Deneddylation and fungal development

Regulation of Nedd8 protein modification by DenA

and the COP9 signalosome

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Für meine Eltern.

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Summary

Ubiquitin dependent protein degradation is a common principle in eukaryotic organisms to control appropriate temporal and spatial protein levels. Abnormal regulation leads to embryonic lethality in plants, insects and mammals and is responsible for the development of certain types of cancer in humans. Substrate specificity of degradation is mediated by E3-ubiquitin ligases, of which most contain a cullin as the core component. Cullin containing ligases are regulated by covalent modification with the ubiquitin-like protein Nedd8. The COP9 signalosome (CSN) and the deneddylating protein 1 (DEN1) are two abundant deneddylases capable of removing Nedd8 from CRLs. This study employs the fungal model organism *Aspergillus nidulans* to explore the function of deneddylation for development. (i) The homolog of DEN1 in the fungus, DenA, was identified and the developmental function was investigated. Genetic experiments revealed a crucial function of *denA* for the promotion of asexual development and the repression of sexual fruit body formation in the presence of light. Biochemical studies, as well as molecular and genetic analysis clearly indicate a deneddylase activity of DenA. Double deletion of *denA* and *csnE* results in a strain that can only grow vegetative. Impairment of asexual as well as sexual development in the double knock-out suggests that some developmental functions are shared between the two deneddylases. Both, *denA* and *csnE* deletion strains display distinct phenotypic characteristics, but overlap in their inability to inhibit the initiation of sexual development in light. This converges in a physical interaction between DenA and the CSN complex that is presumably involved in controlling the balance between the two deneddylases on the protein level. The results indicate a crucial function of deneddylation for the coordination of fungal development. This requires distinct functions of DenA in terms of asexual spore formation and of CSN to proceed beyond the primordial stage of sexual development. Orchestrated function of both deneddylases is required for the light-dependent inhibition of sexual fruit body formation. (ii) Furthermore the CSN complex was studied with regard to the dynamics of complex formation *in vivo*. Functional, tagged versions of CsnA and CsnD were explored for their ability to recruit the remaining subunits of the CSN in co-purification experiments. CsnA recruited six additional subunits in most experiments, but failed to pull-down the catalytic active subunit CsnE. CsnD co-purified with all other seven subunits when expressed in the wild type background. Deletion of *csnG* in the corresponding strain completely abolished the ability of CsnD to recruit any of the additional CSN subunits. These data suggest that the fungal CSN exists primarily as holo-complex, whereas sub-complexes are hardly maintained *in vivo*.

Zusammenfassung

Der Abbau von Proteinen durch das Ubiquitin-Proteasome System ist ein Mechanismus mit zentraler Bedeutung für die korrekte Steuerung unterschiedlicher zelluläre Prozesse. Fehlregulation führt bei Pflanzen, Insekten und Säugetieren bereits im Embryonalstadium zum Absterben des Organismus und ist an der Entstehung einiger Formen von Krebs beteiligt. Die spezifische Markierung von Zielproteinen mit Ubiquitin benötigt die Aktivität von E3-Ligasen. Die größte Gruppe dieser Enzyme enthält ein Cullin als zentrales Element. Diese Art von Ligasen wird in ihrer Aktivität und Stabilität durch das kleine, Ubiquitin-ähnliche Protein Nedd8 reguliert. Das COP9 Signalosome (CSN) und die humane Deneddylase 1 (DEN1) sind die häufigsten Proteasen, die diese Modifikation rückgängig machen. In dieser Arbeit wurde die Rolle der beiden Deneddylasen für die Entwicklung des filamentösen Pilzes *Aspergillus nidulans* untersucht. (i) Das homologe Protein zu humanem DEN1 im Pilz, DenA, konnte identifiziert werden und seine Rolle für die Fruchtkörperbildung wurde untersucht. DenA spielt eine wichtige Rolle bei der lichtabhängigen Förderung asexueller Entwicklung und bei der Repremierung der sexuellen Fruchtkörperbildung im Licht. Genetische Studien, sowie Ergebnisse aus biochemischen und molekularen Experimenten zeigen deutlich das DenA Deneddylaseaktivität besitzt. Deletiert man beide Deneddylasen, *csnE* und *denA*, ist der entsprechende Stamm zu keiner Fruchtkörperbildung mehr fähig, wächst jedoch noch vegetativ, was auf eine teilweise Funktionsüberschneidung zwischen beiden Proteasen hinweist. Beide Einzelmutanten zeigen spezifische Phänotypen, aber gleichen sich in der Eigenschaft, die Initiierung sexueller Entwicklung im Licht nicht mehr unterdrücken zu können. Diese Verbindung zeigt sich auch in Form einer physischen Interaktion zwischen dem CSN Komplex und DenA welche möglicherweise dazu dient die Balance zwischen beiden Proteasen zu kontrollieren. Die Ergebnisse dieser Arbeit zeigen das Deneddylierung eine wichtige Rolle bei der Koordinierung von Entwicklung im Pilz spielt. DenA beeinflusst vorrangig die asexuelle Sporenbildung, während das CSN für die sexuelle Fruchtkörperentwicklung wichtig ist. Beide Deneddylasen beeinflussen die Lichtregulation der sexuellen Entwicklung. (ii) Der CSN Komplex wurde hinsichtlich der Dynamik des Zusammenbaus untersucht. Hierzu wurden Aufreinigungsexperimente mit modifizierten, funktionellen Versionen der Untereinheiten CsnA und CsnD durchgeführt. In Aufreinigungen mit CsnA konnten die meisten CSN Untereinheiten identifiziert werden, mit Ausnahme der enzymatisch aktiven Untereinheit CsnE. CsnD interagierte, ohne Ausnahme, mit allen anderen CSN Untereinheiten, verlor diese Interaktion jedoch komplett nachdem *csnG* im entsprechenden Stamm deletiert wurde. Dies lässt vermuten, dass das CSN im Pilz primär als Gesamtkomplex vorliegt, während kleinere Unterkomplexe in der Zelle nicht stabil sind.

1 Introduction

1.1 Ubiquitin dependent protein degradation

The coordinated cutback of proteins is a common principle in all living organisms. Degradation is required to remove wrongly synthesized or misfolded polypeptides and to control the temporal and spatial presence of proteins. Especially regulatory proteins like kinases, transcription factors, proteases, receptors, intracellular signal molecules and others are often tightly regulated by degradation. In multi-cellular organisms the latter is important for controlled differentiation and proliferation while disruption leads to severe phenotypes and is involved in certain types of cancer in humans (Ciechanover et al, 2000).

Regulatory proteins are most abundant in the nucleus or in the cytoplasm. Their degradation is generally facilitated through the 26S proteasome pathway. Therefore proteins are marked selectively with a small peptide that ubiquitously exists among all eukaryotes and therefore is referred to as ubiquitin (Ub). To emphasize the importance of protein ubiquitination as a prerequisite for degradation by the 26S proteasome the pathway is also named ubiquitin-proteasome system (Hershko & Ciechanover, 1998). The endoplasmatic reticulum associated protein degradation (ERAD) also uses ubiquitin-dependent proteasomal degradation to remove unassembled or misfolded proteins inside the endoplasmatic reticulum. Proteins are transported back from the endoplasmatic reticulum into the cytoplasm, followed by ubiquitination and subsequent degradation by the proteasome (Meusser et al, 2005).

Proteins within organelles, distinct cellular compartments or complexes can also be degraded *en bloc* by autophagy. The targets are enclosed in a double membrane vesicle that subsequently fuses with the vacuole or lysosomes, both containing hydrolytic enzymes (Kundu & Thompson, 2008). Ubiquitination plays only an indirect role for this degradation pathway through its role for trafficking or endocytosis of target proteins (Hicke, 2001; Welchman et al, 2005). Both pathways, autophagy and proteasomal degradation are suggested to have cross-communication abilities regarding intra-cellular protein breakdown (Kundu & Thompson, 2008).

1.2 The Ubiquitin family (UbF) of proteins

1.2.1 Ubiquitin

Ubiquitin was initially described by lymphocyte differentiation experiments in rat liver (Goldstein et al, 1975). These experiments revealed that it binds to histone H2A via an isopeptide linkage between the ubiquitin C-terminus and the side chain amino group of a histone lysine residue (Goldknopf & Busch, 1977; Hunt & Dayhoff, 1977). In 1980 Avram Hershko and Aaron Ciechanover investigated energy dependence of protein breakdown. They found a polypeptide in reticulocyte lysate fractionation which could be covalently attached to other proteins, but only in the presence of certain co-factors and ATP (Ciechanover et al, 1980; Hershko et al, 1980). Therefore the protein was named ATP-dependent proteolysis factor 1 (APF-1). APF-1 was then recognized to be identical to the previously described ubiquitin (Wilkinson et al, 1980).

Ubiquitin became the common name of this 76 amino acid long protein. In the following years of research it turned out that there are variations in the ubiquitin modification of substrate proteins. Mono-ubiquitination means attachment of a single ubiquitin moiety to one lysine residue (Figure 1A). Modification of several lysine residues within a substrate protein with a single ubiquitin is referred to as multi-ubiquitination (Figure 1B). Poly-ubiquitination leads to the formation of chains of several ubiquitin molecules connected via internal lysine residues to a primary ubiquitin, bond to the lysine residue of the substrate protein (Figure 1C/D). Ubiquitin contains seven internal lysines which are all involved in chain formation. Chains formed via internal lysines K48 (Figure 1C) or K29 generally target the recipient protein to degradation by the 26S-proteasome (Kim et al, 2007; Welchman et al, 2005). Other forms of ubiquitin modification are involved in activity control of substrate proteins, trafficking, endocytosis, DNA repair and transcriptional regulation (reviewed by (Hicke, 2001; Welchman et al, 2005). In 2004 Aaron Ciechanover, Avram Hershko and Irwin Rose were jointly awarded with the Nobel Prize in Chemistry for the discovery of ubiquitin-dependent protein degradation (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2004/index.html).

Figure 1: Different ubiquitin modifications of substrate proteins.

A Mono-ubiquitination. **B** Multi-ubiquitination. **C** Ubiquitin chains are formed in different shapes. K48 linked chains target the substrate for degradation. **D** K63 linked ubiquitin chains result in different consequences for the substrate protein. Ub=ubiquitin; The Ub silhouette originates from structural data by PDB entry 2ZVO.

1.2.2 Ubiquitin-like proteins

In the years after the discovery of ubiquitin a number of other small polypeptides, covalently modifying proteins were identified. The family of ubiquitin-like proteins evolved and became an emerging field in molecular biology of eukaryotic organisms in recent years. Often these proteins significantly differ from ubiquitin in amino acid sequence (Hershko et al, 1983), but they are all covalently attached to other proteins by a similar mechanism (Kerscher et al, 2006) and share structural properties (Dye & Schulman, 2007) (Figure 2).

Nedd8 (Neuronal precursor cell developmentally down-regulated gene 8) is the closest relative of ubiquitin within the group of ubiquitin-like (Ubl) proteins (Kamitani et al, 1997). The most prominent targets for Nedd8 modification are the Cullin subunits of CRLs (Cullin-RING E3-ligases) (Deshaies et al, 2010). Nedd8 modification of Cullins is an important regulatory mechanism for ubiquitin-dependent protein degradation (see chapter 1.5.1).

Another well conserved representative of the ubiquitin-like family is SUMO (small ubiquitin-like modifier) (Mahajan et al, 1997). Modification of proteins with SUMO controls their sub-cellular localization and it is required for protein-protein interactions (Lomeli & Vazquez, 2011). Recently sumoylation of proteins was also shown to be involved in ubiquitin-dependent breakdown by the proteasome (Geoffroy & Hay, 2009).

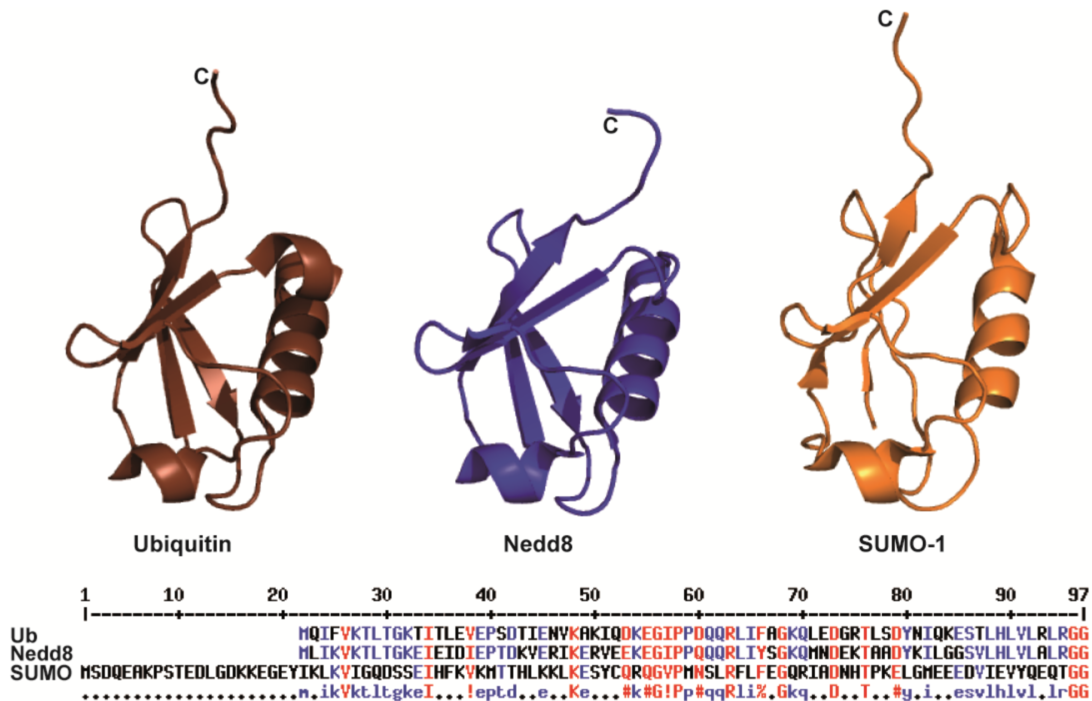

Figure 2: Alignment and structure of mammalian ubiquitin, Nedd8 and SUMO-1.

3D structures of ubiquitin (PDB: 3NHE), Nedd8 (PDB: 2KO3) and SUMO-1 (PDB: 2UYZ) aligned with PyMOL. C-termini are indicated (C). Multiple alignment of the amino acid sequences of the mature forms of mammalian ubiquitin (PRO_0000396477), Nedd8 (PRO_0000042767) and SUMO-1 (PRO_0000035939). High consensus residues (>90%) are highlighted in red and low consensus (>50%) in blue.

Modification of a protein with FAT10 (human leukocyte antigen F-associated), another member of the ubiquitin-like family, also targets the substrate for proteasomal degradation, but in an ubiquitin-independent manner (Pelzer & Groettrup, 2010). FAT10 is also involved in activity regulation of p53 and NFκB (Gong et al, 2010; Li et al, 2011a).

Atg8 (autophagy related gene 8) and Atg12 are two ubiquitin-like proteins also involved in cellular protein turnover. Modification with these polypeptides does not lead to proteasomal degradation, but is required for membrane fusion during autophagosomal protein breakdown (Chung et al, 2010; Geng & Klionsky, 2008; Nakatogawa et al, 2007; Radoshevich & Debnath, 2011).

1.3 Ubiquitin modification and proteasomal degradation

Ubiquitination of proteins as a hallmark for proteasomal degradation is a general mechanism to ensure degradation of key factors for cellular regulation. It is further required to remove misfolded proteins. The ubiquitin-proteasome system is

involved in numerous processes such as cell cycle regulation, differentiation, stress response, secretion, transcriptional regulation and DNA repair. Furthermore circadian rhythm, immune response and organelle biogenesis require a functional ubiquitin-proteasome system. To form the ubiquitin chain which targets the substrate protein to the proteasome, monomeric ubiquitin is required. This is generated from precursor forms by the activity of UCH proteases (ubiquitin C-terminal hydrolase) such as UCH-L1 and 3 (Larsen et al, 1996; Wada et al, 1998).

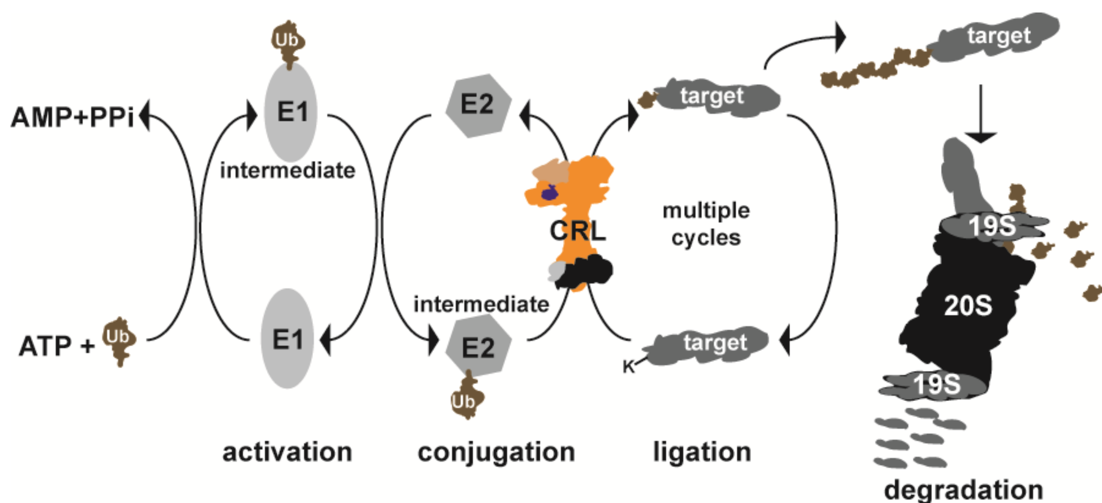

Figure 3: The ubiquitination cascade.

In the first step of the ubiquitin pathway, the C-terminal glycine residue of ubiquitin is activated by an E1-activating enzyme in an ATP dependent reaction (activation). Thereafter, ubiquitin is transferred to a reactive cysteine of an E2-conjugating enzyme (conjugation). E2-conjugating enzymes generally require the aid of an E3-ubiquitin–ligase to form the isopeptide linkage at the target protein (ligation). The CRL (cullin-RING based E3-ligases) functions as an adaptor, juxtaposing the E2-conjugating enzyme loaded with ubiquitin and the substrate to allow transfer of ubiquitin directly from the E2 to a conserved lysine residue (K) at the target protein. After several repetitions of ubiquitin ligation a chain is formed at the target protein marking it for degradation by the 26S proteasome (degradation). Ubiquitin is recycled before breakdown of the target. (modified after (Watson & Irwin, 2006)

Mature ubiquitin is attached to the lysine residue of the recipient protein in a three step enzyme cascade (Figure 3). Initially, ubiquitin needs to be activated in an ATP dependent step which is carried out by an E1-activating enzyme (Haas et al, 1982). This is followed by the transfer of the protein to an E2-conjugating enzyme (Sommer & Seufert, 1992). Finally it is attached to the ε-amino group of a lysine residue within the recipient protein. Formation of the primary isopeptide linkage between the C-terminal glycine of the first ubiquitin and the lysine of the target protein requires an additional E3-ligase activity, as well as the subsequent linkages of following ubiquitin molecules to the internal K48 residue of the prior ubiquitin

(Kerscher et al, 2006). The K48 linked ubiquitin chain is finally recognized by a receptor subunit within the 26S proteasome leading to breakdown of the substrate protein and recycling of the ubiquitin molecules (Kisselev et al, 1999) (Figure 3).

In mammals ubiquitination involves two E1-enzymes, Uba1 (Handley-Gearhart et al, 1994) and Uba6 (Groettrup et al, 2008), several E2-enzymes and a mass of E3-ligases reflecting the broad range of substrate proteins to be ubiquitinated (Kerscher et al, 2006) (see chapter 1.4).

The 26S proteasome, in brief, is a complex combined of the 20S core-particle and the 19S regulatory-complex. The 19S molecule can be subdivided into two nine-subunit protein complexes. The base contains mainly ATPase and chaperone activity and is in direct contact with the 20S core, and the lid-complex (LID) is on top of it and at least two of its subunits display de-ubiquitination activity. The regulatory complex is attached on both sites of the 20S core, destabilizing the tertiary structure of the substrate proteins in an energy consuming way (Enchev et al, 2010).

1.4 Ubiquitin-E3-ligases

Substrate specificity of ubiquitination, and thereby specificity of proteasomal degradation, is mediated by the E3-ubiquitin ligases. Currently, two different E3-based mechanisms for substrate ubiquitination are described. The HECT E3-ubiquitin ligases and the RING E3-ubiquitin ligases (Kerscher et al, 2006). The two E3-ubiquitin ligase families differ by the way the ubiquitin molecule is transferred from the E2-conjugating enzyme to the lysine side chain of the substrate protein.

E3-ubiquitin ligases of the HECT family adopt the ubiquitin from the E2-conjugating enzyme by forming a thioester bond and then transfer it onto the recipient protein (Schwarz et al, 1998). Contrary, RING-E3-ubiquitin ligases (Kamura et al, 1999; Ohta et al, 1999; Seol et al, 1999; Skowyra et al, 1999; Tan et al, 1999) support the transfer of the ubiquitin molecule without binding it. They are only assisting isopeptide bond formation between the C-terminal glycine of ubiquitin, which is still at the E2-conjugating enzyme, and the ε-amino group of the lysine residue at the substrate protein (Ozkan et al, 2005).

The cullin RING E3-ubiquitin ligases (CRL) are the biggest group within the family of E3-ubiquitin ligases (Pan et al, 2004). All of them form heterogeneous complexes with cullin as scaffold protein. Seven cullins are described in mammals

(Dias et al, 2002; Kipreos et al, 1996; Mathias et al, 1996), while the filamentous ascomycete *A. nidulans* contains orthologs for three of them, CulA (CUL1), CulC (CUL3) and CulD (CUL4) (Galagan et al, 2005) (Table I).

Table I: Cullins and corresponding cullin-RING ligases (CRL).

