon-Chung, K.J., and Ebizuka, Y. (2004) Hydrolytic polyketide shortening by ayg1p, a novel enzyme involved in fungal melanin biosynthesis. *J Biol Chem* **279**: 44613-44620.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M., and Vierstra, R.D. (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. *Proc Natl Acad Sci U S A* **99**: 11519-11524.

Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L.J., Wortman, J.R., Batzoglou, S., Lee, S.I., Basturkmen, M., Spevak, C.C., Clutterbuck, J., Kapitonov, V., Jurka, J., Scazzocchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G.H., Draht, O., Busch, S., D'Enfert, C., Bouchier, C., Goldman, G.H., Bell-Pedersen, D., Griffiths-Jones, S., Doonan, J.H., Yu, J., Vienken, K., Pain, A., Freitag, M., Selker, E.U., Archer, D.B., Penalva, M.A., Oakley, B.R., Momany, M., Tanaka, T., Kumagai, T., Asai, K., Machida, M., Nierman, W.C., Denning, D.W., Caddick, M., Hynes, M., Paoletti, M., Fischer, R., Miller, B., Dyer, P., Sachs, M.S., Osmani, S.A., and Birren, B.W. (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438**: 1105-1115.

Galan, J.M., and Peter, M. (1999) Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism. *Proc Natl Acad Sci U S A* **96**: 9124-9129.

Gan-Erdene, T., Nagamalleswari, K., Yin, L., Wu, K., Pan, Z.Q., and Wilkinson, K.D. (2003) Identification and characterization of DEN1, a deneddylase of the ULP family. *J Biol Chem* **278**: 28892-28900.

Gill, G. (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev* **18**: 2046-2059.

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

Glickman, M.H., and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82**: 373-428.

Glickman, M.H., and Maytal, V. (2002) Regulating the 26S proteasome. *Curr Top Microbiol Immunol* **268**: 43-72.

Glickman, M.H., and Adir, N. (2004) The proteasome and the delicate balance between destruction and rescue. *PLoS Biol* **2**: E13.

Glickman, M.H., and Raveh, D. (2005) Proteasome plasticity. *FEBS Lett* **579**: 3214-3223.

Glombitza, K.-W. (1977) Antibakteriell wirksame Substanzen aus Süß- und Meerwasseralgen. *Nachr Chem Tech* **25**: 133-136.

Goehring, A.S., Rivers, D.M., and Sprague, G.F., Jr. (2003) Urmylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Mol Biol Cell* **14**: 4329-4341.

Goldenberg, S.J., Cascio, T.C., Shumway, S.D., Garbutt, K.C., Liu, J., Xiong, Y., and Zheng, N. (2004) Structure of the Cand1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. *Cell* **119**: 517-528.

Goldknopf, I.L., and Busch, H. (1977) Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. *Proc Natl Acad Sci U S A* **74**: 864-868.

Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D.H., Niall, H.D., and Boyse, E.A. (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci U S A* **72**: 11-15.

Golemis, E., and Brent, R. (1996) Protein interaction studies. In *Current protocols in molecular biology* Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidmann, J.G., Smith, A.J. and Struhl, K. (eds). Massachusetts: Harvard Medical School, pp. 429-454.

Gong, L., and Yeh, E.T. (1999) Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. *J Biol Chem* **274**: 12036-12042.

Goubeaud, A., Knirr, S., Renkawitz-Pohl, R., and Paululat, A. (1996) The *Drosophila* gene *alien* is expressed in the muscle attachment sites during embryogenesis and encodes a protein highly conserved between plants, *Drosophila* and vertebrates. *Mech Dev* **57**: 59-68.

Guthrie, C., and Fink, G. (1991) Guide to yeast genetics and molecular biology. *Methods Enzymol* **194**: 1-863.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**: 791-803.

Hamerman, J.A., Hayashi, F., Schroeder, L.A., Gygi, S.P., Haas, A.L., Hampson, L., Coughlin, P., Aebersold, R., and Aderem, A. (2002) Serpin 2a is induced in activated macrophages and conjugates to a ubiquitin homolog. *J Immunol* **168**: 2415-2423.

Han, K.H., Han, K.Y., Yu, J.H., Chae, K.S., Jahng, K.Y., and Han, D.M. (2001) The *nsdD* gene encodes a putative GATA-type transcription factor necessary for sexual development of *Aspergillus nidulans*. *Mol Microbiol* **41**: 299-309.