Cullin#	Cullin homologs in *A. nidulans* (Helmstaedt et al, 2011)	subunit organization	names
CUL1 [Cdc53]	CulA (AN1019)	F-Box protein/SKP1/CUL1/RING	SCF; CDL1
CUL2		SOCS/BC-box protein/elonginBC/CUL2/RING	VDC; CBC; ECS; SCF2; CDL2
CUL3 [Cul3]	CulC (AN3939)	BTB-domain protein/CUL3/RING	BCR3; SCF3; CDL3
CUL4A	CulD (AN10008)	Receptor/DDB1/CUL4A/RING	VDC; DCX; SCF4; CDL4
CUL4B		DDB1/CUL4/RING(Li et al, 2011b)	none
CUL5		SOCS/BC-protein/elonginBC/CUL5/RING	SCF5; CDL5
CUL7		FBX29/SKP1/CUL7/RING	SCF7; CSDL7

#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 SCF (Skp1/CUL1/F-Box) E3-ubiquitin ligases, based on CUL1 as a scaffold are the best known group within the family of CRLs. CUL1 and the C-terminally associated RING (really interesting new gene) protein ROC1/Rbx1 (regulator of cullins-1/RING box protein-1) form the core complex of this type of ligases. ROC1/Rbx1 is important for the recruitment of the E2-ubiquitin-conjugating enzyme to the ligase complex (Seol et al, 1999; Wu et al, 2000; Zheng et al, 2002b). Furthermore ROC1/Rbx1 is suggested to be an E3-ligase for the ubiquitin-like protein Nedd8 (Dharmasiri et al, 2003; Morimoto et al, 2003).

At the N-terminus of CUL1 the Skp1 (S-phase kinase-associated protein 1) protein is recruited to the complex. Skp1 is responsible for the recruitment of the substrate specificity mediating F-Box protein (Skowyra et al, 1997). Together the two proteins form the substrate specific part of the SCF E3-ubiquitin ligase (Figure 4). It is suggested that the interaction of F-Box proteins with Skp1 stabilizes the conformation of the F-Box protein thereby enhancing substrate binding of the ubiquitin ligase (Yoshida et al, 2011).

Regarding the large number of possible substrates to be ubiquitinated, it is not surprising that the F-Box family of adaptor proteins is quite large and diverse. The human genome includes almost 70 proteins containing a F-Box domain (Jin et al, 2004), as does that of *A. nidulans* (Busch et al, 2007; Galagan et al, 2005), whereas in plants actually about 700 F-Box proteins are described (Gagne et al, 2002).

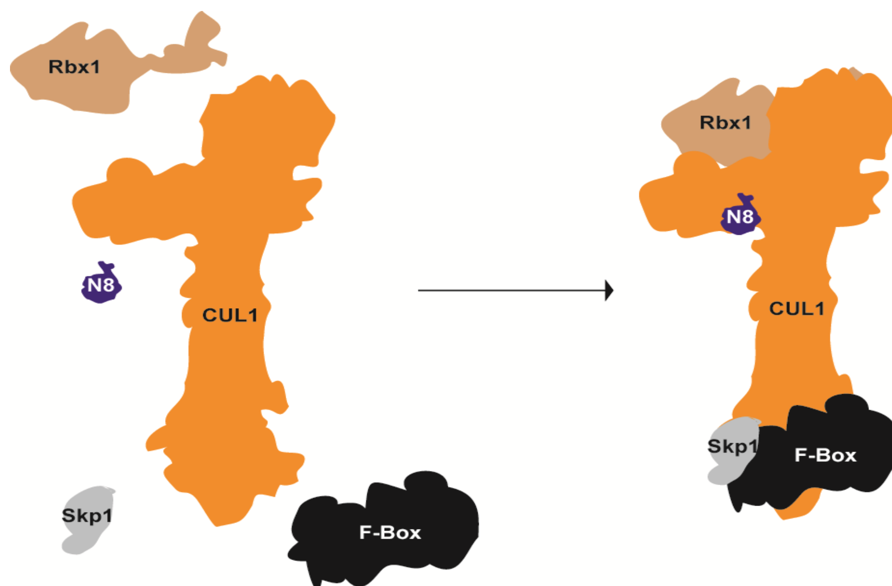

Figure 4: Scheme of SCF complex composition.

CUL1 and the C-terminally associated RING protein Rbx1 form the core complex of a SCF-E3-ubiquitin ligase. The situation appears similar for other CRL, but the cullin is different from CUL1. Additionally the SCF ligase contains the Skp1 adaptor protein at the N-terminus of CUL1, which recruits the F-Box protein to the complex, mediating substrate specificity. Modification of the SCF ligase with the ubiquitin-like protein Nedd8 (N8) enhances the ubiquitination activity and stabilizes the complex (Saha & Deshaies, 2008). Left: "blown apart" view of the components; right: assembled and active ligase complex. Silhouettes originate from structural data by PDB entry 1LDK (SCF components) and 1XT9 (Nedd8).

Adjacent to the large and well investigated group of SCF E3s further CRLs based on other cullins exist in the cell. CUL3 based CRL complexes, for example, also contain ROC1/Rbx1. But they differ from SCFs by their substrate recognition subunit which binds directly to the N-terminus of CUL3 utilizing a BTB/POZ domain (Furukawa et al, 2003). In CRL containing CUL4 as a scaffold the adaptor protein DDB1 is responsible for the recruitment of substrate recognition subunits. These generally contain WD-repeats, which mediate interaction with DDB1 (Higa et al, 2006), but also this class contains ROC1/Rbx1 within its core complex. All other cullins are also involved in CRL formation (Table I; for detailed review see Petroski & Deshaies (2005)).

1.5 Cycles of neddylation and deneddylation regulate CRL activity

1.5.1 Neddylation enhances CRL activity

Nedd8 modification of the cullin subunit of CRL ligases at a conserved lysine residue within the C-terminal part of the protein (Figure 4) enhances ubiquitination activity and stabilizes the E3 complex (Saha & Deshaies, 2008). Neddylation was first discovered for the *S. cerevisiae* cullin Cdc53 (Lammer et al, 1998). Neddylation enhances ubiquitin ligase activity by assisting the charged E2-ubiquitin conjugating enzyme in positioning at the CRL complex at the site of Rbx1 (Sakata et al, 2007). Nedd8 further induces a conformational change that allows the initiator ubiquitin to bridge a gap between the E2 and the substrate to be ubiquitinated (Duda et al, 2008).

Similar to ubiquitination (compare chapter 1.3 and Figure 3) the 81 amino acid protein Nedd8 is translated as a precursor molecule that requires hydrolytic cleavage. Processing of the precursor removes the extension of a few amino acids releasing the C-terminal glycine residue which is required for substrate modification. This function is generally dedicated to proteins of the UCH (ubiquitin C-terminal hydrolase) family, such as UCH-L3 in mammals (Wada et al, 1998) or Yuh1 (yeast ubiquitin hydrolase 1) in yeast (Linghu et al, 2002). In addition the ubiquitin-like protease 1 (ULP1) family protein DEN1 in mammals was shown to be capable of cleaving the Nedd8 C-terminus *in vitro* (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003).

Akin ubiquitin, mature Nedd8 is activated by an E1-enzyme, the heterodimeric APP-BP1/Uba3, in an ATP dependent reaction (Walden et al, 2003). Following this activation it is transferred to the E2-conjugating protein Ubc12 (Huang et al, 2005), which is unique to Nedd8. The Nedd8 E3 ligase forming the isopeptide bond between the C-terminal glycine 76 of Nedd8 and the amino side chain of the lysine residue within the substrate protein is yet controversial. Most studies assign this function to the RING subunit ROC1/Rbx1 within the CRL (Dharmasiri et al, 2003; Morimoto et al, 2003), but others also demonstrated a crucial role for the DCN-1/Dcn1p (deficient in cullin neddylation) protein of *C. elegans* and *S. cerevisiae* in Nedd8 ligation (Kurz et al, 2008; Kurz et al, 2005; Yang et al, 2007). In *Aspergillus nidulans* deletion of the ortholog *dcnA* results in a

decreased level of protein neddylation, but neither fungal viability nor development are affected (von Zeska Kress et al, 2012).

1.5.2 Deneddylation inactivates CRL complexes and allows re-composition

The high specificity of the ubiquitin-proteasome system is mainly achieved by the broad spectrum of distinct substrates that can be addressed. This is represented by the large number of CRL adaptors, such as the F-Box proteins (Jin et al, 2004; Kipreos & Pagano, 2000; Lechner et al, 2006). However, only a small fraction of the cellular pool of CRLs is thought to exist in the neddylated, highly active form to allow subtle stability control of regulative proteins. Temporal and spatial regulation of protein degradation therefore requires not only neddylation, but also the removal of Nedd8 from the cullin to facilitate exchange of substrate adaptors within CRL complexes (Bosu & Kipreos, 2008; Deshaies & Joazeiro, 2009).

This process named deneddylation is generally attributed to the MPN+/JAMM metalloprotease motif within the fifth subunit of the COP9 signalosome (CSN) (Cope et al, 2002). The CSN is a heterogenic protein complex consisting of eight subunits described for a variety of eukaryotic organisms since its initial discovery in the plant *Arabidopsis thaliana* (Wei & Deng, 1999) (chapter 1.5.3). Beside the CSN, another protein, previously classified as SUMO specific protease, was described to be a deneddylase in mammals. *In vitro* biochemical experiments revealed that the human DEN1 (deneddylase1; also referred to as NEDP1 for Nedd8 specific protease) has a much higher affinity to cleave Nedd8 modified conjugates than SUMO or ubiquitin (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003) (chapter 1.5.4). An additional level of complexity is added to the regulation of CRL through CAND1 (cullin associated Nedd8 dissociated protein 1) which selectively binds to non-neddylated cullins (Helmstaedt et al, 2011; Liu et al, 2002) (chapter 1.5.5).

1.5.3 The COP9-signalosome (CSN)

The COP9-signalosome is highly conserved from fungi to humans. It was first described in plants where mutants displayed a constitutive photomorphogenic (COP) phenotype (Wei et al, 1994b). In mammals the CSN was independently isolated by two groups, as the ortholog of *A. thaliana* CSN (Wei & Deng, 1998) and as a byproduct in purifications of 26S proteasomes (Seeger et al, 1998). The complex

was also found in the fruit fly *Drosophila melanogaster* (Freilich et al, 1999). The filamentous ascomycete *A. nidulans* is up to date the simplest organism containing a complete eight subunit CSN (Busch et al, 2007). Other amenable fungal model organisms like *Neurospora crassa*, *Schizosaccharomyces pombe* or the baker's yeast *Saccharomyces cerevisiae* contain only partial signalosomes or even single subunits. The filamentous fungus *N. crassa* lacks the smallest subunit CSN8 and its CSN is thus composed of seven subunits only (He et al, 2005; He & Liu, 2005). In *S. pombe* only six subunits have conserved orthologs; CSN6 and CSN8 are missing (Mundt et al, 1999). However, *S. cerevisiae* contains only one ortholog for the catalytic active subunit CSN5, referred to as Rri1 in yeast, and shows only poor conservation for any of the other CSN subunits (Maytal-Kivity et al, 2003; Wee et al, 2002). In 2000 a unified nomenclature for the COP9 signalosome was introduced (Deng et al, 2000), which is widely accepted today (Table II). However, this study uses the *A. nidulans* nomenclature where applicable.

The CSN complex is involved in diverse cellular functions like cell cycle control, gene expression, DNA repair and development. Defects in CSN activity cause severe phenotypes in multicellular organisms (Chamovitz, 2009). Disruption of CSN function leads to embryonic lethality in plants (Wei & Deng, 1999), insects (Freilich et al, 1999) and mammals (Tomoda et al, 2004). In *A. nidulans* disruption of the CSN complex causes a block in early sexual development and an altered secondary metabolism, but mutants are viable (Braus et al, 2010; Busch et al, 2003; Busch et al, 2007; Nahlik et al, 2010). Overexpression of CSN subunits is involved in the development of certain types of cancer in humans (Kato & Yoneda-Kato, 2009). This demonstrates the importance of the CSN complex in the development of eukaryotic organisms.

The COP9 signalosome shares certain similarities in structure and subunit composition with the LID of the proteasome and with eIF3 (eukaryotic translation initiation factor 3) (Enchev et al, 2010). The regulatory LID of the proteasome, the CSN as well as eIF3 contain six subunits with a domain (PCI) characterized by a conserved region of about 200 amino acids forming a globular winged helix (WH) sub-domain composed of several curved, bi-helical repeats (Dessau et al, 2008; Pick et al, 2009; Scheel & Hofmann, 2005). The PCI domains are suggested to be important in protein-protein interaction and thereby for assembly of the

corresponding complex, but also for the interaction with associated proteins (Scheel & Hofmann, 2005).

Table II: Unified and original nomenclature of the CSN complex.

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/Rri2, Csn11p/Pci8p, Csn12p
CSN2	Sgn1, 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	COPP8, FUS4	**CsnD**	CSN-4	Sgn4[sp]	
CSN5	Sgn5, JAB1	COPS5, Jab1	DCH5	AJH1, AJH2	**CsnE**	CSN-5	subunit5	MPN domain protein: Rri1p (Csn5)
CSN6	Sgn6, HVIP	COPS6	DCH6	subunit 6	**CsnF**	CSN-6	-	-
CSN7	Sgn7	COPS7a, COPS7b	DCH7	FUS5	**CsnG**	CSN-7	subunit7	
CSN8	Sgn8, hCOP9	COPS8	DCH8	COP9	**CsnH**	-	-	-

[a] (Chamovitz & 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 & Deng, 1998); [c] (Freilich et al, 1999; Goubeaud et al, 1996); [d] (Castle & Meinke, 1994; Chamovitz et al, 1996; Karniol et al, 1998; Kwok et al, 1998; Serino et al, 1999; Wei et al, 1994a); [e] (Braus et al, 2010; Busch et al, 2007; Nahlik et al, 2010); [f] (He et al, 2005); [g] (Mundt et al, 2002; Mundt et al, 1999), Genebank; [h] (Maytal-Kivity et al, 2003). Modified from (Petroski & Deshaies, 2005; Schwier, 2007)

The two residual subunits contain a MPN (Mpr1p, Pad1p N-terminal) domain with a JAMM (Jab1/MPN domain metalloenzyme) motif that presumably originated from a metal-binding motif and is even more conserved than the PCI domains. Incidentally, the MPN+ domains in CSN5 or Rpn11 are catalytically active metalloproteases, mediating deneddylation or de-ubiquitination activity, respectively (Chamovitz, 2009). Deneddylase activity is coupled to formation of the CSN holo-complex (Cope et al, 2002).

The CSN structure was initially reported at the rather poor resolution of an electron microscopy density map, suggesting a dynamic particle displaying a certain similarity to the 19S regulatory LID of the proteasome (Kapelari et al, 2000). In recent days these data were improved by a study using electron microscopy (EM) combined with single particle analysis which verified the conservation of the basic structure of CSN, LID, and eIF3 already suggested previously (Enchev et al, 2010; Sharon et al, 2009; Sharon et al, 2006). However structural information beyond 25Å is not yet available for any of the three PCI complexes. There are publications,

reporting evidence for the existence of independently existing CSN subunits and CSN sub-complexes. Recently mass spectrometry data and results from single particle EM lead to the idea of a split COP9-signalosome. Two sub-complexes are postulated in these publications. One contains CSN4, CSN5, CSN6 and CSN7 and the other is composed of CSN1, CSN2, CSN3 and CSN8. Both parts are connected via CSN1 and CSN6 in the holo-complex (Enchev et al, 2010; Sharon et al, 2009) (Figure 5).

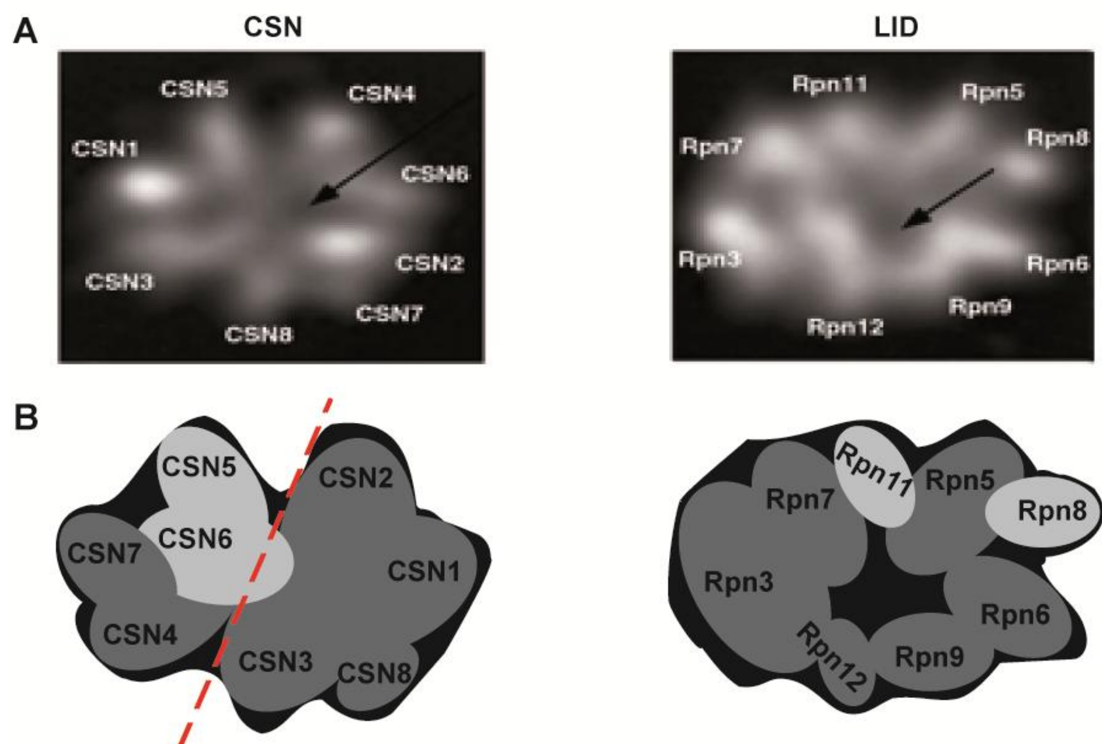

Figure 5: Subunit composition of the COP9-signalosome and the 19S regulatory LID of the proteasome.

A Electron density maps for both complexes (Kapelari et al, 2000). **B** Graphical illustration of both particles derived from the corresponding electron density map. The CSN subunits are rearranged with respect to the data from MS (Sharon et al, 2009) and single particle electron microscopy (Enchev et al, 2010). The dashed, red line represents the proposed axis between the two sub-complexes suggested by Enchev et al. (2010) and Sharon at al. (2009). Subunits with an MPN domain are light grey and PCI subunits are dark grey in both complexes. Details are given in the text.

Accordingly, CSN4 and CSN7 can be found in smaller fractions than the holo-complex, independently of CSN1, in plants or flies (Karniol et al, 1999; Oron et al, 2002; Serino et al, 1999; Wang et al, 2002). In *S. pombe* subunits CSN4 and CSN5 can be found in smaller fractions in the absence of CSN1 or CSN2 (Mundt et al, 2002). In mammalian cells formation of a cytoplasmic sub-complex composed of

CSN4,-5,-6,-7b and -8 is described (Tomoda et al, 2002). However, it is not clear whether these sub-complexes are functionally relevant.

Studies in plants, vertebrates and fungi suggest that deletion of one CSN subunit leads to the loss of the CSN holo-complex *in vivo* (Busch et al, 2007; Serino & Deng, 2003). *A. nidulans* tandem affinity purification (TAP) applying tagged CsnE (CSN5) recruits all other seven subunits in co-purification experiments in a wild type background, but when *csnA* (*CSN1*) or *csnD* (*CSN4*) are deleted only CsnE (CSN5) can be enriched by purification from crude extracts (Busch et al, 2007).

CSN5 in mammals is able to exist alone as a relative stable protein and thought to have functions apart from the CSN complex. For example nuclear export and degradation of p27, as well as the degradation of CFTR (cystic fibrosis transmembrane conductance regulator) are CSN5 dependent, but do not require the deneddylase activity of the CSN holo-complex. Similarly, the function of CSN5 in apoptosis and cell proliferation is thought to be not fully CSN dependent (reviewed by (Wei et al, 2008). However, the fifth subunit harbors the only intrinsic enzymatic activity within the CSN complex which removes Nedd8 from substrate proteins.

Additionally the CSN is thought to act as an assembly platform recruiting a variety of other proteins involved in protein modification, e.g. kinases or the de-ubiquitinating enzyme USP15/Ubp12 (Huang et al, 2009; Zhou et al, 2003). Furthermore CRL can be associated to the CSN complex via the N-terminal domains of CSN1,-2 and -6 (Lyapina et al, 2001; Schwechheimer et al, 2001; Yang et al, 2002). This and further interactions observed with the two other PCI complexes, the LID and eIF3, suggest the formation of super-complexes involved in the regulation of ubiquitin-dependent protein degradation (Fu et al, 2001; Pick et al, 2009).

1.5.4 The human deneddylase 1 (DEN1)

The human protein DEN1 (also referred to as NEPD1) was initially annotated as SUMO specific isopeptidase of the SENP group (sentrin/SUMO-specific protease) named SENP8 (Gan-Erdene et al, 2003). Several groups could purify the protein in 2003 and performed *in vitro* activity assays. These experiments revealed that the protein has significantly higher affinity for Nedd8 than for SUMO or ubiquitin. The other seven members of the SENP group (SENP1-7) are specific for SUMO and are required for processing of the SUMO precursor and de-sumoylation of proteins (Johnson, 2004). All of them belong to the Ulp1 family of cysteine proteases

characterized by a conserved triad of the amino acids histidine (H), aspartate (D) and cysteine (C) amended by an invariant glutamine (Q) residue. Additionally these experiments showed that DEN1/NEDP1 can not only remove the isopeptide linkage between Nedd8 and the substrate protein, but is also capable of processing linear Nedd8 chains to achieve the mature Nedd8 variant (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003) (Figure 6).

Differentiation between Nedd8 and ubiquitin by the protease requires the alanine at position 72 in Nedd8, which is an arginine in ubiquitin (Shen et al, 2005). Structural studies revealed a crucial role of the N-terminus of DEN1 for interaction and recognition of Nedd8 (Reverter et al, 2005).

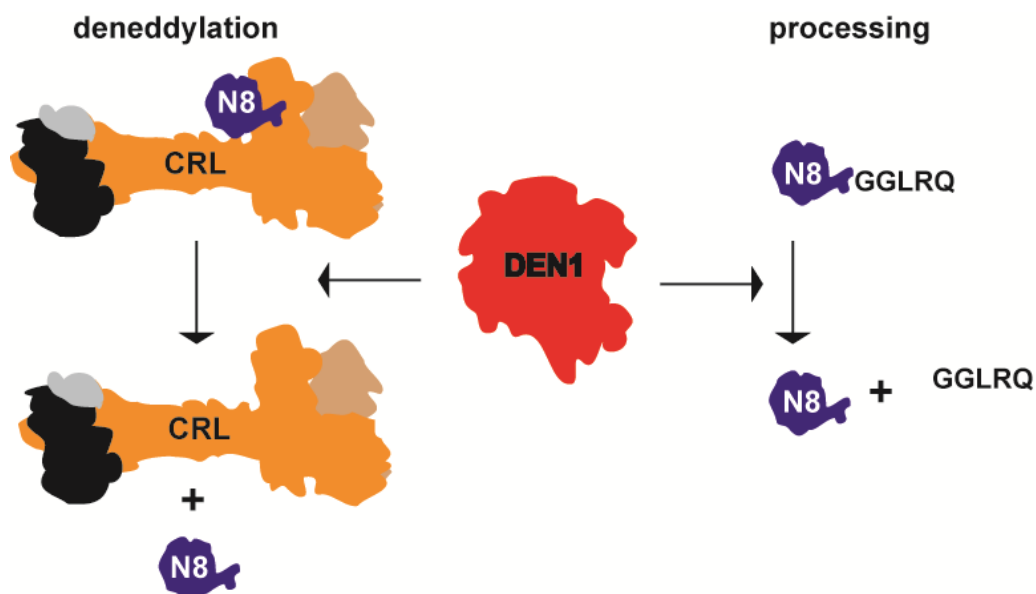

Figure 6: Mammalian DEN1 is a dual functional protease.

DEN1 is capable of cleaving Nedd8 (N8) off from a modified substrate, e.g. the cullin of a CRL (deneddylation). On the other hand DEN1 can also remove the C-terminal tail from the Nedd8 precursor protein producing the mature molecule, which can be attached to substrate proteins (processing). The latter function is referred to as processing activity. Silhouettes originate from structural data by PDB entry 1LDK (SCF components) and 1XT9 (Nedd8 and DEN1).

Orthologs of mammalian DEN1 are found in *Drosophila melanogaster* and *Schizosaccharomyces pombe*. The *Drosophila* DEN1 protein was reported to deneddylate non-cullin proteins and the corresponding *DEN1* deletion mutant can overcome *Nedd8* mutant lethality (Chan et al, 2008). The two *S. pombe* isoforms Nep1 and Nep2, similar to the mammalian deneddylase, display deneddylation activity *in vitro*, whereas the corresponding deletion mutants did not show obvious phenotypes with regard to cell viability. *nep* mutants in yeast accumulate neddylated

proteins which do not correspond to modified cullins, similar to the observations obtained from the fly mutants (Zhou & Watts, 2005).

In mammals, DEN1 is involved in the stability control of MDM2 (murine double minute 2) and subsequent regulation of p53 (Watson et al, 2010), as well as in the regulation of apoptosis. Caspase activity promoting apoptosis can be blocked by IAP (inhibitor of apoptosis) mediated neddylation, thus deneddylation of these caspases by DEN1 positively regulates apoptosis (Broemer et al, 2010).

1.5.5 The cullin associated Nedd8 dissociated protein 1 (CAND1)

CAND1 was initially discovered as TIP120A (TBP (TATA binding protein) interacting protein) in nuclear extracts of mammalian cells (Yogosawa et al, 1996). The protein is conserved in eukaryotes and mostly encoded by a single gene. Interestingly, the ascomycete *A. nidulans* contains two genes for *cand1*, referred to as *candA* in the fungus. The larger ORF encodes for a protein similar to the C-terminal part of CAND1 from other organisms, whereas the second gene is smaller and corresponds to the N-terminus. Both proteins, CandA-C and CandA-N interact and assemble to a fungal CandA protein that resembles those encoded by a single gene in other organisms (Busch et al, 2007; Helmstaedt et al, 2011). All CAND1 orthologs are reported to exclusively associate to unneddylated cullins (Bosu et al, 2010; Chuang et al, 2004; Feng et al, 2004; Helmstaedt et al, 2011; Hwang et al, 2003; Liu et al, 2002; Min et al, 2003; Oshikawa et al, 2003; Schwier, 2007; Zheng et al, 2002a).

Structural studies on mammalian proteins revealed that the CAND1 N-terminus wraps around the C-terminal region of CUL1, thereby blocking the neddylation site at CUL1 lysine 720. The CAND1 C-terminus interacts to the CUL1 N-terminus and obstructs the binding surface for the substrate adaptor (Goldenberg et al, 2004) (Figure 7).

CAND1 and CSN mediated deneddylation of CRLs are reported to be required for sufficient ubiquitination activity of the E3 ligases *in vivo*. In contrast, both proteins are found to inhibit CRLs *in vitro*, a situation that is also known as the CAND1/CSN paradox (Bosu & Kipreos, 2008; Busch et al, 2003; Busch et al, 2007; Feng et al, 2004; Liu et al, 2002).

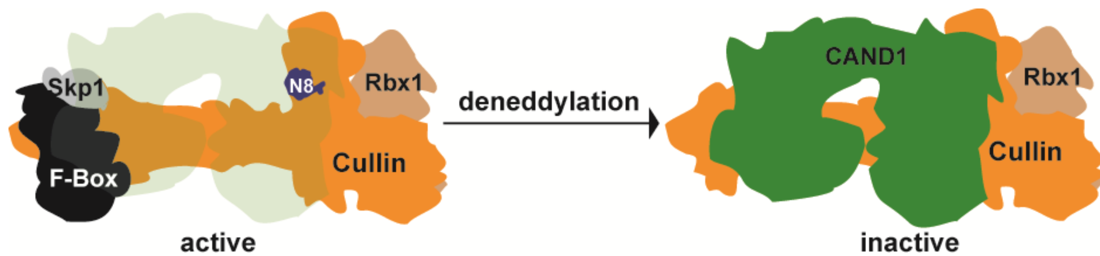

Figure 7 CAND1 associates with CRL core complexes.

Deneddylation by CSN or DEN1 destabilizes the CRL complex and Skp1 and the F-Box protein fall apart. This allows binding of CAND1 which blocks the substrate adaptor part of the cullin with its C-terminal part and the neddylation site with the N-terminal region (compare shaded illustration on the left hand side) resulting in a completely inactive ligase. Silhouettes originate from structural data by PDB entry 1LDK (SCF components) and 1XT9 (Nedd8) and 1UG6 (CAND1).

A cycling model has been proposed, for the situation *in vivo*, by which CRLs change their composition through controlled rounds of assembly and disassembly (Figure 8). Thus, Nedd8 modification of the E3 complexes promotes ligase activity, whereas deneddylation leads to disassembly and inactive cullin-RING sub-complexes. CAND1 can bind to a small fraction of these sub-complexes and allows stability independent recycling of CRL substrate adaptors (Lo & Hannink, 2006). CAND1 is dissociated from the cullin-RING core-complex by Skp1 and an F-Box protein inducing a new round of CRL assembly (Bornstein et al, 2006; Siergiejuk et al, 2009).

CAND1 replacement through the substrate adaptor might be further promoted by other factors, like neddylation of CAND1 itself, as observed for the CAND1 ortholog Lag2 in baker's yeast (Siergiejuk et al, 2009). The recent model is that CRLs change from a CAND1 to CSN cycle, when substrate adaptors become available. The CSN cycle starts with substrate binding followed by neddylation, activating ubiquitin ligase activity. CRLs are re-introduced to the CAND1 cycle after deneddylation to allow incorporation of scarce adaptors into CRLs. CAND1 association might also protect the cullin-RING core complex from degradation (Bornstein et al, 2006; Dubiel, 2009; Schmidt et al, 2009).

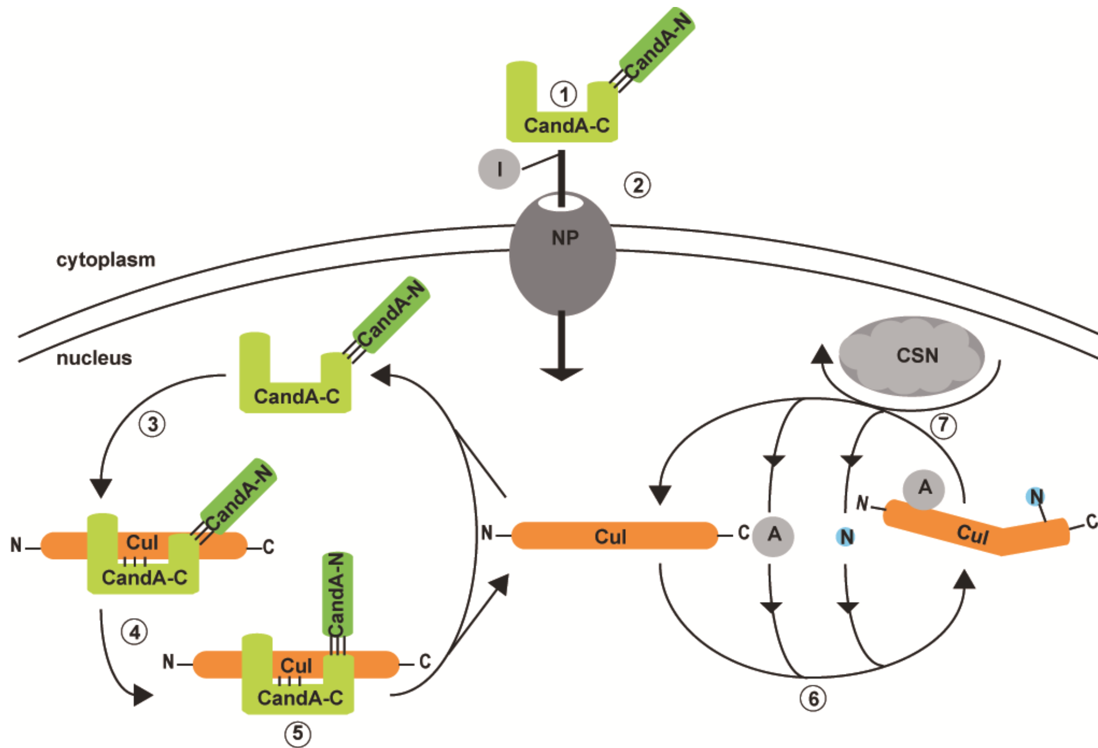

Figure 8: Regulation of CRL activity by cycles of neddylation/deneddylation and CAND1 association.

Molecular function of split, fungal CandA. The situation appears similar in other organisms, except that CAND1 is a single protein. [1] CandA-C/N forms a heterodimer in the cytoplasm. [2] CandA-C nuclear localization signal interacts with importins (I) for transport through the nuclear pore (NP). [3] CandA-C site initiates cullin (Cul) interaction, which [4] mediates binding between CandA-N and cullin's C-terminal domain. [5] This leads to full inhibition of cullin-E3 ligase activity. [6] Release of CandA from cullin allows the formation of new cullin-E3 ligase complexes through recruitment of an adapter protein (A) and subsequent neddylation (N). [7] Removal of Nedd8 through CSN activity destabilizes the cullin complexes and starts the CandA cycle (Helmstaedt et al, 2011).

1.6 The model organism *Aspergillus nidulans*

A. nidulans is a filamentous ascomycete of the order Eurotiales originating from soil borne material, where it lives as a saprotroph. The group of *Aspergilli* includes several other species, of which some are of medical or industrial importance.

A. fumigatus and *A. terreus*, for example, are opportunistic human pathogens that became a thread especially for immune-compromised persons, such as AIDS patients or cancer patients in chemo-/radiotherapy. Aflatoxin, a product of *A. flavus* and *A. parasiticus* secondary metabolism is a very potent poison occurring in spoiled food (Brakhage, 2005; Varga et al, 2011).

Contrary, there are other species which are economically relevant, such as *A. niger* which is used in citric acid production (Bodie et al, 1994). *A. oryzae* is of similar industrial importance especially in Asian countries. It is extensively used in fermentation and food refinement, such as for soybean paste (miso), soy sauce (shoyu) or the well noted rice wine (sake). Additionally *A. oryzae*, similar to *A. niger,* is used for the industrial production of enzymes and metabolites (Abe et al, 2006). In 2005 the genomes of *A. oryzae*, *A. fumigatus* and *A. nidulans* were sequenced (Galagan et al, 2005; Machida et al, 2005) followed by the sequence of *A. niger* two years later (Jones, 2007; Pel et al, 2007).

A. nidulans is the scientific representative of this family of fungi. It was established as a model organism for genetics in the 1950's (Pontecorvo et al, 1953). Since then it became a versatile instrument to study molecular biology, as well as the development and proliferation of eukaryotic cells. Implications on development and metabolism gathered in *A. nidulans* can be transferred to other *Aspergillus* species and help to improve their use in industry. Additionally they aid to a better understanding of the mechanisms underlying fungal pathogenicity. Furthermore these implications can be used to gain a better understanding of cell cycle control and cell polarity in higher eukaryotic organisms.

1.7 The life-cycle of *A. nidulans*

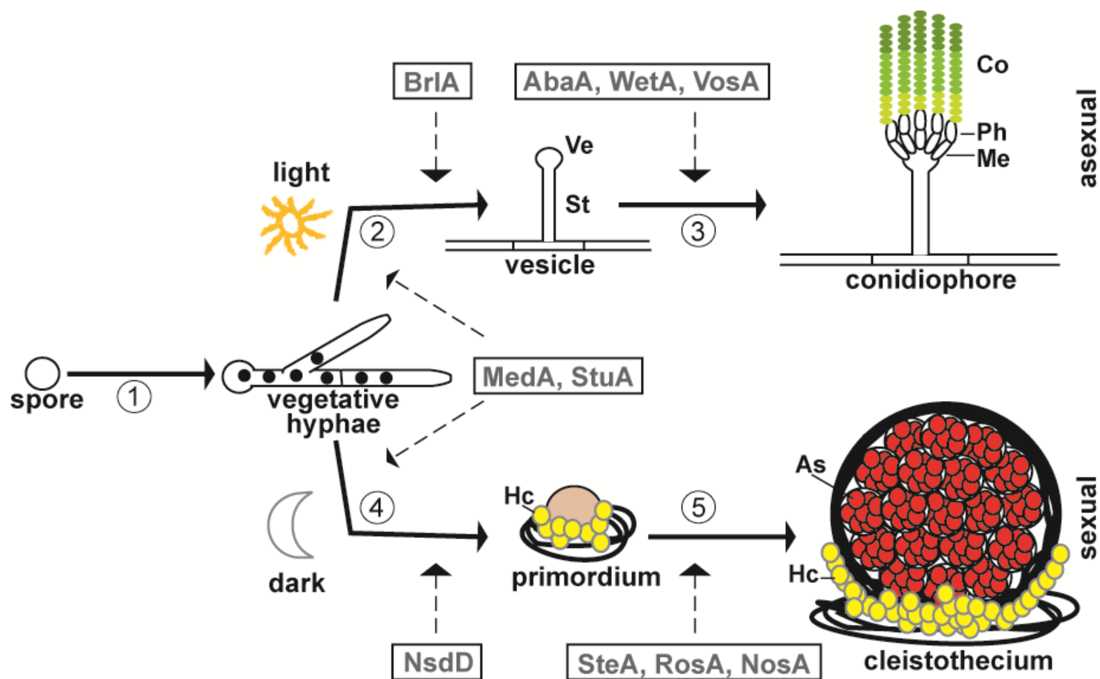

Figure 9: *Aspergillus nidulans* development.

16-20 hours after spore germination the vegetative mycelium achieves developmental competence [1]. In the presence of light [2] a stalk (St) with an apical vesicle (Ve) is formed. [3] The primary (metulae; Me) and secondary sterigmata (phialides; Ph) are formed by budding events. The phialides finally produce the asexual conidiospores (Co, green) which are released into the air. In darkness [4] specialized tissue and Hülle cells (Hc; yellow) are formed to nurse the evolving developmental structure (primordium), which maturates within 7 days [5] to the sexual fruit body (cleistothecium). The cleistothecium is enclosed by melanized hyphae, protecting the globular asci (As) containing eight bi-nucleate ascospores (red) each. Both kinds of spores, asexual conidiospores and sexual ascospores, again give rise to a new mycelium. A couple of transcription factors are required to coordinate both developmental pathways. Dashed lines indicate which pathway is affected (for details see chapter 1.7.4).

1.7.1 Vegetative hyphae

The soil borne mycelium of *A. nidulans* originates from a spore arrested in G1 phase. At first the spore increases volume by isotropic swelling and then changes to polarized growth. The forming germ tube extends by adding newly synthesized cell wall material to the tip. This apical extension of the growing hyphae is accompanied by mitotic multiplication of nuclei and branching events, forming the vegetative mycelium. Hyphae within the mycelium are separated by permeable septa resulting in separated cells of about 40µm length. These hyphal compartments harbor several nuclei and are arrested for mitosis and growth.

The fast growing vegetative mycelium is able to develop either mitotically, or meiotically derived spores by forming rather complex fruiting structures (Momany et

al, 1999). After 16-20 hours of vegetative growth the mycelium achieves developmental competence and, upon reaching a medium-air interface starts either of the two developmental pathways (Axelrod et al, 1973) (Figure 9; chapter 1.7.2 and 1.7.3). Adjacent to various other circumstances, especially the environmental factors light and carbon dioxide partial pressure play a key role for the decision which reproductive cycle is chosen (Adams et al, 1998; Clutterbuck, 1974) (see also chapter 1.7.5).

1.7.2 Asexual conidiation

In the presence of light and at normal carbon dioxide pressure competent *A. nidulans* mycelia prefer the formation of asexual fruiting structures (Adams et al, 1998). The development of the so called conidiophores starts with an approximately 70µm long stalk, originating from the vegetative hyphae. At its tip the stalk starts swelling and generates a vesicle that contains a large number of nuclei. Several budding events constrict the primary, uni-nucleate sterigmata, which are also termed metulae. These again undergo budding to form the secondary sterigmata, named phialides. Finally the phialides start producing long rows of asexual conidiospores by consecutive, asymmetric cell division (Adams et al, 1998; Bennett, 2009) (Figure 9). Those spores contain a single haploid nucleus.

Pigmentation of the spores prevents UV damage to the material inside, when the spores are released to the air. It is these airborne asexual spores which are inhaled by humans and animals, causing allergies in case of *A. nidulans*, but also severe systemic aspergillosis in case of *A. fumigatus* or *A. flavus* (Pasqualotto, 2009).

1.7.3 Sexual fruit body formation

Vegetative mycelia growing at a surface without light and at elevated carbon dioxide levels will hardly produce conidiophores, but will direct development towards the formation of sexual fruit bodies (Clutterbuck, 1974). This sexual or teleomorph form of the fungus is also referred to as *Emericella nidulans*.

The first visible step in the development of sexual fruit bodies is the establishment of a structure called "nest" by specialized hyphae, whereof the species name is derived from (*nidulans* = nest builder). Upon proceeding sexual development these specialized hyphae produce globular, laccase II containing cells by budding. Those are termed Hülle cells and are characterized by a thick cell wall.

They are thought to have a function in nursing the evolving fruit body (Sarikaya Bayram et al, 2010; Scherer & Fischer, 1998; Zonneveld, 1977). The hyphae simultaneously start to form a globular structure by sticking together. This primordium (Figure 9) develops further to become the µ-cleistothecium surrounded by three layers of hyper branched hyphae. Inside this small fungal container fusion of specialized hyphae is followed by crozier formation and synchronized mitosis leading to the differentiation of dikaryotic cells. From those cells the asci are generated through karyogamy, meiosis and two steps of mitosis. Each ascus contains eight binuclear ascospores, whereas each nucleus is haploid. In the end, the mature sexual fruit body, named cleistothecium, contains 80.000 to 100.000 ascospores and has a diameter of about 200µm (Braus, 2002; Champe et al, 1994; Dyer & O'Gorman, 2011; Hermann et al, 1983; Kirk & Morris, 1991) (Figure 9).

1.7.4 Molecular regulation of fungal development

The formation of specialized developmental structures forming fertile spores requires exact spatial and temporal regulation of a number of consecutive events. This involves tight control of many cellular factors. In previous years *A. nidulans* research extensively analyzed fungal development and the regulatory cascades needed.

On the molecular level the control of asexual development requires a central cascade, dominated by two main transcription factors. BrlA (bristle) and AbaA (abacus) are specifically required for conidia formation but not for vegetative growth (Clutterbuck, 1969). *brlA* mutants are blocked at the stage of stalk formation and cannot go further in asexual development. A couple of developmentally regulated genes are activated in a BrlA dependent manner, what renders expression of *brlA* the major and essential control point of asexual development (Han et al, 1993; Prade & Timberlake, 1993). Expression of *brlA* is induced by the orchestrated activity of two upstream transcription factors FlbB and FlbD (Garzia et al, 2010).

Later in asexual development transcription of *abaA* is required for the switch from sterigmata budding to conidiospore formation. The encoded transcription factor represses *brlA* expression (Han & Adams, 2001) and induces *wetA* (wet) transcription.

The *wetA* gene product regulates spore-specific gene expression in developing conidia. Activity of the WetA transcription factor is essential for cell wall composition and conidia maturation (Marshall & Timberlake, 1991).

Another transcription factor influencing conidiophore formation is VosA (Ni & Yu, 2007). When the *vosA* gene is deleted the corresponding strain permanently goes to asexual development even in liquid culture. VosA activity is also linked to the velvet complex (chapter 1.7.5) as it forms a complex with VelB in a light dependent manner (Sarikaya Bayram et al, 2010) (Figure 10).

NsdD (never in sexual development) and SteA (sterile 12-like) are two essential and well characterized transcription factors, which exclusively act on sexual development. Deletion of *nsdD* impairs cleistothecia formation while overproduction leads to a highly increased number of sexual fruit bodies (Han et al, 2001).

The zinc-finger transcription factor SteA is the homolog of *S. cerevisiae* Ste12. The corresponding *steA* deletion mutants are viable and form conidiophores, as well as Hülle cells, but are unable to generate mature cleistothecia (Vallim et al, 2000).

RosA is a further transcription factor that functions in the repression of sexual development. *rosA* deletion strains increase transcription of the sexual developmental regulators *nsdD*, *veA* and *stuA*. RosA also represses NosA, another putative zinc-finger transcription factor essential for primordia maturation (Vienken & Fischer, 2006; Vienken et al, 2005).

MedA (medusa) and StuA (stunted) are two developmental factors that modify sexual, as well as asexual development (Clutterbuck, 1969). The *stuA* transcript is up regulated 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 cannot form cleistothecia (Miller et al, 1991; Vallim et al, 2000).

Mutants defective in the *medA* gene can form conidia, but conidiophore morphology is disturbed. The wild type forms only two rows of sterigmata, metulae and phialides, while four or more rows of sterigmata are formed by *medA* deletion strains. The strains further produce only unorganized Hülle cells during sexual development but never produce cleistothecia or ascospores (Clutterbuck, 1969). In both cases asexual development is affected by regulative function of *brlA* and *abaA*. MedA controls the correct temporal expression of *brlA* and is a co-activator of *abaA*

expression, whereas StuA controls the correct spatial distribution of BrlA and AbaA (Busby et al, 1996; Miller et al, 1992).