Han, S., Navarro, J., Greve, R.A., and Adams, T.H. (1993) Translational repression of *brlA* expression prevents premature development in *Aspergillus*. *EMBO J* **12**: 2449-2457.

Hanahan, D., Jessee, J., and Bloom, F.R. (1991) Plasmid transformation of *Escherichia coli* and other bacteria. *Methods Enzymol* **204**: 63-113.

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

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

Hellmann, H., Hobbie, L., Chapman, A., Dharmasiri, S., Dharmasiri, N., del Pozo, C., Reinhardt, D., and Estelle, M. (2003) *Arabidopsis* AXR6 encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis. *EMBO J* **22**: 3314-3325.

Hermann, T.E., Kurtz, M.B., and Champe, S.P. (1983) Laccase localized in hülle cells and cleistothecial primordia of *Aspergillus nidulans*. *J Bacteriol* **154**: 955-964.

Hershko, A., Ciechanover, A., Heller, H., Haas, A.L., and Rose, I.A. (1980) Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proc Natl Acad Sci U S A* **77**: 1783-1786.

Hicke, L. (2001) Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol* **2**: 195-201.

Hochstrasser, M. (1996) Ubiquitin-dependent protein degradation. *Annu Rev Genet* **30**: 405-439.

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

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

Holmberg, C., Fleck, O., Hansen, H.A., Liu, C., Slaaby, R., Carr, A.M., and Nielsen, O. (2005) Ddb1 controls genome stability and meiosis in fission yeast. *Genes Dev* **19**: 853-862.

Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, K. (1999) Covalent modification of all members of human cullin family proteins by NEDD8. *Oncogene* **18**: 6829-6834.

Huang, X., Hetfeld, B.K., Seifert, U., Kahne, T., Kloetzel, P.M., Naumann, M., Bech-Otschir, D., and Dubiel, W. (2005) Consequences of COP9 signalosome and 26S proteasome interaction. *Febs J* **272**: 3909-3917.

Hunt, L.T., and Dayhoff, M.O. (1977) Amino-terminal sequence identity of ubiquitin and the nonhistone component of nuclear protein A24. *Biochem Biophys Res Commun* **74**: 650-655.

Hwang, J.W., Min, K.W., Tamura, T.A., and Yoon, J.B. (2003) TIP120A associates with unneddylated cullin 1 and regulates its neddylation. *FEBS Lett* **541**: 102-108.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) A ubiquitin-like system mediates protein lipidation. *Nature* **408**: 488-492.

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

Itabashi, T., Nozawa, K., Nakajima, S., and Kawai, T. (1993) A new azaphilone, Falconensin H, from *Emericella falconensis*. *Chem Pharm Bull* **41**: 2040-2041.

Jahn, B., Koch, A., Schmidt, A., Wanner, G., Gehringer, H., Bhakdi, S., and Brakhage, A.A. (1997) Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence. *Infect Immun* **65**: 5110-5117.

James, S.W., Bullock, K.A., Gygax, S.E., Kraynack, B.A., Matura, R.A., MacLeod, J.A., McNeal, K.K., Prasauckas, K.A., Scacheri, P.C., Shenefiel, H.L., Tobin, H.M., and Wade, S.D. (1999) *nimO*, an *Aspergillus* gene related to budding yeast *Dbf4*, is required for DNA synthesis and mitotic checkpoint control. *J Cell Sci* **112 (Pt 9)**: 1313-1324.

Jin, J., Cardozo, T., Lovering, R.C., Elledge, S.J., Pagano, M., and Harper, J.W. (2004) Systematic analysis and nomenclature of mammalian F-box proteins. *Genes Dev* **18**: 2573-2580.

Jones, M.G. (2007) The first filamentous fungal genome sequences: *Aspergillus* leads the way for essential everyday resources or dusty museum specimens? *Microbiology* **153**: 1-6.

Käfer, E. (1965) Origins of translocations in *Aspergillus nidulans*. *Genetics* **52**: 217-232.

Kaldis, P. (2007) Another piece of the p27Kip1 puzzle. *Cell* **128**: 241-244.

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

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

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22**: 159-180.

Kim, H., Han, K., Kim, K., Han, D., Jahng, K., and Chae, K. (2002) The *veA* gene activates sexual development in *Aspergillus nidulans*. *Fungal Genet Biol* **37**: 72-80.

Kim, H.T., Kim, K.P., Lledias, F., Kisselev, A.F., Scaglione, K.M., Skowyra, D., Gygi, S.P., and Goldberg, A.L. (2007) Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. *J Biol Chem* **282**: 17375-17386.

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

Kisselev, A.F., Akopian, T.N., Woo, K.M., and Goldberg, A.L. (1999) The sizes of peptides generated from protein by mammalian 26 and 20S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem* **274**: 3363-3371.

König, W.A., Pfaff, K.-P., Loeffler, W., Schanz, D., and Zähner, H. (1978) Ethericin A; Isolierung, Charakterisierung und Strukturaufklärung eines neuen, antibiotisch wirksamen Diphenylethers. *Liebigs Ann Chem*: 1289-1296.

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

Krappmann, S., Jung, N., Medic, B., Busch, S., Prade, R.A., and Braus, G.H. (2006) The *Aspergillus nidulans* F-box protein GrrA links SCF activity to meiosis. *Mol Microbiol* **61**: 76-88.

Kroetz, M.B. (2005) SUMO: a ubiquitin-like protein modifier. *Yale J Biol Med* **78**: 197-201.

Kumar, S., Yoshida, Y., and Noda, M. (1993) Cloning of a cDNA which encodes a novel ubiquitin-like protein. *Biochem Biophys Res Commun* **195**: 393-399.

Kurz, T., Ozlu, N., Rudolf, F., O'Rourke, S.M., Luke, B., Hofmann, K., Hyman, A.A., Bowerman, B., and Peter, M. (2005) The conserved protein DCN-1/Dcn1p is required for cullin neddylation in *C. elegans* and *S. cerevisiae*. *Nature* **435**: 1257-1261.

Kwok, S.F., Solano, R., Tsuge, T., Chamovitz, D.A., Ecker, J.R., Matsui, M., and Deng, X.W. (1998) *Arabidopsis* homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic *cop/det/fus* mutations. *Plant Cell* **10**: 1779-1790.

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

Lammer, D., Mathias, N., Laplaza, J.M., Jiang, W., Liu, Y., Callis, J., Goebl, M., and Estelle, M. (1998) Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCF^{Cdc4} complex. *Genes Dev* **12**: 914-926.

Langfelder, K., Jahn, B., Gehringer, H., Schmidt, A., Wanner, G., and Brakhage, A.A. (1998) Identification of a polyketide synthase gene (*pksP*) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. *Med Microbiol Immunol* **187**: 79-89.

Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhage, A.A. (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genet Biol* **38**: 143-158.

Lee, B.S., and Taylor, J.W. (1990) Isolation of DNA from fungal mycelia and single spores. In *PCR protocols: A guide to methods and applications*. Innis, M.A., Gelfand, D.H., Sninsky, J.S. and White, T.J. (eds). San Diego: Academic Press Inc pp. 282-287.

Lee, J.W., Choi, H.S., Gyuris, J., Brent, R., and Moore, D.D. (1995) Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. *Mol Endocrinol* **9**: 243-254.

Lewis, C., and Champe, S.P. (1995) A pre-induction sporulation gene from *Aspergillus nidulans*. *Microbiology* **141 (Pt 8)**: 1821-1828.

Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998) A novel protein modification pathway related to the ubiquitin system. *EMBO J* **17**: 2208-2214.

Lima, J.F., Malavazi, I., von Zeska Kress Fagundes, M.R., Savoldi, M., Goldman, M.H., Schwier, E., Braus, G.H., and Goldman, G.H. (2005) The *csnD*/*csnE* signalosome genes are involved in the *Aspergillus nidulans* DNA damage response. *Genetics* **171**: 1003-1015.

Linghu, B., Callis, J., and Goebl, M.G. (2002) Rub1p processing by Yuh1p is required for wild-type levels of Rub1p conjugation to Cdc53p. *Eukaryot Cell* **1**: 491-494.

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

Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (2002) NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. *Mol Cell* **10**: 1511-1518.