1.7.5 Regulation of fungal development by environmental factors

Mooney and Yager showed that asexual development depends on aeration and light and spore formation varies at different wavelength of red light. When vegetative mycelia are exposed to red light, during a specific time frame, conidiation is induced, while it is repressed by an immediate shift to far red light (Mooney & Yager, 1990). Such behavior is also observed in plants and bacteria and requires a phytochrome. *A. nidulans* contains an ortholog protein, FphA that localizes to the cytoplasm. It is reminiscent to the bacterial phytochromes acting as a red-light sensor (Blumenstein et al, 2005). Blue light is recognized by the white collar homologues IreA and IreB in *A. nidulans* (Purschwitz et al, 2008). Blue light and UV spectra can also be detected by *A. nidulans* through the photolyase-like protein CryA, which is localized to the nucleus. CryA is responsible for the repression of sexual development in the light (Bayram et al, 2008a; Braus et al, 2010).

The *veA* gene product was recognized to be an additional factor of fungal light regulation, connecting the light signal with the downstream developmental response (Purschwitz et al, 2009; Yager, 1992). *veA* expression is significantly increased during sexual development suggesting that the gene product is a positive regulator of sexual development. The majority of VeA localizes to the cytoplasm in the light, while the protein locates to the nucleus in the dark (Stinnett et al, 2007) (Figure 10). Accompanied with the altered localization in the dark, VeA is supposed to form a hetero-trimeric complex with the velvet-like protein VelB and the methyltransferase LaeA in the nucleus that influences development (Bayram et al, 2008b) (Figure 10). The *velvet* complex links light dependent regulation of development to secondary metabolism by connecting VeA and the master regulator of secondary metabolism LaeA (Bayram & Braus, 2011; Bayram et al, 2008b; Bok et al, 2006).

Deletion of *veA* leads to strains unaffected in asexual spore formation, but blocked in sexual fruit body development. Contrary, overproduction drastically enhances sexual development, uncoupling it from light regulation and reduces asexual spore formation. This indicates an additional function for VeA in the repression of asexual spore production (Kim et al, 2002). Accordingly many of the

laboratory strains carry the *veA1* mutant allele encoding an N-terminally truncated form of VeA. A point mutation within the start codon reduces the 572 amino acid protein by 37 amino acids at the N-terminus. This leads to a reduced VeA function resulting in strains that favor asexual sporulation in a light independent manner (Kim et al, 2002; Mooney et al, 1990). This is presumably caused by a decrease of the ability to interact with VelB and translocate it from the cytoplasm into the nucleus. VelB is not only part of the velvet complex but associates also with VosA inside the nucleus. The VelB-VosA dimer represses asexual development in the dark and is required for spore viability (Sarikaya Bayram et al, 2010) (Figure 10). This is possibly achieved through the VosA interacting protein VelC that occurs to be a positive regulator of sexual fruit body formation and a negative regulator of asexual development (Sarikaya Bayram et al, 2010).

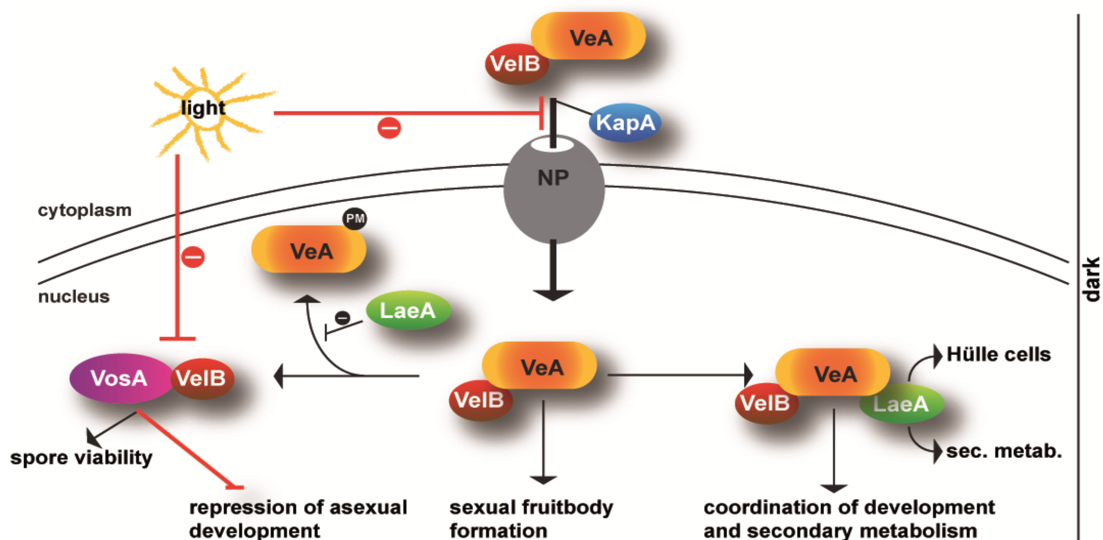

Figure 10: Molecular mechanism of light dependent regulation of fungal development by VeA.

In darkness, VelB primarily enters the nucleus together with VeA by translocation through the nuclear pore (NP). The α-importin KapA supports the entry of the VeA–VelB dimer into the nucleus in the dark. Light inhibits translocation of the dimer (red line). Inside the nucleus VelB is involved in the formation of two distinct complexes. The VosA-VelB dimer represses asexual spore formation and controls spore viability. Light decreases the cellular levels of VosA and VelB (red line) and allows asexual sporulation. VeA-VelB additionally associates with LaeA forming a dimeric and/or trimeric complex controlling sexual development. Association of LaeA with the VelB-VeA complex links secondary metabolism to development. LaeA controls Hülle cell formation, secondary metabolism and protects VeA against posttranslational modification (PM) (adapted from (Sarikaya Bayram et al, 2010).

Interestingly, the third component of the velvet complex, the methyltransferase LaeA, seems to be required for the light regulation of fungal development as well. But this requires the presence of intact VeA. Deletion of *laeA*

in a *veA1* background hardly affects spore formation (Bok & Keller, 2004), while in the *veA* wild type background conidiation is drastically reduced and development is almost exclusively directed towards sexual fruit body formation, even in the presence of light (Sarikaya Bayram et al, 2010).

VeA is also reported to be involved in the maintenance of the cellular oxylipin level, which is a crucial measure for the developmental switch in the fungus. Thus the PpoA protein which regulates the balance of oxylipins, also referred to as *psi* factors in *A. nidulans*, is affected in a VeA dependent manner. PpoA balances the levels of the *psi* factors and dependent on their ratio, formation of asexual or sexual spores is promoted. Deletion of *veA* almost completely abolishes *ppoA* expression (Bayram & Braus, 2011; Brodhun & Feussner, 2011; Dyer & O'Gorman, 2011; Tsitsigiannis et al, 2004). Additionally, the CSN is involved in maintaining the *psi* factor balance by influencing the PpoA and PpoC dioxygenases (Dyer & O'Gorman, 2011; Nahlik et al, 2010).

The zinc-finger domain proteins SilA and SilG are two candidates found by mutant screening, which aimed to identify genes required to repress sexual development in the light. Both deletion strains produce high amounts of cleistothecia in the presence of light (Dyer & O'Gorman, 2011).

Nutrient accessibility is another environmental factor that influences fungal development. Mycelia starving for carbon or nitrogen tend to initiate asexual spore production (Skromne et al, 1995). Phosphate deficiency also leads to induction of asexual development, probably by a signaling cascade including the gene product of *phoA*. The encoded cyclin–dependent kinase is involved in the sensing of environmental signals like phosphate, inoculation density and pH (Bussink & Osmani, 1998).

Formation of sexual fruit bodies is impaired by amino acid starvation (Eckert et al, 1999). In contrast shortage of nutrients like glucose, nitrate and phosphate, induces cleistothecia formation although it is an energy-consuming process. Sufficient energy supply for sexual development is achieved by consumption of the polysaccharide α-1,3-glucan, stored in the hyphal cell wall that is made available by α-1,3-glucanases (Zonneveld, 1972).

1.8 Scope and aim of this study

Ubiquitin dependent protein degradation by the 26S proteasome is an important principle for the tight regulation of a couple of cellular processes, as outlined in the previous chapters. In the filamentous ascomycete *Aspergillus nidulans* deletion of CSN subunits causes a block at the early stage of sexual fruit body formation resembling the embryonic lethal phenotype of higher organisms. However, vegetative growth and asexual development are hardly affected (Busch et al, 2003; Busch et al, 2007). This finding and the fact that all crucial components of the ubiquitin proteasome pathway are conserved within the fungal genome makes *A. nidulans* a versatile model organism to study the role of deneddylation in terms of development. Interestingly, the *nedd8* gene, encoding the fungal Nedd8 ortholog, as well as the genes for the Nedd8 E2-conjugating enzyme UbcL (Ubc12 in yeast), are essential for fungal viability. Similarly, the fungal CRL components CulA (ortholog to CUL1), SkpA (Skp1) or the RING protein RbxA (Rbx1/ROC1) are mandatory for all aspects of the *A. nidulans* life-cycle (von Zeska Kress et al, 2012). Therefore the question arose, why CSN dependent deneddylation is only required for normal sexual development, whereas asexual spore formation and vegetative growth are hardly affected. This lead to the hypothesis that another protein capable of deneddylation might exist within the fungal genome. This study examines the role of the cysteine protease DenA, which is the ortholog of human DEN1, for fungal development and the interplay with the CSN complex of *A. nidulans*.

The first part of this thesis is dedicated to the characterization of *denA* and its gene product trying to specify the function of the protein, as well as sub-cellular localization and interacting proteins.

In the second part of the work the genetic connection and the physical interaction of DenA and the CSN are investigated.

Finally, in the third section, co-purification is applied to look more closely on CSN complex formation, as well as possibly existing sub-complexes.

2 Materials and Methods

2.1 Growth media and growth conditions

Chemicals used for media, buffers and solutions were purchased from MERCK (Darmstadt, D), CARL ROTH GMBH & COKG (Karlsruhe, D), APLLICHEM GMBH (Darmstadt, D), INVITROGEN GMBH (Karlsruhe, D), FLUKA (Neu-Ulm, D), SIGMA-ALDRICH CHEMIE GMBH (Steinheim, D), VWR INTERNATIONAL GMBH (Darmstadt, D), BD BIOSCIENCES (Heidelberg, D) and SERVA ELECTROPHORESIS GMBH (Heidelberg, D) .

2.1.1 Conditions for *Escherichia coli*

E. coli strains were cultivated in LB (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) medium at 30°C or 37°C modified from Bertani (1951). Gene expression from the *tac* promoter in pGEX4-T1 constructs was induced by addition of 1 mM IPTG. For selection 100 µg/ml ampicillin, 50 µg/ml chloramphenicol or 25 µg/ml kanamycin was used. 2% agar was added for solid media. Liquid cultures were grown at 37°C on a rotary shaker, except indicated otherwise. Plate cultures were incubated headfirst at 37°C.

2.1.2 Conditions for *Saccharomyces cerevisiae*

Saccharomyces cerevisiae strains were grown at 30°C in YEPD rich medium (2% pepton, 1% yeast extract, 2% glucose) for non-selective conditions or 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 lacking uracil, L-histidine, L-tryptophane and L-leucine]. 2% glucose or 2% galactose/1% raffinose was added as carbon source, the appropriate amino acids were supplemented as required and 1.5 % agar was added for solid media. Liquid cultures were incubated at 30°C on a rotary shaker or a rotating platform. Plates were incubated headfirst at 30°C.

2.1.3 Conditions for *Aspergillus nidulans*

Aspergillus nidulans strains were grown at 30°C or 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]

(Bennet & Lasure, 1991) or in complete medium [0.5% yeast extract, 1% peptone, 0.1% casein, 7 mM KCl, 11.2 mM KH_2PO_4 (pH 5.5), 2 mM $MgSO_4$, trace elements] prior to TAP purification. As carbon source 1% glucose was used. Expression of *alcA* promoter constructs was induced by growing the corresponding strains on 0.9% Fructose and 6.25 mM threonine instead of 1% glucose. The nitrogen source was 70 mM $NaNO_3$. Induction of the nitrate promoter for BiFC was performed on London Medium [1% glucose, 2% salt solution (26 g/l KCl, 26 g/l $MgSO_4$, 76 g/l KH_2PO_4, 5% (v/v) trace elements) pH 6.5] plus 70 mM $NaNO_3$ for induction, or 5 mM NH_4-tartrate for repression, respectively. Media were supplemented with 100 µM pyridoxine-HCl and/or 5 mM uridine as required (Käfer, 1977). 2% agar was added for plates. 10 µg/ml phleomycin were implemented to select for strains carrying the dominant *ble* marker gene of *Streptoalloteichus hindustanus* (Austin et al, 1990). For selection of strains carrying the *ptrA* cassette (Kubodera et al, 2000) 100 ng/ml pyrithiamin was added to the medium. 100 ng/ml nourseothricin (ClonNAT) was added to the medium to select for clones carrying the NAT resistance cassette (Krugel et al, 1993). Vegetative cultures were obtained by inoculation of liquid medium in flasks with indentions. $1x10^6$ spores/ml were added to the medium and incubated on a rotary shaker for the indicated time periods. Development was induced by transfer of 20 hours, vegetative grown cultures to plates or direct dispersal of $1x10^6$ spores on agar plates. Strains were grown on plates covered with cellophane foil (MERCK CHEMICALS) when cultivated for harvesting. Asexual sporulation was triggered by incubation of plates in constant white light. Sexual fruit body formation was induced by oxygen limitation on tape-sealed plates and incubation in the dark (Clutterbuck, 1974). For fluorescence microscopy $2x10^5$ spores of the corresponding strains were inoculated in 400 µl liquid medium in an 8-well chambered coverslip (NUNC) and incubated at 30°C or 37°C under the appropriate conditions.

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 (rK^-, mK^+), supE44, λ^-, thi1, gyrA96,

relA1] (Woodcock et al, 1989) was used. The Rosetta^{TM} (DE3) host strain [F$^-$, ompT, hsdS$_B$(r$_B^-$, m$_B^-$), gal, dcm, pRARE(Cam^R)] (MERCK, Darmstadt, D) was used for expression and purification of recombinant *A. nidulans* proteins.

2.2.2 *Saccharomyces cerevisiae* strains

For heterologous expression of *A. nidulans* proteins *S. cerevisiae* strains Y03914 [*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*;*YDL216c*::*kan*MX4] and Y06911 [*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*;*YJR099w*::*kan*MX4] (EUROSCARF strain collection) were transformed with the plasmids pME3278 and pME3279 and subsequently with pME3280. Wild type strain BY4741 [*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*] was transformed with plasmid pME3280 as control. Positive transformants were selected on SC-medium lacking histidine and uracil.

S. cerevisiae strains for yeast-2-hybrid tests were obtained in a similar way. Bait and prey plasmids were subsequently transformed into *S. cerevisiae* EGY48-p1840 [*MATa*, *his3*, *trp1*, *ura3-52*, *leu2*::*pLEU2-LexAop6*,*URA3*::*lacZ-LexAop2*] (Golemis et al, 1999) and transformants were selected on SC-medium lacking uracil, histidine and tryptophane. Strains, transformed with non-integrative plasmids, were not preserved in long term stock solutions.

2.2.3 *Aspergillus nidulans* strains

A. nidulans strains including their genotypes used and constructed in this study are summarized in table III. Details on construction are given below.

Table III: *A. nidulans* strains used in this study.

Name	Genotype	Reference
A4	Glasgow wild type	FGSC[1]
AGB152	*pyrG89;pyroA4*	(Busch et al, 2003)
TNO2a3	*pyrG89;pyroA4;argB2;ΔnkuA::argB*	(Nayak et al, 2006)
AGB195	*pyrG89;pyroA4;ΔcsnD::pyr4*$^+$	(Busch et al, 2003)
AGB197	*pyrG89;pyroA4;*^{P}*alcA::csnD::gfp::his2B*[1]	(Busch et al, 2003)
AGB209	*pyrG89;pyroA4;ΔcsnE::pyr4*$^+$	(Busch et al, 2003)
AGB223	*pyrG89;pyroA4;ΔcsnA::pyr4*$^+$	(Busch et al, 2007)
AGB238	*pyrG89;pyroA4;ΔcsnB::pyr4*$^+$	(Busch et al, 2007)
AGB244	*pyrG89;pyroA4;csnE(D*^{147}*N)::pyr4*$^+$	(Busch et al, 2007)

Table III continued

AGB245	*pyrG89;pyroA4;csnE(H*^{134}*A, H*^{136}*A, D*^{147}*N)::pyr4*$^{+}$	(Busch et al, 2007)
AGB316	*pyrG89;pyroA4;ΔdenA::pyr4*$^{+}$	this study
AGB318	*pyrG89;pyroA4;ΔdenA::pyr4*$^{+}$*;denA*$^{+}$*;bleo*^{R}	this study
AGB457	*pyrG89;pyroA4;argB2;ΔnkuA::argB;* ^{P}*nedd8:ntap:nedd8:pyroA*$^{+}$*:nedd8*^{T}	M. v. Z. Kress, p.c.
AGB461	*pyrG89;pyroA4;argB2;ΔnkuA::argB;* ^{P}*nedd8::pyroA*$^{+}$*::ntap::nedd8::nedd8*^{T}*; ΔdenA::pyr4*$^{+}$	M. v. Z. Kress, p.c.
AGB466	*pyrG89;pyroA4;argB2;ΔnkuA::argB;ΔcsnE::ptrA*^{R}	M. v. Z. Kress, p.c.
AGB596	*pyrG89;pyroA4;*^{P}*gpdA::gfp::his2B*^{T}	Ö. Bayram, p.c.
AGB630	*pyrG89;pyroA4; ΔdenA::pyr4*$^{+}$*;* ^{P}*niaD::cyfp::niaD*^{T}*;* ^{P}*niiA::nyfp::denA*^{cDNA}*::niiA*^{T}*;ptrA*^{R}	this study
AGB631	*pyrG89;pyroA4;ΔcsnG::ptrA*^{R}	this study
AGB632	*pyrG89;pyroA4;argB2;ΔnkuA::argB;* *ΔcsnE::ptrA*^{R}*;ΔdenA::pyr4*$^{+}$	this study
AGB633	*pyrG89;pyroA4;ΔcsnG::ptrA*^{R}*;csnG*$^{+}$*::bleo*^{R}	this study
AGB634	*pyrG89;pyroA4;*^{P}*denA::denA::gfp::nat*^{R}*::denA*^{T}	this study
AGB635	*pyrG89;pyroA4;ΔcsnA::pyr4*$^{+}$*;* ^{P}*denA::denA::gfp::nat*^{R}*::denA*^{T}	this study
AGB636	*pyrG89;pyroA4;*^{P}*denA::denA::ctap::nat*^{R}*::denA*^{T}	this study
AGB640	*pyrG89;pyroA4;*^{P}*denA:denA::gfp::nat*^{R}*::denA*^{T}*;* ^{P}*gpdA::mrfp::h2A::hisB*^{T}*;pyrG*$^{+}_{af}$	this study
AGB641	*pyrG89;pyroA4;* ^{P}*gpdA::mrfp::h2A::hisB*^{T}*;phleo*^{R}	this study
AGB642	*pyrG89;pyroA4;ΔcsnA::pyr4*$^{+}$*;* ^{P}*denA::denA::gfp::nat*^{R}*::denA*^{T} ^{P}*gpdA::mrfp::h2A::hisB*^{T}*;phleo*^{R}	this study
AGB643	*pyrG89;pyroA4;*^{P}*denA::gfp::denA::denA*^{T}*;* ^{P}*gpdA::mrfp::h2A::hisB*^{T}*;phleo*^{R}	this study
AGB644	*pyrG89;pyroA4;ΔdenA:pyr4*$^{+}$*;* ^{P}*niaD::csnG*^{cDNA}*::cyfp::niaD*^{T}*;* ^{P}*niiA::nyfp::denA*^{cDNA}*::niiA*^{T}*;ptrA*^{R}	this study
AGB645	*pyrG89;pyroA4;* ^{P}*denA::gfp::denA::denA*^{T}	this study
AGB646	*pyrG89;pyroA4;*^{P}*alcA::csnD::gfp::his2B*^{T}*;* *pyrG89;pyroA4;ΔcsnG::ptrA*^{R}	this study
AGB647	*pyrG89;pyroA4;ΔcsnA::pyr4*$^{+}$*;* ^{P}*csnA::ntap::csnA::csnA*^{T}	this study

[1]Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, KS, USA); p.c. (personal communication); P promoter; T terminator; R resistance; af *Aspergillus fumigatus.*

2.2.3.1 Construction of *Aspergillus nidulans* strains for *denA* characterization

Transformation and selection for uridine/uracil prototrophic clones was applied to obtain homologous integration of the linear *denA* deletion cassette. The fragment was excised by digestion of pME3275 with *Cla*I/*Not*I and introduced into AGB152 resulting in the *denA* deletion strain AGB316. Integration was verified by Southern analysis with a probe detecting the 3' flanking region of *denA* revealed by *Spe*I/*Eco*RI digestion of pME3267. Digestion of genomic DNA with *Eco*RV generated a signal at about 5.2 kb for the wild type gene and 3.3 kb for the integrated *denA* deletion cassette.

Complementation of the *denA* deletion strain AGB316 was achieved by ectopic integration of the linearized pME3267 in AGB316. The plasmid contains a genomic *denA* fragment downstream of the phleomycin resistance cassette. Transformation and selection for phleomycin resistant clones resulted in strain AGB318. Integration of the plasmid was proven by PCR (MC2/MC20; wt=2.5 kb; Δ*denA*=3.3 kb; $denA^{comp}$=both) and Southern hybridization. The Southern probe and digestion of genomic DNA was similar to that of the deletion strain. Ectopic integration of the plasmid resulted in an additional band, different from that of the deletion cassette integrated at the endogenous *denA* locus (3.3 kb).

Strain AGB645, carrying an N-terminal GFP fusion construct of *denA* at the endogenous locus was achieved by transformation of the *Not*I/*Cla*I fragment from pME3277 into AGB316 and selection on minimal medium containing uridine, uracil, pyridoxine-HCl and 5-fluoroorotic acid. AGB645 was further transformed with plasmid pME3857 for ectopic integration of the *mrfp::h2A* construct together with a *phleo* marker cassette mediating resistance to phleomycin. Clones were selected on medium containing phleomycin and checked for fluorescent nuclei by microscopy. Integration of the *gfp::denA* construct at the locus of AN10456 was verified by Southern analysis resulting in strain AGB643. Probe and restriction enzyme were similar to the deletion strain. Successful integration of the fusion cassette generated a 6 kb fragment instead of the 3.3 kb fragment of the deletion strain.