Lo, S.C., and Hannink, M. (2006) CAND1-mediated substrate adaptor recycling is required for efficient repression of Nrf2 by Keap1. *Mol Cell Biol* **26**: 1235-1244.

Lu, K.P., Osmani, S.A., and Means, A.R. (1993) Properties and regulation of the cell cycle-specific NIMA protein kinase of *Aspergillus nidulans*. *J Biol Chem* **268**: 8769-8776.

Luders, J., Pyrowolakis, G., and Jentsch, S. (2003) The ubiquitin-like protein HUB1 forms SDS-resistant complexes with cellular proteins in the absence of ATP. *EMBO Rep* **4**: 1169-1174.

Lundgren, J., Masson, P., Realini, C.A., and Young, P. (2003) Use of RNA interference and complementation to study the function of the *Drosophila* and human 26S proteasome subunit S13. *Mol Cell Biol* **23**: 5320-5330.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A., and Deshaies, R.J. (2001) Promotion of NEDD8-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**: 1382-1385.

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

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* **88**: 97-107.

Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., Kao, G.D., Muschel, R.J., and Weiner, D.B. (1998) HIV-1 Vpr interacts with a human 34-kDa mov34 homologue, a cellular factor linked to the G2/M phase transition of the mammalian cell cycle. *Proc Natl Acad Sci U S A* **95**: 3419-3424.

Makino, Y., Yogosawa, S., Kayukawa, K., Coin, F., Egly, J.M., Wang, Z., Roeder, R.G., Yamamoto, K., Muramatsu, M., and Tamura, T. (1999) TATA-Binding protein-interacting protein 120, TIP120, stimulates three classes of eukaryotic transcription via a unique mechanism. *Mol Cell Biol* **19**: 7951-7960.

Malakhov, M.P., Kim, K.I., Malakhova, O.A., Jacobs, B.S., Borden, E.C., and Zhang, D.E. (2003) High-throughput immunoblotting. Ubiquitin-like protein ISG15 modifies key regulators of signal transduction. *J Biol Chem* **278**: 16608-16613.

Marshall, M.A., and Timberlake, W.E. (1991) *Aspergillus nidulans wetA* activates spore-specific gene expression. *Mol Cell Biol* **11**: 55-62.

Mayorga, M.E., and Timberlake, W.E. (1990) Isolation and molecular characterization of the *Aspergillus nidulans wA* gene. *Genetics* **126**: 73-79.

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

Maytal-Kivity, V., Pick, E., Piran, R., Hofmann, K., and Glickman, M.H. (2003) The COP9 signalosome-like complex in *S. cerevisiae* and links to other PCI complexes. *Int J Biochem Cell Biol* **35**: 706-715.

McGahren, W.J., Andres, W.W., and Kunstmann, M.P. (1970) A Novel Diaryl Ether, LL-V125α, from a Fungus of the Order *Sphaeropsidales*. *J Org Chem* **35**: 2433-2435.

McGoldrick, C.A., Gruver, C., and May, G.S. (1995) *myoA* of *Aspergillus nidulans* encodes an essential myosin I required for secretion and polarized growth. *J Cell Biol* **128**: 577-587.

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

Mendoza, H.M., Shen, L.N., Botting, C., Lewis, A., Chen, J., Ink, B., and Hay, R.T. (2003) NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. *J Biol Chem* **278**: 25637-25643.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, K.Y., Toennis, T.M., Adams, T.H., and Miller, B.L. (1991) Isolation and transcriptional characterization of a morphological modifier: the *Aspergillus nidulans* stunted (*stuA*) gene. *Mol Gen Genet* **227**: 285-292.

Miller, K.Y., Wu, J., and Miller, B.L. (1992) StuA is required for cell pattern formation in *Aspergillus*. *Genes Dev* **6**: 1770-1782.

Min, K.W., Hwang, J.W., Lee, J.S., Park, Y., Tamura, T.A., and Yoon, J.B. (2003) TIP120A associates with cullins and modulates ubiquitin ligase activity. *J Biol Chem* **278**: 15905-15910.

Min, K.W., Kwon, M.J., Park, H.S., Park, Y., Yoon, S.K., and Yoon, J.B. (2005) CAND1 enhances deneddylation of CUL1 by COP9 signalosome. *Biochem Biophys Res Commun* **334**: 867-874.

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

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., and Ohsumi, Y. (1998) A protein conjugation system essential for autophagy. *Nature* **395**: 395-398.

Momany, M., Westfall, P.J., and Abramowsky, G. (1999) *Aspergillus nidulans swo* mutants show defects in polarity establishment, polarity maintenance and hyphal morphogenesis. *Genetics* **151**: 557-567.

Mooney, J.L., and Yager, L.N. (1990) Light is required for conidiation in *Aspergillus nidulans*. *Genes Dev* **4**: 1473-1482.

Mosbach, K., and Schultz, J. (1971) Studies on lichen enzymes. Purification and properties of orsellinate decarboxylase obtained from *Lasallia pustulata*. *Eur J Biochem* **22**: 485-488.

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

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

Nahlik, K. (2007) The COP9 signalosome of *Aspergillus nidulans*: Regulation of protein degradation and transcriptional pathways in sexual development. Dissertation, *Georg-August-Universität Göttingen*.

Nair, M.S., and Carey, S.T. (1979) Metabolites of pyrenomycetes: XII. Polyketides from the Hypocreales. *Mycologia* **71**: 1089-1096.

Nakamura, M., Xavier, R.M., Tsunematsu, T., and Tanigawa, Y. (1995) Molecular cloning and characterization of a cDNA encoding monoclonal nonspecific suppressor factor. *Proc Natl Acad Sci U S A* **92**: 3463-3467.

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001) Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* **414**: 514-521.

Naumann, M., Bech-Otschir, D., Huang, X., Ferrell, K., and Dubiel, W. (1999) COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK. *J Biol Chem* **274**: 35297-35300.

Nayak, T., Szewczyk, E., Oakley, C.E., Osmani, A., Ukil, L., Murray, S.L., Hynes, M.J., Osmani, S.A., and Oakley, B.R. (2006) A versatile and efficient gene-targeting system for *Aspergillus nidulans*. *Genetics* **172**: 1557-1566.

Ni, M., and Yu, J.H. (2007) A Novel Regulator Couples Sporogenesis and Trehalose Biogenesis in *Aspergillus nidulans*. *PLoS ONE* **2**: e970.

Nielsen, O. (2003) COP9 signalosome: a provider of DNA building blocks. *Curr Biol* **13**: R565-567.

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

Oshikawa, K., Matsumoto, M., Yada, M., Kamura, T., Hatakeyama, S., and Nakayama, K.I. (2003) Preferential interaction of TIP120A with Cul1 that is not modified by NEDD8 and not associated with Skp1. *Biochem Biophys Res Commun* **303**: 1209-1216.

Papagianni, M. (2007) Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. *Biotechnol Adv* **25**: 244-263.

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

Petroski, M.D., and Deshaies, R.J. (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6**: 9-20.

Pettersson, G. (1964). On the Biosynthesis of Toluquinones from *Aspergillus fumigatus*. I. The Biogenetic Role of Orsellinic Acid and Orcinol. *Acta Chem Scand* **18**: 1202-1207.

Pettersson, G. (1965) New Phenolic Metabolites from *Gliocladium Roseum*. *Acta Chem Scand* **19**: 414-420.

Pirt, S.J., and Rowley, B.I. (1969) Melanin production in *Aspergillus nidulans*. *Biochem J* **114**: 9P-10P.

Plonka, P.M., and Grabacka, M. (2006) Melanin synthesis in microorganisms - biotechnological and medical aspects. *Acta Biochim Pol* **53**: 429-443.

Podust, V.N., Brownell, J.E., Gladysheva, T.B., Luo, R.S., Wang, C., Coggins, M.B., Pierce, J.W., Lightcap, E.S., and Chau, V. (2000) A Nedd8 conjugation pathway is essential for proteolytic targeting of $p27^{Kip1}$ by ubiquitination. *Proc Natl Acad Sci U S A* **97**: 4579-4584.

Pontecorvo, G., Roper, J.A., Hemmons, L.M., Macdonald, K.D., and Bufton, A.W. (1953) The genetics of *Aspergillus nidulans*. *Adv Genet* **5**: 141-238.

Prade, R.A., and Timberlake, W.E. (1993) The *Aspergillus nidulans brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiophore development. *EMBO J* **12**: 2439-2447.