AGB640 containing a C-terminal fusion of *denA* with *gfp* and the *mrfp::h2A* construct was obtained by transformation of AGB152 with pME3900 resulting in AGB634 and subsequent transformation with pME3858. Homologous recombination of *denA* with the *gfp* fusion construct was proven by Southern hybridization and ectopic integration of the *mrfp::h2A* construct was verified by microscopy. The

3' flanking region of *denA* again served as probe applied on genomic DNA digested with *Eco*RV. Successful integration of the fusion construct resulted in detection of a 7.2 kb fragment instead of the 5.2 kb wild type fragment.

To investigate a possible impact of CSN integrity on DenA protein stability the *denA::gfp::nat*^{R} cassette of pME3900 was also transformed into the *csnA* deletion strain AGB223 (Busch et al, 2007). Homologous integration of the fusion construct at the endogenous *denA* locus was proven by Southern analysis, as described above, resulting in strain AGB635. Additionally the *csnA* genotype was reviewed by Southern hybridization of a probe (Elke11/MC90 from genomic DNA) detecting the 3' flanking region of *csnA*. The *csnA* deletion strain showed a 1.2 kb fragment instead of the 2.7 kb fragment of the wild type strain after digestion with *Xho*I. A smaller fragment of 0.85 kb was detected for both strains. AGB635 was further transformed with pME3857 for ectopic integration of an *mrfp::h2A* construct, to visualize nuclei. Positive clones were determined by microscopy and *denA*, as well as *csnA*, genotypes were reviewed by Southern analysis, as described above resulting in strain AGB642.

Co-purification of DenA interacting proteins was achieved by introducing the *denA::ctap::nat*^{R} fusion cassette from plasmid pME3901 at the endogenous *denA* locus. The fragment was excised from the plasmid with *Xho*I and transformed into AGB152. Transformants were selected on medium containing ClonNAT and verified by Southern hybridization resulting in strain AGB636. Genomic DNA was digested with *Eco*RV and probed similar to the *denA::gfp* fusion strain.

Strain AGB461 containing a processed variant of *nedd8* at the endogenous locus combined with the deletion for *denA* was obtained by genetic crossing of strains AGB457 and ABG316, accomplished by Marcia von Zeska Kress. Clones were verified by Southern analysis of each locus. The *denA* genotype was analyzed as described above. A probe detecting the *nedd8* 5' flanking region was amplified from genomic DNA with primers MK067/MK082. Digestion of candidate DNA with *Xho*I generated fragments of 3.8 kb for the wild type gene and of 6.1 kb for the *ntap::nedd8GG* construct in Southern analysis.

The *denA/csnE* double knock-out AGB632 was obtained by transformation of AGB466, kindly provided by Marcia von Zeska Kress, with the *denA* deletion cassette excised with *Cla*I/*Not*I from pME3275. Deletion of both genes *csnE* and *denA* was verified by Southern hybridization. Successful integration of the *denA*

deletion cassette was verified as described above. The *csnE* genotype was analyzed with a probe against the 5' flanking region of *csnE* amplified with primers SB114/ES21 from genomic DNA. DNA of candidate clones and control strains was digested with EcoRI, resulting in detection of a 3.3 kb fragment (Δ*csnE*) or a 5.8 kb fragment (wild type).

BiFC plasmids pME3885 and pME3886 were transformed into AGB316 resulting in AGB630 and AGB644, respectively. Clones were selected on medium containing phleomycin, lacking uridine and uracil. Ectopic integration was verified by Southern analysis. A probe against the nitrate promoter detected a fragment of 3.9 kb for pME3885, 4.8 kb for pME3886 and 7.4 kb for the endogenous nitrate promoter. The probe was PCR amplified with primers OLKM86/OLKM87 from plasmid pSK409. The *denA* genotype was reviewed as described above.

2.2.3.2 Construction of *A. nidulans* strains for CSN studies

The *csnG* deletion strain AGB631 constructed in the study was obtained by transformation of a 5.7 kb *Xho*I fragment, excised from pME3887 into AGB152 (Busch et al, 2003). Homologous recombination was verified by Southern analysis with a probe detecting the 5' flanking region of *csnG*. The probe was amplified with primers MC125/MC126 from genomic DNA and detected a fragment of 5.2 kb for the wild type and of 3.2 kb for the *csnG* deletion in *Bam*HI digested samples. For complementation of Δ*csnG*, plasmid pME3890, containing a genomic copy of *csnG* and the *ble* marker, was transformed into AGB631 for ectopic integration. Clones were selected on medium containing phleomycin and integration of the plasmid was verified by Southern hybridization resulting in strain AGB633. The above mentioned probe recognized a second band in addition to that of the *csnG* deletion in case of a successful ectopic integration of the complementation plasmid.

AGB647 containing the N-terminal *tap* tagged version of *csnA* at the endogenous locus was received by transformation of the *csnA* deletion strain AGB223 (Busch et al, 2007) with a linear fragment excised with *Cla*I/*Not*I from pME3883. Transformants were selected on plates containing 5-fluororotic acid, allowing growth only to those clones without the *pyr-4* cassette of the deletion construct. Homologous replacement of the *csnA* deletion cassette with the transformed *ntap::csnA* fusion construct was verified by Southern hybridization. The above mentioned *csnA* probe generated a signal for a 3.2 kb fragment in *Xho*I

digested samples instead of the 1.2 kb fragment of the deletion or the 2.7 kb fragment of the wild type.

The strain carrying the *csnD::gfp* fusion in the Δ*csnG* background is a derivative of AGB197 (Busch et al, 2003) transformed with the *csnG* deletion cassette excised by *Xho*I digestion from pME3887. Transformation and selection for pyridthiamin resistance resulted in strain AGB646. Successful deletion of *csnG* was verified by Southern hybridization as described above. Presence of the *csnD::gfp* fusion was verified by fluorescence microscopy and western hybridization with a GFP antibody.

2.3 Genetic manipulation

2.3.1 Transformation

Transformations of *A. nidulans, S. cerevisiae* and *E. coli* were performed following existing protocols (Eckert et al, 2000; Elble, 1992; Inoue et al, 1990).

2.3.2 Constructs for genetic manipulation

Plasmids constructed in this study are listed in table IV and details on construction are given below. Primer sequences from this study are given in table V.

Table IV: Plasmids used and cloned during this study.

Name	Description	Reference
pBluescript®II SK+	cloning vector	(STRATAGENE, La Jolla, CA, USA)
pYES2.1 TOPO-TA	Yeast expression vector with *GAL1*^{P} and C-terminal V5/6xHIS tandem tag	(INVITROGEN, Karlsruhe, D)
TOPO-Blunt®II	cloning vector	(INVITROGEN, Karlsruhe, D)
pJET1.2 Blunt	cloning vector	(FERMENTAS, St. Leon-Rot, D)
pGEX4-T1	*E. coli* expression vector	(GE HEALTHCARE, Freiburg, D)
pRG3	expression construct for *pyr4* gene from *Neurospora crassa*	(Waring et al, 1989)
pEG202	yeast-2-hybrid bait vector	(Golemis & Brent, 1996; Gyuris et al, 1993)

Table IV *continued*

pJG4-5	yeast-2-hybrid prey vector	(Golemis & Brent, 1996; Gyuris et al, 1993)
pSK409	${}^{P}niaD::niaD^{T};{}^{P}niiA::niiA^{T};ptrA^{R}$ overexpression vector	S. Krappman, p.c.
pME2357	*csnD* cDNA in pJG4-5	(Busch et al, 2007)
pME2501	*csnA* cDNA in pJG4-5	(Busch et al, 2007)
pME2968	plasmid containing *ntap* adapted to fungi	(Busch et al, 2007)
pME2978	*csnB* cDNA in pJG4-5	(Busch et al, 2007)
pME2979	*csnC* cDNA in pJG4-5	(Busch et al, 2007)
pME2980	*csnE* cDNA in pJG4-5	(Busch et al, 2007)
pME2981	*csnF* cDNA in pJG4-5	(Busch et al, 2007)
pME2982	*csnG* cDNA in pJG4-5	(Busch et al, 2007)
pME2983	*csnH* cDNA in pJG4-5	(Busch et al, 2007)
pME3123	*gfp* in pBlue II SK+	E. Schwier, p.c.
pME3267	$5'UTR^{denA}::denA::3'UTR^{denA}$ in pME3281	this study
pME3269	$3'UTR^{denA}$ -PCR-Fragment in TOPO-Blunt®II	this study
pME3270	$denA::5'UTR^{denA}$ -PCR-Fragment in TOPO-Blunt®II	this study
pME3271	$5'UTR^{denA}$ -PCR-Fragment in TOPO-Blunt®II	this study
pME3272	$denA::3'UTR^{denA}$ -PCR-Fragment in TOPO-Blunt®II	this study
pME3273	*pyr4* -PCR-Fragment in TOPO-Blunt®II	this study
pME3275	$5'UTR^{denA}::pyr4^{+}::3'UTR^{denA}$ in pME3281	this study
pME3276	$5'UTR^{denA}::{}_{SpeI}denA::3'UTR^{denA}$ in pME3281	this study
pME3277	$5'UTR^{denA}::gfp::denA::3'UTR^{denA}$ in pME3281	this study
pME3278	*denA* (cDNA) in pYES2.1	this study
pME3279	$denA^{V5/HIS6}$(cDNA) in pYES2.1	this study
pME3280	*culD* (cDNA) in pEG202	(Helmstaedt et al, 2011)
pME3281	pBluescript®II SK+ with phleomycin resistance cassette	(Helmstaedt et al, 2011)
pME3674	${}^{P}niaD::cyfp::nedd8^{cDNA}::niaD^{T};{}^{P}niiA::dcnA^{cDNA}::nyfp::niiA^{T}$	R. Harting, p.c.
pME3857	${}^{P}gpdA::mrfp::h2A::hisB^{T};\ phleo^{R}$ in pBlueII SK+	Ö. Bayram, p.c.
pME3858	${}^{P}gpdA::mrfp::h2A::hisB^{T};\ pyrG_{af}$ in pBlueII SK+	Ö. Bayram, p.c.

Table IV *continued*

pME3874	*denA* (cDNA) in pEG202	this study
pME3877	*culC* (cDNA) in pJG4-5	this study
pME3879	$nedd8^{precursor}$ (cDNA) in pJG4-5	this study
pME3881	$nedd8^{mature}$ (cDNA) in pJG4-5	this study
pME3883	$5'UTR^{csnA}::ntap::csnA::3'UTR^{csnA}$ in pJET1.2	this study
pME3884	$denA(N^{50}S, H^{123}A)$ (cDNA) in pJET1.2	this study
pME3885	$cyfp; nyfp::denA^{cDNA}$ in pSK409	this study
pME3886	$cyfp::csnG^{cDNA}; nyfp::denA^{cDNA}$ in pSK409	this study
pME3887	$5'UTR^{csnG}::ptrA^{R}::3'UTR^{csnG}$ in pJET1.2	this study
pME3888	$denA(N^{50}S, H^{123}A)$ (cDNA) in pGEX4-T1	this study
pME3889	*denA* (cDNA) in pGEX4-T1	this study
pME3890	$5'UTR^{csnG}::csnG::3'UTR^{csnG}$ in pME3281	this study
pME3891	$5'UTR^{denA}$ -PCR-Fragment 1 [RACE] in pJET1.2 Blunt	this study
pME3892	$5'UTR^{denA}$ -PCR-Fragment 2 [RACE] in pJET1.2 Blunt	this study
pME3893	$5'UTR^{denA}$ -PCR-Fragment 3 [RACE] in pJET1.2 Blunt	this study
pME3894	$3'UTR^{denA}$ -PCR-Fragment 1 [RACE] in pJET1.2 Blunt	this study
pME3895	$3'UTR^{denA}$ -PCR-Fragment 4 [RACE] in pJET1.2 Blunt	this study
pME3900	$5'UTR^{denA}::denA::gfp::nat^{R}::3'UTR^{denA}$ in pJET1.2 Blunt	this study
pME3901	$5'UTR^{denA}::denA::ctap::nat^{R}::3'UTR^{denA}$ in pJET1.2 Blunt	this study
pME3913	$denA^{cDNA\Delta 1\text{-}23}$ in pEG202	this study
pME3914	$denA^{cDNA\Delta 1\text{-}45}$ in pEG202	this study
pME3915	$denA^{cDNA\Delta 236\text{-}258}$ in pEG202	this study
pME3916	$denA^{cDNA\Delta 1\text{-}23,\Delta 236\text{-}258}$ in pEG202	this study
pME3917	$denA^{cDNA\Delta 1\text{-}45,\Delta 236\text{-}258}$ in pEG202	this study
pME3922	*denA* (cDNA) in pJET1.2	this study
pME3923	*culC* (cDNA) in pJET1.2	this study
pME3924	$nedd8^{mature}$ (cDNA) in pJET1.2	this study
pME3925	$nedd8^{precursor}$ (cDNA) in pJET1.2	this study
pME3928	$ctap::nat^{R}$ cassette in pJET1.2 Blunt	Ö. Bayram, p.c.
pME3929	$gfp::nat^{R}$ cassette in pJET1.2 Blunt	Ö. Bayram, p.c.

p.c. (personal communication); P promoter; T terminator; R resistance; af *Aspergillus fumigatus*.

2.3.2.1 Cloning of RACE fragments from *denA* (AN10456)

Gene specific primers MC133 and MC134 together with the primers included in the GeneRacer™ Kit (INVITROGEN) were used to amplify the 5'UTR or the 3'UTR of AN10456, respectively. PCR Fragments obtained from RACE experiments were cloned into pJET1.2 giving pME3891 to pME3895 and subjected to sequencing.

2.3.2.2 Constructs for deletion and complementation of *denA*

Deletion constructs for *A. nidulans* contained the *pyr-4* marker from *Neurospora crassa*. This was amplified from plasmid pRG3 (Waring et al, 1989) by PCR with the primers MC5/MC9 and inserted into TOPO-Blunt®II resulting in plasmid pME3273.

Plasmid pME3267 contained the genomic sequence of *denA* with 1200bp flanking region to both 5' and 3' ends. The plasmid was constructed by PCR amplification of the 5' flanking region with primers MC1/MC3 and the *denA* open reading frame in addition with the 3' flanking region by oligonucleotides MC2/MC4 from genomic DNA of *A. nidulans*. PCR fragments were cloned into TOPO-Blunt®II for the 5' flanking region to give plasmid pME3271 and into pME3281 via *Not*I/*Eco*RV restriction sites for the ORF in addition with the 3' flanking region resulting in pME3272. pME3281 is the pBluescript®II SK+ vector carrying a *phleo* resistance cassette. The 5' flanking region was excised from pME3271 by cutting with the restriction enzymes *Bst*EII/*Cla*I and ligated into the corresponding restriction sites in pME3272 giving pME3267. Digestion with restriction enzymes *Mfe*I/*Hpa*I removed the *denA* coding sequence from pME3267 but left the flanking regions required for homologous recombination. The *pyr4* marker cassette was excised by digestion with *Eco*RI/*Hpa*I from pME3273 and introduced into the *Mfe*I/*Hpa*I restriction sites of plasmid pME3267 giving plasmid pME3275.

2.3.2.3 Construction of *denA*::*gfp* fusions

N-terminal fusion of DenA with GFP was achieved by cutting the *gfp* sequence from plasmid pME3123 (Elke Schwier, personal communication) with *Spe*I and introducing it into the *Spe*I site, upstream of the *denA* coding sequence in plasmid pME3276 giving plasmid pME3277.

A construct encoding the *denA::gfp* fusion was obtained by PCR mediated fusion (Szewczyk et al, 2006) and subsequent ligation of the obtained fragment into the pJET1.2 cloning vector. The *gfp* module combined with a downstream *nat* resistance cassette (Goldstein & McCusker, 1999) was amplified by PCR with primers OZG207 and OZG192 from plasmid pME3929. PCR on genomic DNA of *A. nidulans* with primers MC178 and MC179 revealed 2402bp combined of the 5' flanking region and the *denA* ORF with 3' sequence overhang corresponding to the *gfp* sequence. Amplification with MC175 and MC176 on fungal genomic DNA revealed the 3' flanking region of *denA* with 5' sequence overhang for the *nat* resistance cassette. All three fragments were used as template for a fusion PCR with nested primers MC1 and MC2 resulting in the final construct *5'UTR*$^{\text{denA}}$*::denA::gfp::nat*^{R}*::3'UTR*^{denA}, that was introduced into pJET1.2 giving pME3900.

2.3.2.4 Construction of a C-terminal *tap* tag fusion of *denA*

The *denA* sequence was fused with the downstream *ctap* by PCR mediated fusion (Szewczyk et al, 2006). Amplification with primers OZG209 and OZG192 on plasmid pME3928 resulted in a fragment combined of *ctap* and the *nat* resistance cassette (*ctap::nat*^{R}). The 3' flanking region of *denA* with 5' sequence overhang for the *nat* resistance cassette (Goldstein & McCusker, 1999) was obtained from a PCR with primers MC175 and MC176 on fungal genomic DNA. Similarly, the 5' flanking region and the *denA* ORF with 3' sequence overhang corresponding to the *ctap* sequence was amplified with primers MC177 and MC178. All fragments were fused together in PCR with primers MC1 and MC2 and the PCR product was introduced into pJET1.2 resulting in plasmid pME3901.

2.3.2.5 Construction of yeast-2-hybrid plasmids

The *denA* bait plasmid was cloned by amplifying the cDNA with primers MC71/MC72 introducing *Eco*RI restriction sites at both ends. The PCR fragment was sub-cloned into pJET1.2 resulting in pME3922, digested with *EcoR*I and introduced into the *Eco*RI site of pEG202 resulting in plasmid pME3874. The same strategy was used to obtain the N- and C-terminally truncated versions of *denA*. The fragment of primers MC72 and MC191 in pEG202 gave pME3913 (Δ1-23), MC72 and MC192 gave pME3914 (Δ1-45), MC71 and MC193 pME3915 (Δ236-258),

MC191 and MC193 pME3916 (Δ1-23; Δ236-258) and MC192 and MC193 pME3917 (Δ1-45; Δ236-258). Accordingly the *nedd8* and *culC* cDNAs were amplified with primers MC91/MC92 (*nedd8* precursor form), MC91/MC93 (mature *nedd8*) and MC73/MC74 (*culC*), but with flanking *Mfe*I restriction sites and introduced into pJET1.2 resulting in pME3925, pME3924 and pME3923, respectively. After digestion with *Mfe*I they were ligated into the *Eco*RI site of pJG4-5 resulting in pME3879, pME3881 and pME3877. Derivatives of the 2-hybrid prey plasmid pJG4-5 containing the cDNA of each *csn* subunit (pME2501, pME2978-79, pME2357, pME2980-83) were used from a previous study (Busch et al, 2007).

2.3.2.6 Constructs for BiFC studies

Protein fusions with one half of a split YFP, for BiFC interaction studies, were obtained by combined fusion PCR (Szewczyk et al, 2006) and restriction site mediated cloning. The C-terminal part of *yfp* (*cyfp*) was amplified with primers OLKM86 and OLKM87 and the N-terminal *yfp* (*nyfp*) was obtained by primers OLKM91 and MC94 from plasmid pME3674. *csnG* cDNA was amplified from plasmid pME2982 (Busch et al, 2007) with MC96/MC97 and *denA* cDNA from pME3874 with MC32/MC94. The C-terminal half of *yfp* was fused to the 5' end of *csnG* in a fusion PCR with primers MC97/OLKM86 and the N-terminal part of *yfp* to the 5' end of *denA* in a PCR with MC31/OLKM91. Plasmid pME3885 derived from subsequent introduction of the C-terminal half of *yfp* into the *Pme*I site and the fusion of the N-terminal half of *yfp* with *denA* into the *Swa*I site of pSK409, serving as control for unspecific interaction of split YFP. pME3886 originates as well from pSK409, subsequently added with *cyfp::csnG* fusion to the *Pme*I site and *nyfp::denA* to the *Swa*I site.

2.3.2.7 Construction of plasmids for expression of *culD* and *denA* in *S. cerevisiae*

For heterologous expression in yeast the *denA* cDNA was amplified with MC30/MC31 or MC30/MC32 lacking the *denA* stop codon, respectively. Both fragments were cloned by TA overhangs into pYES2.1 TOPO-TA to give plasmids pME3278 and pME3279.

Plasmid pME3280, expressing the *lexA::culD* fusion was obtained from a previous study (Helmstaedt et al, 2011).

2.3.2.8 Constructs for purification of recombinant DenA from *E. coli*

For overexpression and purification of recombinant *denA* the corresponding cDNA fragment was excised from plasmid pME3874 with *Eco*RI and ligated into the accordingly linearized pGEX4-T1 resulting in plasmid pME3889. The correct reading frame was verified by sequence analysis. The mutant version of DenA ($N^{50}S$, $H^{123}A$) was generated incidentally during amplification of *denA* cDNA with MC71/MC72. The fragment was introduced into pJET1.2 (pME3884) and sequenced. For expression in *E. coli* the sequence was excised from pME3884 with *Eco*RI and introduced into the corresponding restriction site of pGEX4-T1 resulting in pME3888. Similar to pME3889 the correct reading frame was proven by sequence analysis.

2.3.2.9 Construction of an N-terminal *ntap::csnA* construct

Upstream fusion of *csnA* with the *tap* tag sequence was achieved by several, none preserved sub-cloning steps finally resulting in plasmid pME3883. In short, the *csnA* ORF together with the downstream 3' flanking region was amplified from genomic DNA with primers MC81/MC82 and introduced into pJET1.2. Amplification introduced an *Eco*RI site upstream of the *csnA* gene. This restriction site was used to introduce the *csnA* 5' flanking region together with the *5'UTR*. This was cut with *Eco*RI from an intermediate plasmid obtained from introducing the PCR fragment of MC83/MC84 amplified from genomic DNA into pJET1.2. The resulting vector then contained a genomic copy of *csnA* with corresponding flanking regions and an *Eco*RI restriction site in front of the *csnA* gene. The vector plasmid was digested with *Eco*RI and the *ntap* sequence, obtained by PCR with MC85/MC86 on pME2968 and subsequent *Eco*RI digestion of the up- and downstream ends was inserted, resulting in pME3883.