Punt, P.J., Dingemanse, M.A., Kuyvenhoven, A., Soede, R.D., Pouwels, P.H., and van den Hondel, C.A. (1990) Functional elements in the promoter region of the *Aspergillus nidulans gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. *Gene* **93**: 101-109.

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

Quail, P.H. (1994) Photosensory perception and signal transduction in plants. *Curr Opin Genet Dev* **4**: 652-661.

Raasi, S., Schmidtke, G., and Groettrup, M. (2001) The ubiquitin-like protein FAT10 forms covalent conjugates and induces apoptosis. *J Biol Chem* **276**: 35334-35343.

Sakata, E., Yamaguchi, Y., Miyauchi, Y., Iwai, K., Chiba, T., Saeki, Y., Matsuda, N., Tanaka, K., and Kato, K. (2007) Direct interactions between NEDD8 and ubiquitin E2 conjugating enzymes upregulate cullin-based E3 ligase activity. *Nat Struct Mol Biol* **14**: 167-168.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press.

Schaefer, L., Beermann, M.L., and Miller, J.B. (1999) Coding sequence, genomic organization, chromosomal localization, and expression pattern of the signalosome component *Cops2*: the mouse homologue of *Drosophila alien*. *Genomics* **56**: 310-316.

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

Scherer, M., and Fischer, R. (1998) Purification and characterization of laccase II of *Aspergillus nidulans*. *Arch Microbiol* **170**: 78-84.

Schier, N., Liese, R., and Fischer, R. (2001) A Pcl-like cyclin of *Aspergillus nidulans* is transcriptionally activated by developmental regulators and is involved in sporulation. *Mol Cell Biol* **21**: 4075-4088.

Schier, N., and Fischer, R. (2002) The *Aspergillus nidulans* cyclin PclA accumulates in the nucleus and interacts with the central cell cycle regulator $NimX^{Cdc2}$. *FEBS Lett* **523**: 143-146.

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

Schwechheimer, C. (2004) The COP9 signalosome (CSN): an evolutionary conserved proteolysis regulator in eukaryotic development. *Biochim Biophys Acta* **1695**: 45-54.

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

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

Serino, G., and Deng, X.W. (2003) The COP9 signalosome: regulating plant development through the control of proteolysis. *Annu Rev Plant Biol* **54**: 165-182.

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

Sewall, T.C., Mims, C.W., and Timberlake, W.E. (1990) *abaA* controls phialide differentiation in *Aspergillus nidulans*. *Plant Cell* **2**: 731-739.

Shen, L.N., Liu, H., Dong, C., Xirodimas, D., Naismith, J.H., and Hay, R.T. (2005) Structural basis of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1. *EMBO J* **24**: 1341-1351.

Shen, Z., Pardington-Purtymun, P.E., Comeaux, J.C., Moyzis, R.K., and Chen, D.J. (1996) UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics* **36**: 271-279.

Skromne, I., Sanchez, O., and Aguirre, J. (1995) Starvation stress modulates the expression of the *Aspergillus nidulans brlA* regulatory gene. *Microbiology* **141 (Pt 1)**: 21-28.

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

Spain, B.H., Bowdish, K.S., Pacal, A.R., Staub, S.F., Koo, D., Chang, C.Y., Xie, W., and Colicelli, J. (1996) Two human cDNAs, including a homolog of *Arabidopsis* FUS6 (COP11), suppress G-protein- and mitogen-activated protein kinase-mediated signal transduction in yeast and mammalian cells. *Mol Cell Biol* **16**: 6698-6706.

Stinnett, S.M., Espeso, E.A., Cobeno, L., Araujo-Bazan, L., and Calvo, A.M. (2007) *Aspergillus nidulans* VeA subcellular localization is dependent on the importin alpha carrier and on light. *Mol Microbiol* **63**: 242-255.

Su, W., Li, S., Oakley, B.R., and Xiang, X. (2004) Dual-Color imaging of nuclear division and mitotic spindle elongation in live cells of *Aspergillus nidulans*. *Eukaryot Cell* **3**: 553-556.

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

Swart, K., van Heemst, D., Slakhorst, M., Debets, F., and Heyting, C. (2001) Isolation and characterization of sexual sporulation mutants of *Aspergillus nidulans*. *Fungal Genet Biol* **33**: 25-35.

Takenaka, Y., Tanahashi, T., Nagakura, N., and Hamada, N. (2003) Phenyl ethers from cultured lichen mycobionts of *Graphis scripta* var. *serpentina* and *G. rikuzensis*. *Chem Pharm Bull (Tokyo)* **51**: 794-797.

Taniguchi, M., Kaneda, N., Shibata, K., and Kamikawa, T. (1978) Isolation and biological activity of aspermutarubrol, a self-growth inhibitor from *Aspergillus sydowi*. *Agric Biol Chem* **42**: 1629-1630.

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

Tomoda, K., Kubota, Y., and Kato, J. (1999) Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. *Nature* **398**: 160-165.

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

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

Tomoda, K., Kato, J.Y., Tatsumi, E., Takahashi, T., Matsuo, Y., and Yoneda-Kato, N. (2005) The Jab1/COP9 signalosome subcomplex is a downstream mediator of Bcr-Abl kinase activity and facilitates cell-cycle progression. *Blood* **105**: 775-783.

Tsai, H.F., Washburn, R.G., Chang, Y.C., and Kwon-Chung, K.J. (1997) *Aspergillus fumigatus arp1* modulates conidial pigmentation and complement deposition. *Mol Microbiol* **26**: 175-183.

Tsai, H.F., Wheeler, M.H., Chang, Y.C., and Kwon-Chung, K.J. (1999) A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *J Bacteriol* **181**: 6469-6477.

Tsai, H.F., Fujii, I., Watanabe, A., Wheeler, M.H., Chang, Y.C., Yasuoka, Y., Ebizuka, Y., and Kwon-Chung, K.J. (2001) Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. *J Biol Chem* **276**: 29292-29298.

Tsitsigiannis, D.I., Zarnowski, R., and Keller, N.P. (2004) The lipid body protein, PpoA, coordinates sexual and asexual sporulation in *Aspergillus nidulans*. *J Biol Chem* **279**: 11344-11353.

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

Turner, W.B. (1971) *Fungal metabolites*. London/New York: Academic Press Inc.

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

Vallim, M.A., Miller, K.Y., and Miller, B.L. (2000) *Aspergillus* SteA (sterile12-like) is a homeodomain-C_2/H_2-Zn^{+2} finger transcription factor required for sexual reproduction. *Mol Microbiol* **36**: 290-301.

van Gorcom, R.F., Punt, P.J., Pouwels, P.H., and van den Hondel, C.A. (1986) A system for the analysis of expression signals in *Aspergillus*. *Gene* **48**: 211-217.

Veith, D., Scherr, N., Efimov, V.P., and Fischer, R. (2005) Role of the spindle-pole-body protein ApsB and the cortex protein ApsA in microtubule organization and nuclear migration in *Aspergillus nidulans*. *J Cell Sci* **118**: 3705-3716.

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

Vienken, K., Scherer, M., and Fischer, R. (2005) The $Zn(II)_2Cys_6$ putative *Aspergillus nidulans* transcription factor repressor of sexual development inhibits sexual development under low-carbon conditions and in submersed culture. *Genetics* **169**: 619-630.

Vienken, K., and Fischer, R. (2006) The $Zn(II)_2Cys_6$ putative transcription factor NosA controls fruiting body formation in *Aspergillus nidulans*. *Mol Microbiol* **61**: 544-554.

Vijay-Kumar, S., Bugg, C.E., and Cook, W.J. (1987) Structure of ubiquitin refined at 1.8 Å resolution. *J Mol Biol* **194**: 531-544.

Wada, H., Kito, K., Caskey, L.S., Yeh, E.T., and Kamitani, T. (1998) Cleavage of the C-terminus of NEDD8 by UCH-L3. *Biochem Biophys Res Commun* **251**: 688-692.

Waring, R.B., May, G.S., and Morris, N.R. (1989) Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin-coding genes. *Gene* **79**: 119-130.

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

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

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

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

Wei, N., and Deng, X.W. (1998) Characterization and purification of the mammalian COP9 complex, a conserved nuclear regulator initially identified as a repressor of photomorphogenesis in higher plants. *Photochem Photobiol* **68**: 237-241.

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

Wei, N., and Deng, X.W. (1999) Making sense of the COP9 signalosome. A regulatory protein complex conserved from *Arabidopsis* to human. *Trends Genet* **15**: 98-103.