2.3.2.10 Construct for *csnG* studies

The *csnG* deletion cassette was generated by PCR mediated fusion (Szewczyk et al, 2006) of the *csnG* flanking regions to the *ptrA* resistance cassette (Kubodera et al, 2000). 1.2 kb 5' flanking region of *csnG*, containing a downstream overhang for the *ptrA* cassette were amplified with MC125/MC126 from genomic DNA. PCR with primers MC129/MC130 on genomic DNA generated a 2.1 kb fragment of the *csnG* 3' flanging region with an upstream overhang for *ptrA*. The

ptrA cassette with overhangs for each *csnG* flanking region was amplified from pSK409 with primers MC127/MC128. All three PCR fragments were assembled in a fusion PCR reaction with primers MC125 and MC130. The deduced fragment was introduced into pJET1.2 resulting in plasmid pME3887. Plasmid pME3890 was obtained by amplification of a genomic *csnG* fragment with primers MC187 and MC190 and introduction into the *Eco*RV digested pME3281 (Helmstaedt et al, 2011).

Table V: Sequences of primers used in this study.

Name	5'-sequence-3'
MC1	GTAATCGATGTCATCGCTGAAAAGGG
MC2	CCTGCGGCCGCTCTACATGGGTATGACTAGAG
MC3	GTTGGTCACCGATGGTCTAATCACGAACCTC
MC4	CAAGGTGACCATGCGCGACGGAGGGCTAGG
MC5	GTAGTTAACTATGCGGCATCAGAGCAG
MC9	CCTACTAGTGCGGTATTTCACACCGCATAC
MC30	CAATGCGCGACGGAGGGCTAGG
MC31	TCACTCAATACGCGGCGGACTC
MC32	CTCAATACGCGGCGGACTCC
MC71	AAGAATTCATGCGCGACGGAGGGCTAGG
MC72	TAGAATTCTCACTCAATACGCGGCGGACT
MC73	ATCAATTGATGGTAATGAGAGCGCGACAG
MC74	TACAATTGTCACGCAACATAACCGTATGTCG
MC81	TTGAATTCATGGAGCCCATGTTACCAGAAGC
MC82	CTGTCGAGTCGCCGGGTCG
MC83	AACAATTGAGGCCTCATGCATTCTGG
MC84	GTGAATTCTGCGTCTATGCTGGACAGCTC
MC85	TTGAATTCATGGCAGGCCTTGCGCAACAC
MC86	TAGAATTCGATAAGCTTATCGTCATCATCAAG
MC90	ACTAGTCTGTCGAGTCGCCGGGTCG
MC91	GTCAATTGATGTTGATCAAGGTCCGTACAC
MC92	GTCAATTGCTACTGAAGGGCGGCGCAGC
MC93	GTCAATTGCTAGCCGCCACGGAGAGCAAGA
MC94	TCATGCGCTCCATCGCCACGCGCGACGGAGGGCTAGGAAAGC
MC96	ACAGAAGGTCATGAACCACGACCAAGTCCACCATAGGGC
MC97	CTATGACTTCTTCCCAAGAAAACG
MC125	TACCGAGACTATCAAGGGAC
MC126	CATCTAGGCCTCGTGGCTGGTGTTGTTGG

Table V *continued*	
MC127	ACCAGCCACGAGGCCTAGATGGCCTCTTGC
MC128	ACAATGAGATGGGCCACTCAGGCCAATTGA
MC129	CTGAGTGGCCCATCTCATTGTACGGTTCAGG
MC130	TACTCGAGCGCTGCAAAACGAAACACCA
MC133	TCGCCGAATCGCCTCATCCTCTTC
MC134	GTATGAGTTTGGGTGGCTGGAAGGT
MC175	ATCAAGACCCGAGGCAATTTGAC
MC176	CAGGCGCTCTACATGAGCATGCCCTGCCCCTGAATAGTTGGCCCGACCGCTT CTAC
MC177	CTTTTTCCATCTTCTCTTACCACCGCTACCACCCTCAATACGCGGCGGACTCC TC
MC178	ATCGCCGAATCAGAGGCCAATGT
MC179	GCCCTTGCTCACCATACCACCGCTACCACCCTCAATACGCGGCGGACTCCTC
MC187	GCTAATATTGGAGACTGCCATCGTACCG
MC190	GCTAATATTTCGGATCAACGTTGAGGC
MC191	AAGAATTCCCGGACGATGCATACCTTAGC
MC192	AAGAATTCTGGCTAACGGATAACATTATCTC
OZG192	TCAGGGGCAGGGCATGCTCATGTAGAG
OZG207	GGTGGTAGCGGTGGTATGGTGAGC
OZG209	GGTGGTAGCGGTGGTAAGAGAAGATGGAAAAAGAATTTCATAG
OLKM67	CCATAACCCTATTGCCACTAG
OLKM68	GTATGGGATAGGAAAATAATATAG
OLKM86	ATGGCCGACAAGCAGAAGAAC
OLKM87	GTGGTTCATGACCTTCTGTTTCAGGTCGTTCAGGATCTTGCAGGCCGGGCGCT TGTACAGCTCGTCCATG
OLKM91	ATGGTGAGCAAGGGCGAGG
Elke11	GTGCATCCCGGTGGAGAGATC
ES21	TGGCTTTCTCGTCAACC
SB114	AGCTGTTGCGGAGAAGCATC
MK067	CCTGCGGCCGCCCCGGGAGACTGATATTCTCGAAACGA
MK082	GCCGAAAATTAACCGCTGCATGATATCCTTGAGAATGCGATGCTCTA
RT-MC1	GACGATTCACCAACCCAAGAGA
RT-MC2	CTACCTTCCAGCCACCCAAACT
HO89	TGACCCTGATTGGGAGAGACTTG
HO90	GTTGCGTGAGTGCCGTAGTGAC

2.4 Molecular methods

2.4.1 Computational sequence analysis

Gene and Protein accession numbers were obtained from, and BLAST searches were performed at the National Center for Biotechnology Information webpage (NCBI; http://www.ncbi.nlm.nih.gov/). *A. nidulans* proteins were identified from the *A. nidulans* genome sequence (Galagan et al., 2005) at AspGD (http://www.aspgd.org) or CADRE (http://www.cadre-genomes.org.uk) genome browsers. Protein family affiliation and domains were analyzed by InterPro Scan (http://www.ebi.ac.uk/Tools/pfa/iprscan). Sequence alignments were performed using Clustal W (http://npsa-pbil.ibcp.fr/) and the multiple alignment tool (Corpet, 1988) at http://multalin.toulouse.inra.fr/multalin/multalin.html. Phosphorylation sites were predicted using the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos). Automated structure homology-modeling (Arnold et al, 2006; Bordoli et al, 2009; Kiefer et al, 2009) was performed using the SWISS model pipeline (http://swissmodel.expasy.org). DNA sequencing was performed at the "Labor für Genomanalyse" in Göttingen. Sequences were analyzed with the Lasergene 8.0 software (DNASTAR).

2.4.2 Recombinant DNA methods

Recombinant DNA technologies were performed according to the standard methods (Sambrock & Russel, 2000). DNA fragments for hybridization probes, plasmid construction or sequencing were amplified by PCR with the *Taq*- (FERMENTAS), *Pfu*- (PROMEGA), or Phusion- (FINZYMES) polymerase, respectively. *A. nidulans* cDNA was generated from total RNA using the Omniscript RT Kit (QIAGEN) following the user's manual. Rapid amplification of cDNA ends (RACE) was achieved by using the GeneRacer™ Kit (INVITROGEN) together with the SuperScript®II reverse transcriptase (INVITROGEN) following the protocol provided by the company.

2.4.3 DNA isolation and hybridization

Isolation of plasmid DNA from *E.coli* was performed using the QIAGEN-tip 100 MIDI Kit or QIAGEN-tip 20 Plasmid MINI Kit, respectively, referring to the

producer's manual. DNA gel extraction was performed using the QIAquick Gel Extraction Kit (QIAGEN). To obtain genomic DNA of *A. nidulans* homogenized cell material was processed as described previously (Lee & Taylor, 1990). Southern hybridization was carried out with non-radioactive probes using the AlkPhos Direct™ labeling and detection system from GE HEALTHCARE following the manufactures guidelines. Chemiluminescent signals were detected on Hyperfilm™ ECL (GE HEALTHCARE).

2.4.4 RNA methods

A. nidulans total RNA was isolated from 0.5 ml of ground mycelia with the QIAGEN RNeasy Plant Mini Kit referring to the manufacturer's instructions. RNA integrity was verified by fractionation of 20 µg of RNA in 2.2 M formaldehyde, 1.2% agarose gel, stained with ethidium bromide, and subsequent visualization with UV-light. The presence of intact 28S and 18S ribosomal RNA bands was used to determine RNA integrity. Probes for northern analysis were generated using radioactively labeled ATP and the Prime-It® II Random Labeling Kit (AGILENT) referring to manual's instructions. Northern hybridization was performed according to standard techniques (Rave et al, 1979).

2.4.5 Reverse transcription and quantitative real-time PCR

DNase digestion and subsequent cDNA synthesis was carried out in duplicates for each sample using 0.8 µg of total RNA with the QuantiTect Reverse Transcription Kit (QIAGEN). Amplification was performed in a LightCycler 2.0 (ROCHE) with the RealMaster SYBR Rox Kit (5PRIME) using 1 µl of a 1/10 dilution of the cDNA and gene specific primers. *denA* was amplified using primers RT-MC1/RT-MC2 and *h2A* with primers HO89/HO90. Amplification conditions were as follows: 36 cycles of 15 s at 95°C, 22 s at 64°C, 22 s at 70°C, and an adjacent melting step (42-95°C). Expression of the gene of interest was quantified relative to *histone h2A* expression using the delta ct method (Pfaffl et al, 2002). All qRT-PCR experiments were performed at least in duplicate.

2.5 Protein methods

2.5.1 Protein isolation and analysis

Protein isolation from *S. cerevisiae* was performed with overnight cultures grown at 30°C in SC medium containing 2% glucose or 2% galactose / 1% raffinose as carbon source. Main cultures were inoculated from starter cultures and grown at 30°C until an OD_{595nm} of 1.0. Cells were harvested by centrifugation at 15.000 rcf, at 4°C 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 PIM (Complete protease inhibitor mix (ROCHE))]. The cell suspension was combined with 0.5 ml glass beads, shook for 5 min at 4°C with a Vortex Genie 2 (SCIENTIFIC INDUSTRIES) at maximum speed and centrifuged for 15 min with 16.000 rcf at 4°C. The supernatant containing the soluble protein fraction was stored at -20°C or used immediately for further analyses.

A. nidulans strains were grown under the desired conditions. The mycelium was harvested through Miracloth filters (MERCK) and washed with 0.9% NaCl/ 0.1 mM PMSF, dried with paper towels and frozen in liquid N_2. Cell material was ground by hand with mortar and pestle or a RETSCH MM400 ball mill cooled with liquid N_2. Protein crude extracts from *A. nidulans* were achieved by resuspension of ground mycelium in B-buffer [300 mM NaCl, 100 mM Tris-HCl pH 7.2, 10% glycerol, 0.1% NP-40, freshly added 1 mM DTT, PIM]. 500 µl B-buffer were added to 300 µl grinded, frozen mycelia and vortexed 4 times for 15 sec. After centrifugation with 16.000 rcf at 4°C for 10 min the supernatant was used directly for further analyses. Protein concentrations were determined as described (Bradford, 1976) using the Roti-Quant® assay solution (ROTH) following the manual.

For western hybridization proteins were denatured in 3x sample buffer [250 mM Tris-HCl pH 6.8, 15% β-mercaptoethanol, 30% glycerol, 7% SDS, 0.3% bromphenol blue] by heating at 95°C for 10 minutes and subjected to SDS-PAGE followed by electro-blot transfer to a nitrocellulose membrane (WHATMAN). The PageRuler™ Prestained Protein Ladder (FERMENTAS) was used as standard. Detection was carried out using the Enhanced ChemiLuminescence (ECL) method described by Tesfaigzi et al. (1994). Signals were recorded on Hyperfilm ECL (GE

HEALTHCARE) or with a Fusion-SL 4.2 MP detection system (PEQLAB). Signal intensity was quantified using the Bio1D software (PEQLAB).

2.5.2 Antibodies

Primary antibodies for yeast extracts were directed against Rub1 (N0580-05, US-BIOLOGICAL), Cdc53 (Sc-6716, SANTA CRUZ) and the V5 epitope (R960-25, INVITROGEN). GFP fusion proteins were detected using α-GFP antibody (sc-9996, SANTA CRUZ) and His tagged proteins by α-His-Tag antibody (70796-4, NOVAGEN). Expression of TAP fusion proteins was detected with α-Calmodulin antibody (UPSTATE/MILLIPORE) and α-Tubulin antibody (T0926, SIGMA-ALDRICH) was used for loading control. Polyclonal antibody directed against Nedd8 was obtained by rabbit immunization with an N-terminal peptide of *A. nidulans* Nedd8 (GENSCRIPT). HRP labeled α-mouse (115-035-003, JACKSON IMMUNO RESEARCH) and α-rabbit (G21234, INVITROGEN) were applied as secondary antibodies.

2.5.3 Tandem Affinity Purification (TAP)

TAP was performed by the method modified for *A. nidulans* described earlier (Bayram et al, 2008b; Busch et al, 2007; Helmstaedt et al, 2008). Briefly, total proteins from at least 10 L culture were extracted as described above (chapter 2.4.1). 50 ml crude extracts were incubated with 400 µl IgG-agarose (GE HEALTHCARE) for 2 h on a rotating platform at 4°C. The suspension was poured onto a PolyPrep column (BIO-RAD) separating the beads from the supernatant. Beads were washed twice with 10 ml IPP300 (25 mM Tris-HCl pH 8.0, 300 mM NaCl, 0,1% NP-40, 2 mM DTT), once with 10 ml IPP150 (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40, 2 mM DTT), and once with 10 ml tobacco etch virus (TEV) cleavage buffer (25 ml Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT). Columns were top and bottom closed and beads were incubated with 300 units TEV protease in 1 ml of TEV cleavage buffer on a rotating platform for 16 h at 4°C. Cleaved proteins were eluted into a fresh PolyPrep column containing 300 µl calmodulin affinity resin (STRATAGENE). Calmodulin beads were equilibrated with 5 ml of calmodulin binding buffer (CBB; 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM $CaCl_2$, 10 mM ß-mercaptoethanol) prior to incubation. The elution was repeated once with 1 ml of TEV cleavage buffer. 6 ml CBB and 6 µl of 1 M $CaCl_2$, were added to the solution. The mixture was incubated

on a rotating platform for 2 h at 4°C. After incubation the calmodulin resin was washed twice with 1 ml of CBB containing 0.1% NP-40 and once with 1 ml of CBB containing 0.02% NP-40. Bond proteins were eluted with 3 x 1 ml of Calmodulin elution buffer (CEB- 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% NP-40, 1 mM Mg-acetate, 1 mM imidazole, 20 mM EGTA, 10 mM ß-mercaptoethanol). Eluted proteins were precipitated by addition of trichloroacetic acid (TCA) to a concentration of 25% TCA short mixing and incubation for 16 h at 4°C. Precipitated proteins were collected by centrifugation with 16.000 rcf, for 1 h, at 4°C, and washed with ice-cold acetone/0.05 M HCl and acetone. Precipitated proteins were completely dried in a vacuum exhausted centrifuge. The solid protein residue was resuspended in 30-50 µl 3x sample buffer and separated by gradient (8-20%) SDS-PAGE. Gels were either stained with Coomassie brilliant blue (SERVA) and bands were cut out for subsequent LC-MS analysis or electro-blotted and subjected to western hybridization.

2.5.4 GFP-Trap® purification

Protein Crude extracts of fungal mycelium were obtained as described above (chapter 2.4.1). Crude extracts were added to 50-75µl of washed GFP-Trap® beads (CHROMOTEK) by pouring it through Miracloth filter. Beads were washed twice in 2 ml of dilution buffer [10 mM Tris pH7.5, 300 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, PIM] prior to addition of the protein extract. In each washing step beads were mixed with the buffer and collected by centrifugation (2700 rcf, 2 min, 4°C). The bead protein mixture was incubated on a rotating platform for about 1-2 h at 4°C. Following incubation samples were centrifuged at 2000 rcf for 2 min and at 4°C, to collect the beads. The residue was washed once with ice cold dilution buffer and twice with washing buffer [10 mM Tris pH7.5, 500 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, PIM, 0.02% NP-40]. Washed beads were resuspended in 100 µl 3x sample buffer and bond proteins were dissociated from the beads by boiling at 95°C for 10 min. Samples were centrifuged with 2700 rcf for 2 min and the supernatant was taken for SDS-PAGE and subsequent western and/or LC-MS analysis.

2.5.5 Coomassie staining

Proteins separated by SDS-PAGE were stained with Coomassie brilliant blue solution [40% v/v ethanol, 10% v/v acetic acid, 1 g Coomassie brilliant blue R250 (SERVA)] for 15 min at room temperature (RT) with gentle agitation. Gels were destained in 12.5 % v/v iso-propanol/ 10 % v/v acetic acid. The solution was renewed three to four times and gels were incubated at RT for desired time frames.

2.5.6 Tryptic in-gel digestion of protein samples (Shevchenko et al, 1996)

Gel pieces containing protein samples for tryptic in-gel digestion were cut into approximately 2 mm big pieces and covered with acetonitril for 10 min at RT. Thereafter the liquid was removed and gel pieces were dried for 10 min in a vacuum exhausted centrifuge. 150µl of a 10 mM DTT solution [10 mM DTT in 100 mM NH_4HCO_3] were added to the samples and they were incubated for 1 h at 56°C on a heating block. The DTT solution was replaced then by 150 µl idoacetamid [55 mM idoacetamid in 100 mM NH_4HCO_3] and samples were kept in the dark at RT for 45 min. In the next steps the idoacetamid solution is replaced by 150 µl of 100 mM NH_4HCO_3, incubated for 10 min at RT, then the 100 mM NH_4HCO_3 was removed and 150 µl acetonitril was added to the gel. Both steps were repeated once, followed by drying of the samples in a vacuum exhausted centrifuge for 10 min. Dried gel pieces were covered with trypsin digestion buffer [1:20 sequencing grade modified trypsin (V5111, PROMEGA) in 50 mM NH_4HCO_3] and incubated on ice for 45 min. Remaining digestion buffer was removed and samples were covered with 50 mM NH_4HCO_3 and incubated overnight at 37°C. The next day samples were centrifuged at 11.000 rcf, RT for 1 min. The supernatant was collected in a new tube and the gel pieces were covered with 20 mM NH_4HCO_3 followed by a 10 min incubation at RT. Again liquid was collected in the new tube and gel pieces were covered with 50% acetonitril/ 5% formic acid and incubated for 20 min at RT. Samples were centrifuged (11.000 rcf, 1 min, RT) and the supernatant was collected. Both extraction steps were repeated twice and the collected supernatant was completely evaporated in a vacuum exhausted centrifuge. Precipitated proteins were reconstituted in 20 µl 95% H_2O/ 5% acetonitril/ 0.1% formic acid and subjected to LC-MS analysis.

2.5.7 Protein identification by tandem mass spectrometry

Peptides of in-gel trypsinated proteins were extracted from gel slices of stained protein bands and separated on a NAN75-15-03-C18-PM column with an *ultimate3000* HPLC system (DIONEX) prior to mass analyses with a LCQ DecaXP mass spectrometer (THERMO SCIENTIFIC). Cycles of MS spectra with m/z ratios of peptides and four data-dependent MS2 spectra were recorded by mass spectrometry. The 'peak list' was created with *extractms* provided by the Xcalibur software package (BioworksBrowser 3.3.1SP1). The MS2 spectra were analyzed against the *A. nidulans* genome protein database (http:/www.broad.mit.edu/annotation/genome/ aspergillus_nidulans/Home.html) using the Turbo-SEQUEST program (Lundgren et al, 2005) of Bioworks (THERMO SCIENTIFIC). Protein identification required at least two different high scoring peptides meeting the following criteria: (i) XCorr (1+, 2+, 3+) > 2.0, 2.5, 3.0, (ii) ΔCn > 0.4 and (iii) Sp > 500. MS2 spectra of the highest scoring peptides were individually verified.

2.5.8 Purification of recombinant GST-DenA from *E. coli*

Plasmids carrying the respective fusion construct were transformed into competent *E.coli* Rosetta™ cells and transformants were selected on LB medium containing ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml). 15 ml culture was inoculated with a single colony and grown overnight at 37°C on a rotary shaker. 10 ml of the on-culture were taken to inoculate 1 L of LB medium supplemented with 0.2% glucose and 10 mM PIPES, pH 6.7. Protein expression was induced by adding 1 mM IPTG after growth at 37°C on a rotary shaker to an OD_{600}= 0.4-0.6. Induced cultures were incubated for 21 h at 20°C on a rotary shaker. Cells were harvested by centrifugation for 1 h (4°C, 2200 rcf). Harvested cells were incubated in buffer 1 [20 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EGTA] for 30 min over ice with casual swinging. The pellet was resuspended in 20 ml buffer 1 and cells were broken by sonification (power 07/60%; 10x 30 sec). Centrifugation for 1 h at 4°C and 12.000 rcf removed the un-soluble parts. The supernatant was passed through a 0.45 µm cartridge filter and 1 ml GST beads (GE HEALTHCARE) per liter culture were applied for purification. Beads and supernatant were incubated for 2 h at 4°C with slow rotation. The bead containing solution was given on a PolyPrep column (BIORAD) and the flow through was discarded. The beads were washed four times with 10 ml washing buffer [buffer1 + 0.25% Tween20] and proteins were eluted with

buffer 2 [50 mM Tris, pH 8.0; 5 mM reduced glutathion]. For further concentration of protein samples, and buffer exchange to PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH7.4), AMICON Ultra filter devices (10 K, MILLIPORE) were used following the manufacturer's guidelines.

2.5.9 *In vitro* assays on DenA activity

Activity tests with recombinant DenA were performed on Nedd8-GFP and CUL1-Nedd8 substrates kindly provided by Tilo Schmaler (AG Dubiel, Charité, Berlin). Nedd8-GFP was mixed with H_2O and different amounts of purified GST::DenA on ice in a total sample volume of 40 µl with 10x AB buffer [30 mM Tris, 10 mM KCl, 5 mM DTT, pH 7.8]. Half of the reaction mixture (20 µl) was immediately combined with 3x sample buffer denatured at 98°C for 5 min. The remaining 20 µl were incubated for 30 min at 37°C and subsequently processed, similar to the initial sample. Both, the 0 min and the 30 min samples were then subjected to SDS-PAGE and cleavage of the substrate was analyzed by western hybridization. Similarly, the CUL1-Nedd8 substrate was combined with different amounts of GST::DenA in a total volume of 30 µl containing 3.3 mM DTT and H_2O. The complete reaction mixture was then incubated for 30 min at 37°C. Following incubation, samples were mixed with 3x sample buffer, denatured at 98°C for 5 min and subjected to SDS-PAGE and subsequent western analysis of substrate cleavage.

2.6 Yeast-2-hybrid

A. nidulans protein interactions were tested with the yeast-2-hybrid based interaction trap (Golemis et al, 1999) following existing protocols (Busch et al, 2007; Helmstaedt et al, 2008). Plasmids were transformed into *S. cerevisiae* strain EGY48-p1840. For the interaction tests cells from overnight cultures were collected and washed in 0.9% NaCl. Cells were resuspended to an OD_{546} = 0.2 in 0.9% NaCl and 10 µl cell suspension were dropped on test plates. Plates containing 2% glucose and 2% leucine served as positive control and plates containing 2% glucose and no leucine as negative control. Growth tests were performed on plates containing 2% galactose/1% raffinose without leucine. For β-galactosidase tests SC-plates containing 2% galactose/1% raffinose supplemented with 0.2 g/l leucine were covered with Hybond-C membrane (GE HEALTHCARE) and samples were dropped

onto the membrane. Plates were incubated headfirst for two days at 30°C. For the β-galactosidase activity test, the Hybond membrane was frozen in liquid N_2 and placed on a filter 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 20 µl 2% XGal in DMF (N,N-dimethylformamid). Membranes were incubated at 37°C for 1 to 6 h.

2.7 Microscopic analysis

A. nidulans colonies, hyphae and structures were photographed with an OLYMPUS CS30 digital camera combined with an OLYMPUS SZX-ILLB2-200 binocular or a ZEISS Axiolab microscope. The KL1500-LCD light source (OLYMPUS) was applied for impinging or transmitting light illumination of samples observed with the OLYMPUS SZX-ILLB2-200 binocular. Pictures were edited and calibrated for magnification with the cellSens software (OLYMPUS). Fluorescent microscopy was performed using a ZEISS Axio Observer Z.1 system with ZEISS PlanAPOCHROMAT 63x/1,4$_{oil}$ or ZEISS PlanAPOCHROMAT 100x/1,4$_{oil}$ objective, respectively. Pictures were taken using the QuantEM:512SC (PHOTOMETRICS) camera or a Coolsnap HQ^2 (PHOTOMETRICS) and the SlideBook 5.0 imaging software (INTELLIGENT IMAGING INNOVATIONS INC.). Confocal pictures were obtained using the Yokogawa CSM-X1 spinning disc system (INTELLIGENT IMAGING INNOVATIONS INC.) in combination with the above system. Membranes were visualized by staining with 1 µM FM4-64 (INVITROGEN). Nuclei were stained with DAPI (4',6-diamidin-2-phenylindol), unless indicated otherwise.

2.8 Quantification methods

Colony growth was recorded as colony diameter over time and quantification of conidiospores was performed as described previously (Busch et al, 2003; Bussink & Osmani, 1998). Spores were counted with a THOMA counting chamber. Quantification of cleistothecia was carried out using 6-8 days sexually grown cultures. Surface pictures of plated cultures were acquired at 150 fold magnification using an OLYMPUS SZX12 binocular connected to an OLYMPUS CS30 camera. Cleistothecia within a 4 x 4 field grid of 1 mm^2 in size were counted and multiplied to get the number per cm^2.

3 Results

3.1 Identification and characterization of the fungal deneddylase DenA

3.1.1 AN10456 encodes a cysteine protease similar to human DEN1/NEDP1

The first identification of DenA was obtained by BlastP using the human DEN1/NEDP1 amino acid sequence as query against an *Aspergillus nidulans* genome database (Galagan et al, 2005). Cloning and sequencing of the corresponding fungal cDNA revealed differences in the existing sequence compared to the database annotations. Rapid amplification of cDNA ends (RACE) (Frohman et al, 1988) was applied for a more careful investigation of the coding sequence. These experiments revealed that AN10456 codes for a transcript with seven exons interrupted by six introns, instead of six exons and five introns as suggested on CADRE (Mabey et al, 2004), or 9 exons interrupted by 8 introns presented by the annotation at Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) (Figure 11). The mRNA has a total length of 1469 base pairs including the coding sequence flanked by an un-translated region (UTR) on either site. The corresponding protein consists of 258 amino acids and has a predicted molecular mass of 29,5kDa (Figure 12). The protein encoded by AN10456 belongs to the family of C-terminal Ulp1 peptidases (Pfam PF02902). ClustalW (Higgins & Sharp, 1988) multiple alignment (http://multalin.toulouse.inra.fr/multalin/multalin.html) with homologs from other organisms, using the deduced peptide sequence of AN10456, revealed that the protein is conserved from fungi to humans.

Closest homologs are found in other *Aspergilli* (82% *A. fumigatus*, 81% *A. oryzae*) or *Penicillium chrysogenum* (71%). The protein of the corn smut fungus *Ustilago maydis* shares only 18% identity, whereas the human DEN1/NEDP1 protein is still 32% identical with *A. nidulans* DenA (Figure 12A). The amino acids of the so called catalytic triad, histidine (H), aspartate (D) and cysteine (C) are highly conserved among species (Figure 12).

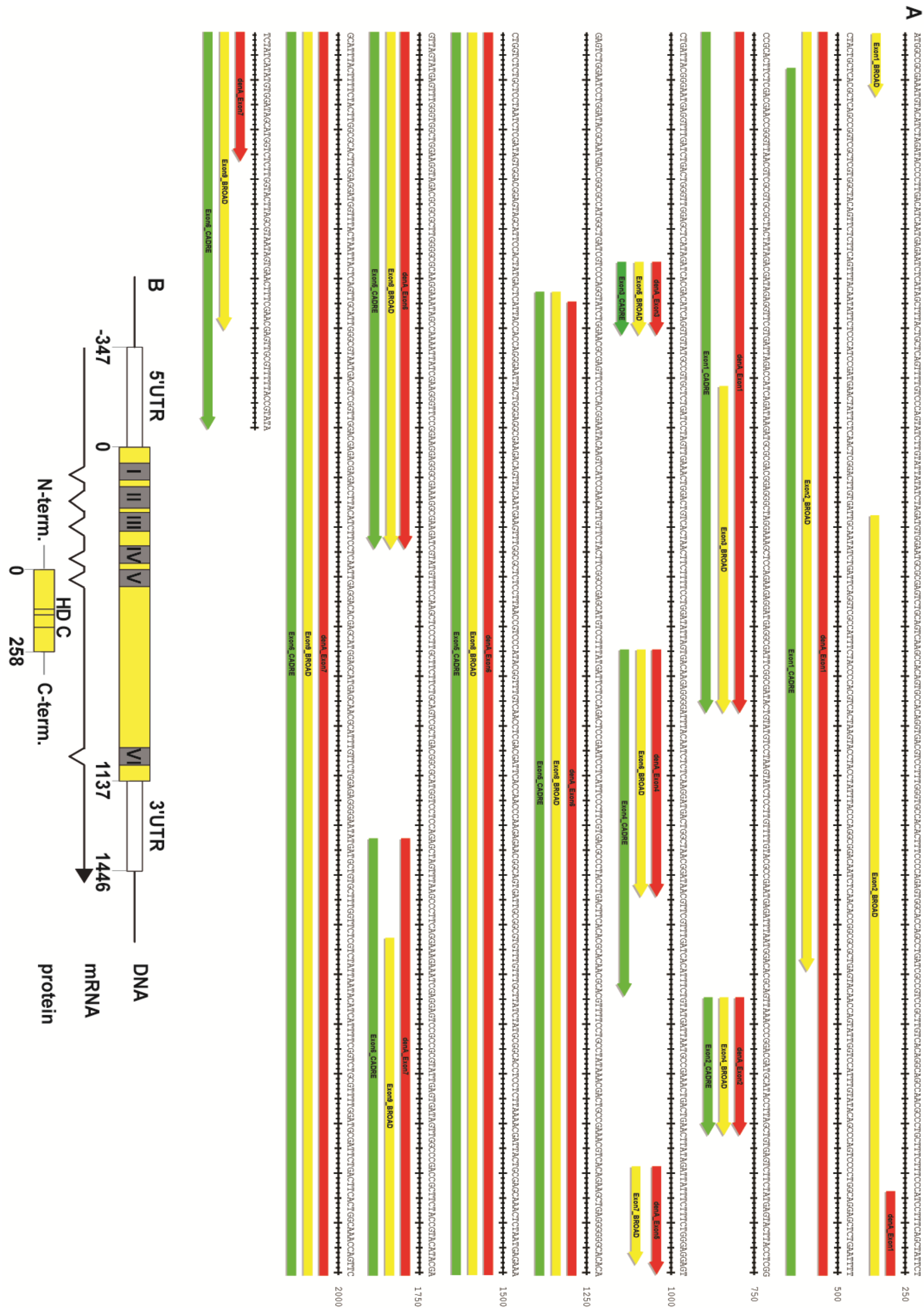

Figure 11: Structural organization of AN10456.

A Genomic sequence of AN10456. The experimentally observed exons are marked in red, whereas those from computational annotations are given in yellow (Broad) or green (Cadre). **B** Schematic view of gene (DNA), transcript (RNA) and protein encoded by AN10456. Introns are grey with roman ciphers and exons, as well as the corresponding transcript and protein, are yellow. Positional specifications for the genomic sequence are given with respect to the adenosine of the start codon. The three conserved amino acids histidine (H), aspartate (D) and cysteine (C), forming the active site are indicated.

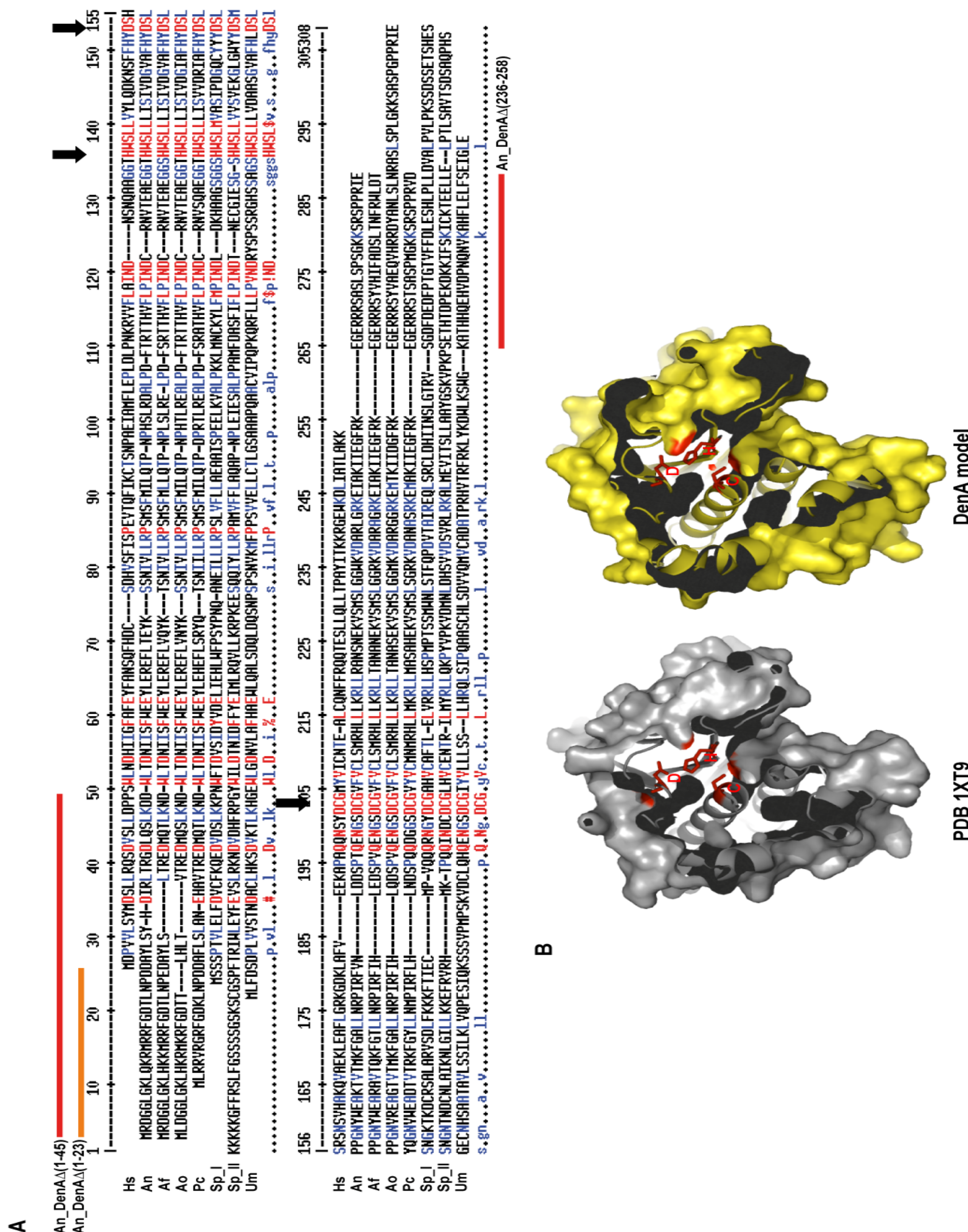

Figure 12: Multiple alignment of DenA gene with related proteins from other organisms.

A *A. nidulans* (An) DenA was aligned to sequences of *A. fumigatus* (Af; XP_749049), *A. oryzae* (Ao; XP_001817262), *Penicillium chrysogenum* (Pc; XP_002567908), *Ustilago maydis* (Um; XP_759519), *Schizosaccharomyces pombe* NEP1 (Sp_I; SPBC17D11.01) and NEP2 (Sp_II; SPBC32H8.02c) and *Homo sapiens* (Hs; NP_001165582). Sequences of *S. pombe* proteins were N- and C-terminally truncated for the alignment. High consensus residues (>90%) are highlighted in red and low consensus (>50%) in blue. Black arrows indicate the conserved residues (H, D, C) forming the catalytic active triad site of the protein. Colored bars correspond to the regions removed from fungal DenA for Y2H interaction experiments (chapter 3.1.3). **B** DenA structure (yellow) based on structure homology modeling to human DEN1/NEDP1 (Reverter et al, 2005) as template (grey). The residues histidine (H), aspartate (D) and cysteine (C) form the catalytic active site of the protein and are highlighted in red.

Automated structure homology-modeling using the structure of human DEN1/NEDP1 (PDB-ID: 1XT9) (Reverter et al, 2005) as template for the SWISS model pipeline (Arnold et al, 2006; Bordoli et al, 2009; Kiefer et al, 2009) supported this finding on the structural level (Figure 12 B). According to the reasons listed above the identified gene AN10456 was named *denA* and the corresponding protein DenA.

3.1.2 *denA* transcript abundance is not coupled to the presence of the protein which exists in different sub-populations in the cell

The knowledge on when a gene product is present in an organism is a first indication for timely regulated processes in which it is involved or how it influences the cellular machinery. Transcript abundance of *denA* was investigated by northern hybridization and the more sensitive quantitative real-time PCR (qRT-PCR).

Northern experiments indicated that the *denA* transcript is made throughout the complete life-cycle of *A. nidulans*. The relative expression level, normalized to ribosomal RNA, occurred to be similar at both time points observed for vegetative growth (Figure 13A veg). Transcription of *denA* was increased at the initial time points observed for asexual, as well as for sexual development, in northern experiments. However, it was reduced again at later time points analyzed for both developmental pathways (Figure 13A asex and sex).

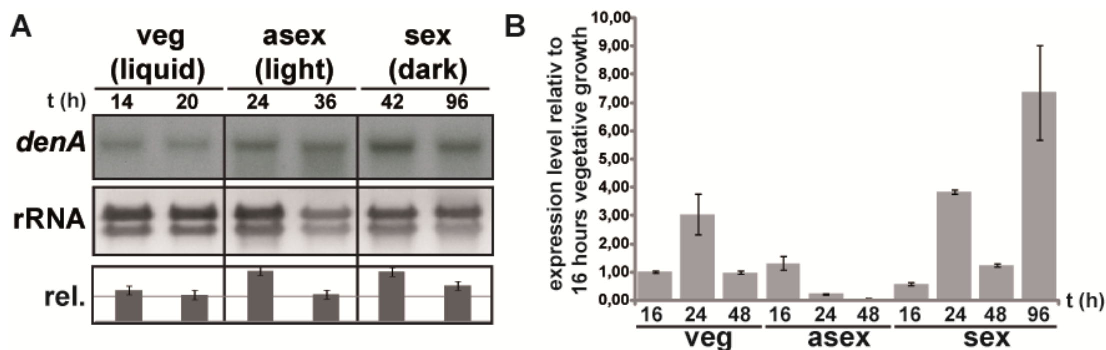

Figure 13: Analysis of *denA* transcription during development.

A Northern hybridization of total RNA from different developmental time points of *A. nidulans* probed with *denA* cDNA probe. *denA* signals were normalized (rel) to ribosomal RNA amounts (rRNA). **B** Transcript analysis by qRT-PCR with *denA* specific primers (RT-MC1/RT-MC2) on cDNA samples obtained from total RNA of different developmental stages of an *A. nidulans* wild type strain.

Data from quantitative real-time PCR (qRT-PCR) support the findings obtained by northern hybridization, even though variations can be observed, that may be due to the higher sensitivity of the qRT-PCR method (Figure 13B). In both experiments the *denA* transcript was present at all observed time points of vegetative growth. Along development expression seemed to increase a little in the beginning, what was seen in the results from both experiments. For vegetative growth this effect was only seen with the qRT-PCR data presumably due to higher sensitivity and differences in the tested time points. During asexual development expression was reduced what was also represented by both experiments, but occurred to be more drastic with the qRT-PCR data. However, the decrease of *denA* transcript at late stages of sexual development (sex 96 hours) observed with the northern data was not seen with the qRT-PCR results. qRT-PCR data showed a wave like course of *denA* transcript abundance with a small peak at 24 hours sexual development, followed by a reduction at 48 hours and a peak, higher than the first one, at 96 hours (Figure 13B sex). Even though there were differences, also the more sensitive qRT-PCR revealed no obvious development specific expression pattern for *denA* (Figure 13). Therefore one can claim that *denA* is transcribed at all observed time points of the fungal life cycle, with a slight preference for development.

To investigate if the protein expression correlates with transcript abundance western hybridization experiments were performed. A C-terminally tagged version of DenA was detected in crude extracts from different time points of fungal development, applying an anti-GFP antibody (Figure 14A). The C-terminally tagged DenA (DenA::GFP) was introduced to the endogenic locus of AN10456 driven by the native *denA* promoter. Detection of the GFP tag with the corresponding antibody generated a signal for the fusion protein (~56 kDa, Figure 14A, upper panel)), as well as for the GFP tag alone, which occurred to be stable towards protein degradation (~27 kDa, Figure 14A lower panel). The DenA::GFP fusion protein was made at all observed stages of vegetative growth, but was degraded with the onset of development. At each time point of asexual development, later than 12 hours, only the stable GFP alone was detected in western experiments (Figure 14A, asex). DenA::GFP occurred to be more stable with regard to the time points observed for sexual development, where the fusion protein was still detectable at 48 hours.

Interestingly, no fusion protein could be detected in western experiments at late stages of sexual development (96 h, sex), even though expression of *denA* detected with qRT-PCR showed a peak there (Figure 13B, Figure 14A). A similar discrepancy between the protein level and the expression pattern detected by qRT-PCR was seen for vegetative growing mycelia. The protein level was almost stable at each time point (Figure 14A, veg), corresponding to the northern data (Figure 13A), while the qRT-PCR data showed an increase of expression at 24 hours (Figure 13B). Only for asexual development the expression pattern observed for the *denA* mRNA correlated with the protein abundance (Figure 13 and 14). Altogether, these results show that the protein abundance of DenA is not completely correlating with mRNA expression, especially during vegetative and sexual development. This suggests that DenA protein amounts can be regulated on the post-transcriptional or post-translational level.

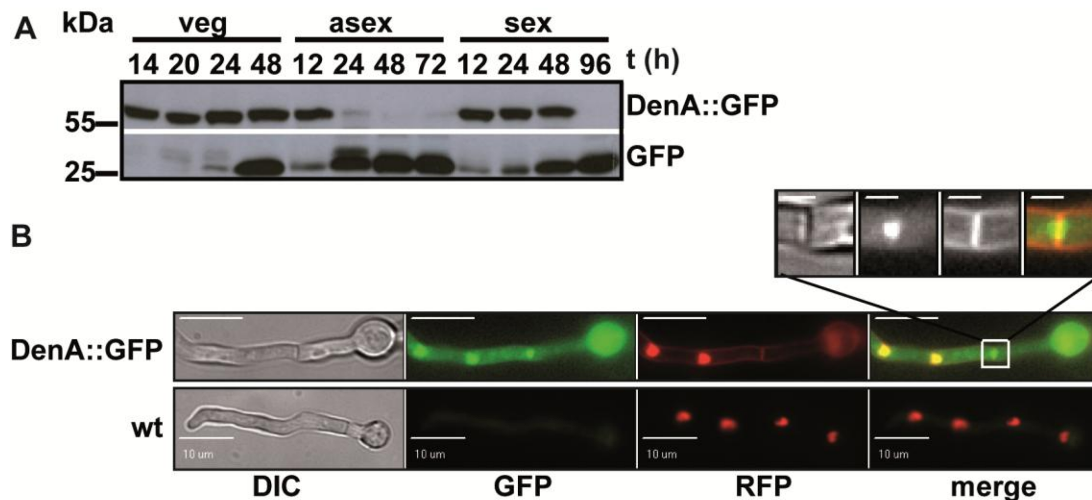

Figure 14: Presence and localization of the DenA protein.

A Analysis of DenA protein abundance during fungal development by detection of a GFP fusion protein. GFP was fused C-terminally with DenA (DenA::GFP). Expression was driven by the native *denA* promoter. Mycelia were harvested after the indicated times of growth under vegetative (veg), asexual (asex), and sexual (sex) growth conditions. kDa: kilo Dalton. **B** Localization of C-terminally GFP tagged DenA in *A. nidulans*. Nuclei are marked by red fluorescence from mRFP::H2A expressed from an ectopically integrated plasmid (scale bar = 10 µm; Exposure time GFP: 1500 ms, RFP: 25 ms, DIC: 100 ms).

The *denA::gfp* strain was also used to determine the cellular localization of the protein. As seen in Figure 14B, DenA::GFP could be detected in different sub-populations within the growing hyphae. Fluorescence was detected in the cytoplasm,

within the nucleus and at the site of septa in ball shaped structures (Figure 14B, detail).