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

Welchman, R.L., Gordon, C., and Mayer, R.J. (2005) Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat Rev Mol Cell Biol* **6**: 599-609.

Westermann, M. (2007) Charakterisierung von Stressmetaboliten aus *Aspergillus*-Mutanten. First state exam work, *Georg-August-Universität Göttingen*.

Wheeler, M.H., and Bell, A.A. (1988) Melanins and their importance in pathogenic fungi. *Curr Top Med Mycol* **2**: 338-387.

Wilkinson, K.D., Urban, M.K., and Haas, A.L. (1980) Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *J Biol Chem* **255**: 7529-7532.

Wilkinson, K.D., and Hochstrasser, M. (1998) *Ubiquitin and the Biology of the Cell.* New York: Plenum Publishing Corp.

Woodcock, D.M., Crowther, P.J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S.S., Michael, M.Z., and Graham, M.W. (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res* **17**: 3469-3478.

Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., Lee, C.G., Wei, N., Wilkinson, K.D., Wang, R., and Pan, Z.Q. (2003) DEN1 is a dual function protease capable of processing the C terminus of Nedd8 and deconjugating hyper-neddylated CUL1. *J Biol Chem* **278**: 28882-28891.

Xirodimas, D.P., Saville, M.K., Bourdon, J.C., Hay, R.T., and Lane, D.P. (2004) Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* **118**: 83-97.

Yamazaki, M., and Maebayashi, Y. (1982) Structure determination of Violaceol-I and -II, new fungal metabolites from a strain of *Emericella violacea*. *Chem Pharm Bull* **30**: 514-518.

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

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

Yang, X., Zhou, J., Sun, L., Wei, Z., Gao, J., Gong, W., Xu, R.M., Rao, Z., and Liu, Y. (2007) Structural basis for the function of DCN-1 in protein Neddylation. *J Biol Chem* **282**: 24490-24494.

Yogosawa, S., Makino, Y., Yoshida, T., Kishimoto, T., Muramatsu, M., and Tamura, T. (1996) Molecular cloning of a novel 120-kDa TBP-interacting protein. *Biochem Biophys Res Commun* **229**: 612-617.

Yogosawa, S., Kayukawa, K., Kawata, T., Makino, Y., Inoue, S., Okuda, A., Muramatsu, M., and Tamura, T. (1999) Induced expression, localization, and chromosome mapping of a gene for the TBP-interacting protein 120A. *Biochem Biophys Res Commun* **266**: 123-128.

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

Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M., Conaway, R.C., Conaway, J.W., Harper, J.W., and Pavletich, N.P. (2002b) Structure of the Cul1-Rbx1-Skp1-F box^{Skp2} SCF ubiquitin ligase complex. *Nature* **416**: 703-709.

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

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

Zhou, P., and Howley, P.M. (1998) Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. *Mol Cell* **2**: 571-580.

Zonneveld, B.J. (1972) Morphogenesis in *Aspergillus nidulans*. The significance of a alpha-1, 3-glucan of the cell wall and alpha-1, 3-glucanase for cleistothecium development. *Biochim Biophys Acta* **273**: 174-187.

Zonneveld, B.J. (1977) Biochemistry and ultrastructure of sexual development in *Aspergillus*. In *Genetics and physiology of Aspergillus*. Smith, J.E., and Pateman, J.A. (eds). London: Academic Press, pp. 59-80.

Curriculum vitae

Personal details

Elke Ute Schwier
born on September 10, 1976
in Herford, Germany

Education

1983-1987: **Primary education**, Grundschule Obernbeck, Löhne, Germany
1987-1996: **Secondary education**, Städtisches Gymnasium Löhne, Germany

Scientific Background

Sept. 1996 – Feb. 2003: **Study** of biology at the Georg-August-University Göttingen, Germany

Aug. 1998 – June 1999: **Study** of biology at the Lund University, Sweden

Mai 2003 – Aug. 2003: **Practical work** at the School of Life and Environmental Sciences, University Nottingham, United Kingdom

from Sept. 2003: **Scientific assistant** at the Department of Molecular Microbiology and Genetics of Prof. Dr. G.H. Braus in the Institute of Microbiology and Genetics, Georg-August-University Göttingen, Germany