3.1.3 The N-terminus of DenA is required to interact with Nedd8 and is involved in DenA stability

Deneddylation is a process predominantly required for the control of cullin based E3-ubiquitin ligases (Bornstein et al, 2006; Wu et al, 2005). The human DEN1/NEDP1 was further described to process the Nedd8 precursor to obtain mature, attachable protein (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003). Therefore yeast-2-hybrid analysis was conducted to test the ability of fungal DenA to interact with the precursor or mature variant of fungal Nedd8, as well as with the three *A. nidulans* cullins, CulA, CulC and CulD.

As depicted in Figure 15A, Nedd8 and DenA were interacting efficiently, independent of the Nedd8 C-terminal extension. Both, the growth test, as well as the more sensitive ß-galactosidase filter test, indicated an interaction. However, neither the growth test, nor the ß-galactosidase assay showed a positive read out for DenA combined with any of the three fungal cullins (Figure 15A). This suggests that a direct protein-protein interaction between DenA and the cullins does not occur.

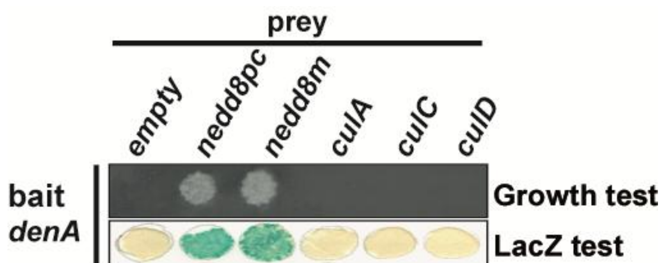

Figure 15: Yeast-2-hybrid interaction studies with *A. nidulans* Nedd8 and cullins.

A. nidulans cullins *culA*, *culC*, *culD* as well as the fungal *nedd8* in its precursor (pc), and mature (m) form were tested growth and ß-galactosidase activity (LacZ) tests. empty: prey vector with activation domain only.

It is known from structural studies of mammalian DEN1/NEDP1 that the N-terminus is crucial for Nedd8 interaction (Reverter et al, 2005). To test whether certain parts of DenA play a role for Nedd8 interaction several derivatives of DenA were produced. Using PCR, *denA* variants, truncated at the N- or C-terminus or at both sides were generated. The corresponding cDNAs were introduced into the yeast-2-hybrid prey plasmid pJG4-5 and the effect on growth and ß-galactosidase

activity was compared to the full length DenA (Figure 16, first panel). Reduction for the fungal specific N-terminal residues (ΔN1-23, Figure 16, second panel; see also Figure 12A for comparison), the C-terminal protein part (ΔC236-258, Figure 16, third panel), or both (Figure 16, fourth panel) did not change the ability of DenA to interact to Nedd8 in yeast-2-hybrid. However, when the N-terminus was reduced for 21 further amino acids (ΔN1-45, Figure 16, third and fifth panel) neither growth on selective medium, nor ß-galactosidase activity was observed anymore.

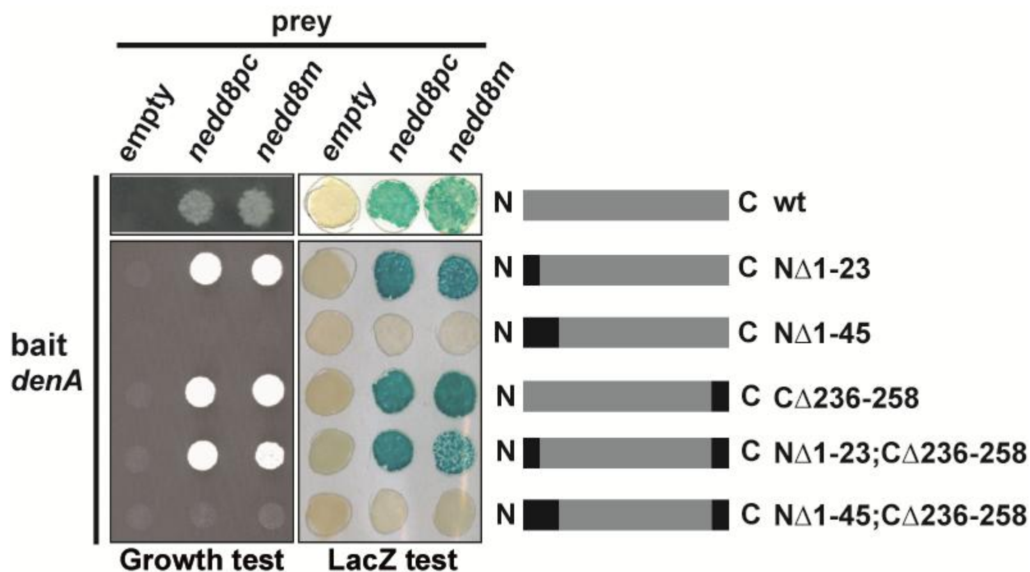

Figure 16: Yeast-2-hybrid interaction with Nedd8 and truncated variants of DenA.

The precursor (pc) and the mature (m) form of Nedd8 were tested for their ability to interact to truncated versions of DenA in growth and β-galactosidase activity (LacZ) test. DenA was truncated N-terminally for 23 residues (NΔ1-23) or 45 residues (NΔ1-45), respectively. C-terminally the protein was reduced by 22 residues (ΔC236-258). Also combinations of N- and C-terminal truncations (NΔ1-23;ΔC236-258 and NΔ1-45;ΔC236-258) were tested. Black areas indicate the protein parts removed and grey areas represent the expressed protein part. N: N-terminus, C: C-terminus, empty: prey vector with activation domain only.

The role of the DenA N-terminus was further investigated in western experiments. A strain containing an N-terminally GFP tagged DenA (GFP::DenA) was compared to the above described *denA::gfp* strain, expressing the C-terminally tagged version of the protein. As seen in Figure 17, the GFP::DenA fusion protein occurred to be less stable than the C-terminally tagged protein. Stability of the N-terminal fusion protein (GFP::DenA) was already decreased during vegetative growth, compared to the C-terminal fusion (DenA::GFP, Figure 17, veg). Also during sexual development the N-terminal GFP tagged version of DenA (GFP::DenA) was degraded faster (Figure 17, sex). However, for asexual

development the situation looked almost similar for both. These data suggest that accessibility of the DenA N-terminus is required for stability of the protein during vegetative growth and sexual development. Furthermore is involved in protein-protein interaction with Nedd8, as seen above (Figure 16).

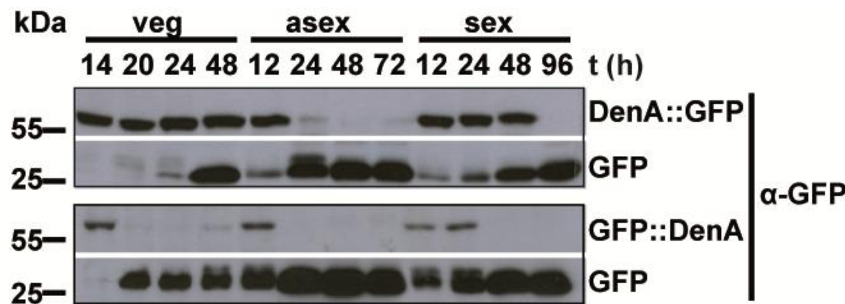

Figure 17: Western experiments investigating the differential effect of C- and N-terminal fusion of GFP to DenA.

GFP was fused either to the C-terminus (DenA::GFP) or the N-terminus (GFP::DenA) of the protein. Expression of both constructs was driven by the native *denA* promoter. Detection with a α-GFP antibody generated a signal for the respective fusion protein (~56 kDa) and GFP tag alone (~26 kDa) which occurred to be more stable. Mycelia were harvested after the indicated times of growth under vegetative (veg), asexual (asex), and sexual (sex) growth conditions. kDa: kilo Dalton.

3.1.4 Deletion of *denA* results in impaired asexual spore formation

The function of the DEN1 deneddylase *in vivo* is yet elusive, because mutant strains with clear phenotypes have not been described. Data from the fruit fly *Drosophila melanogaster* describe *DEN1* mutants that showed defects in cell viability and display an altered neddylation pattern of endogenous proteins (Chan et al, 2008). Results obtained for the *Schizosaccharomyces pombe* DEN1 homolog Nep1 suggests a function in cell cycle progression (Zhou & Watts, 2005) whereas mammalian DEN1/NEDP1 has only been investigated *in vitro* (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003). To investigate the role of DenA for the life and development of *A. nidulans* a knock-out strain was generated. In the Δ*denA* strain the complete *denA* coding sequence was removed. The resulting *denA* deletion strain was viable, but showed severe defects in growth and asexual spore formation (Figure 18). Radial growth, recorded as colony diameter over time, corresponded to only half that of a wild type strain after 10 days of incubation. Re-introduction of a genomic copy of *denA* was sufficient to rescue normal growth (Figure 18A and B).

Macroscopic and microscopic analysis revealed that the Δ*denA* strain exhibited a dramatic decrease in the formation of conidiophores, accompanied with

the reduced colony size, compared to wild type (Figure 18A). A lot of aerial hyphae were formed on the surface but only very few asexual structures could be observed. Quantification experiments were performed to verify the reduction in asexual spore formation. The results strongly support the microscopic observations, as deletion of *denA* lead to a conidiospore production that was not more than 5% that of a wild type strain (Figure 18 C). Similar to the growth phenotype, re-introduction of a genomic copy of *denA* into the deletion strain was able to restore normal conidiation (Figure 18C). Altogether, the results above imply that DenA has a function in the control of asexual spore formation in *A. nidulans*.

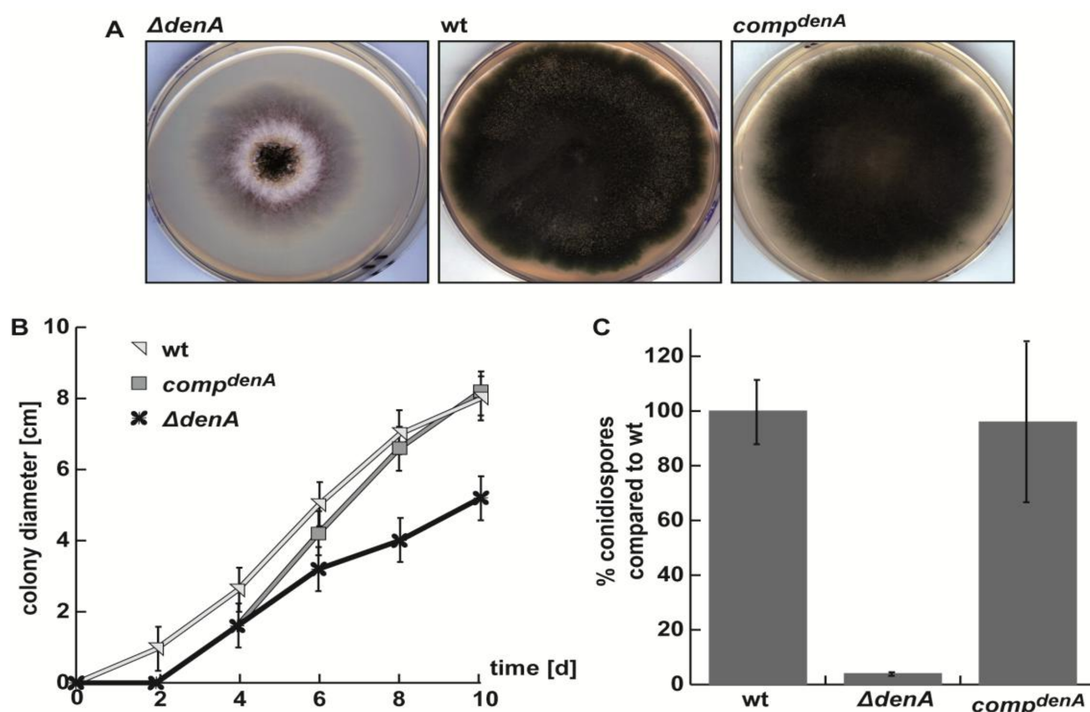

Figure 18: Evaluation of growth and asexual spore formation.

A Surface pictures from plates of a radial growth test after 8 days of growth under asexual conditions. **B** Graphical representation of the increase of colony diameter over time (d). **C** Diagram comparing the amount of conidiospores produced under normal asexual conditions by wild type (wt), *denA* deletion (Δ*denA*), *denA* complementation strain (*comp*^{denA}).

3.1.5 DenA is required for light-inhibition of sexual development

In the absence of light *A. nidulans* favors the formation of sexual fruit bodies named cleistothecia instead of conidiation (Braus, 2002; Dyer & O'Gorman, 2011). When the *denA* deletion strain was analyzed for sexual development, there was no difference observed between the corresponding wild type strain and the *denA* deletion strain when grown in the dark. After 8 to 10 days comparable amounts of

cleistothecia were observed. All were within the normal dimensions of about 200 µm surrounded by Hülle cells. Isolation of ascospores from cleistothecia of the Δ*denA* strain and re-plating them on agar plates showed that the produced sexual spores were viable (data not shown).

A wild type strain normally reduces cleistothecia formation in the presence of light to about 10% that of darkness. However, the Δ*denA* strain was not able to reduce the number of cleistothecia in the presence of light. As illustrated in Figure 19 the *denA* deletion strain formed equal amounts of cleistothecia in the light, as well as in the dark. These cleistothecia did not differ in size and ascospore viability from those produced, by either the wild type or the *denA* deletion strain, in the absence of light. Similar to the observations from growth and asexual development re-introduction of a genomic copy of *denA* was able to restore inhibition of sexual fruit body formation in the presence of light (Figure 19). This suggests an additional function of DenA activity for the light dependent repression of sexual development. Probably DenA promotes sexual development and thereby asexual sporulation is repressed.

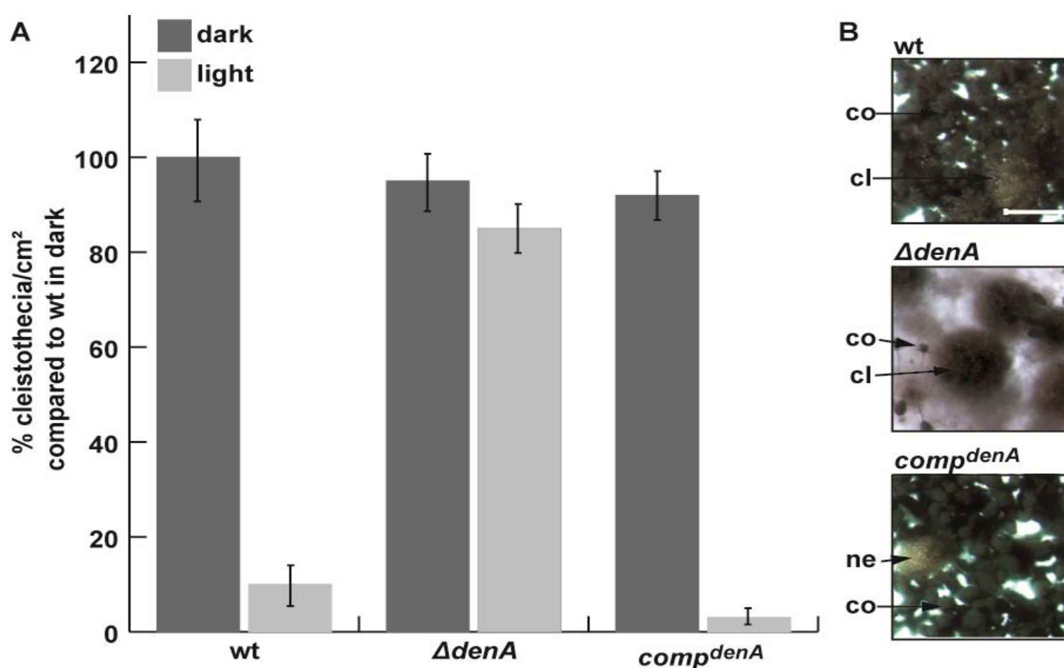

Figure 19: Examination of sexual fruit body formation in the *denA* deletion strain.

A Graphical representation of cleistothecia formation in the absence (dark) or presence (light) of light by wild type (wt), *denA* deletion (Δ*denA*) and *denA* complementation strain (*comp*^{denA}). **B** Surface pictures of the corresponding *A. nidulans* strains taken after the seventh day of growth under asexual conditions. Conidia (co), cleistothecia (cl), nest (ne), scale bar = 250µm.

3.1.6 The *denA* deletion phenotype can be rescued by re-integration of *denA* but not through providing a gene encoding a processed *nedd8*

Human DEN1/NEDP1 was described to have two functions *in vitro*. At first, the protease is capable of processing the Nedd8 precursor removing the C-terminal tail covering the gly-gly motif. Secondly it cleaves the isopeptide bond between Nedd8 and a substrate protein with high affinity (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003). As already shown in Figure 15 by yeast-2-hybrid experiments, DenA interacts with both, the precursor and the mature form of Nedd8, but does not recognize any of the fungal cullins. In order to dissect which function, processing, deneddylation, or both, is responsible for the observed *denA* deletion phenotypes several experiments were performed.

At first, the potential Nedd8 processing activity of DenA was addressed. Therefore a strain carrying the *ntap::nedd8GG* variant at the endogenous locus was used. This Nedd8 construct already lacks the C-terminal amino acid extension and requires no processing activity to gain attachable Nedd8. This strain was combined with the *denA* deletion. As seen in Figure 20, the resulting strain (Δ*denA*;*ntap::nedd8GG*) still showed similar defects in light dependent repression of sexual fruit body formation and lacks proper conidiation, reminiscent to the *denA* deletion. Also the radial growth ability was still decreased (data not shown).

Experiments in yeast were performed attempting to rescue the deletion of the yeast Nedd8 processing enzyme Yuh1. Deletion of *yuh1* in yeast has no phenotype on viability, but in western experiments neddylation of the yeast cullin Cdc53 is abolished, due to the lack of attachable Nedd8 (Rub1 in yeast) (Linghu et al, 2002) (Figure 21, lane 2). As shown in Figure 21 *A. nidulans denA* was expressed in the Δ*yuh1* yeast strain but it was unable to restore neddylation. Detection with an antibody for the yeast cullin Cdc53 (Figure 21, α-Cdc53) revealed a signal at the size of the neddylated protein in wild type extracts (Figure 21, α-Cdc53, lane 1). This modification is lost in the Δ*yuh1* background in agreement with the published data and expression of *A. nidulans denA* did not lead to any detectable change (Figure 21, α-Cdc53, lane 2-4).

Similar results are obtained with an antibody against the yeast Nedd8 ortholog Rub1 (Figure 21, α-Rub1). Neddylated proteins are detected in crude extracts from the wild type (Figure 21, lane 1), but deletion of *yuh1* abolishes the signal and expression of *denA* did not cause any change (Figure 21, lane 2-4).

Expression of *A. nidulans denA* was verified by detection of a *denA::V5* fusion construct with an antibody directed against the V5-epitope (α-V5, lane 4).

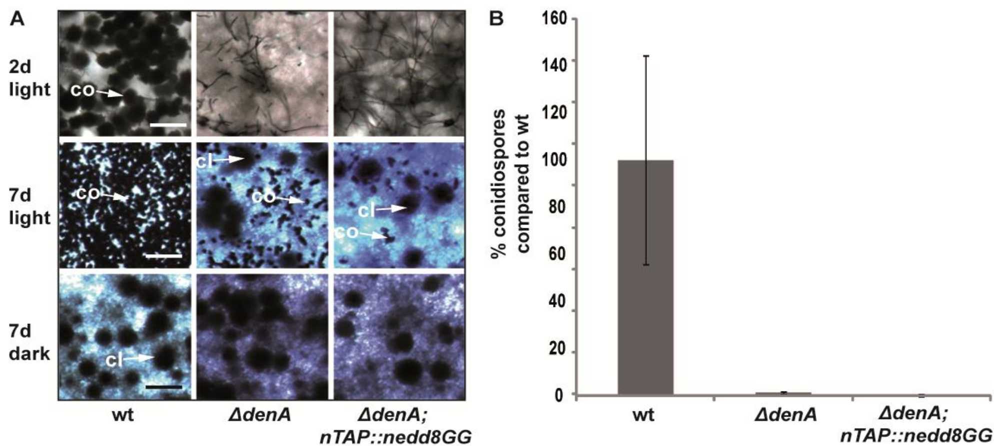

Figure 20: Characterization of a Δ*denA* strain carrying a mature Nedd8 variant (*nTAP::nedd8GG*).

A Microscopic evaluation of developmental structures formed by the wild type (wt), the Δ*denA* strain and the Δ*denA;nTAP::nedd8GG* mutant. Conidiophore (co) formation and light regulation of cleistothecia development (cl) cannot be rescued by *nTAP::nedd8GG*. (scale bar = 50 µm, first row; scale bar = 225 µm, second and third row). **B** Diagram displaying the number of conidiospores produced under normal asexual conditions by the corresponding strains in comparison to the wild type.

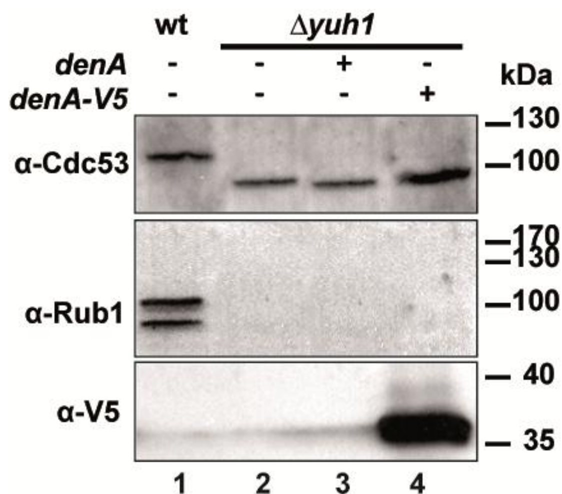

Figure 21: Test for complementation of the *S. cerevisiae yuh1* gene by *denA*.

DenA was expressed as native protein or C-terminally fused with a V5/His6 epitope tag, respectively. *denA* expression was driven by the inducible *GAL1* promoter and monitored by detection of the fusion protein with V5 antibody (lane 4). Using a Cdc53 (α-Cdc53) or Rub1 (α-Rub1) antibody for western hybridization revealed that *denA* expression is not sufficient to produce mature Rub1 species in a *yuh1* deficient *S. cerevisiae* strain and rescue Cdc53 modification (lanes 2- 4). kDa: kilo Dalton.

Furthermore, *in vitro* experiments were performed to investigate the Nedd8 processing activity of DenA. Recombinant GST::DenA was combined with a substrate produced by C-terminal fusion of human Nedd8 with GFP (kindly provided by Tilo Schmaler, AG Dubiel, Charité Berlin). As depicted in Figure 22 addition of different amounts of DenA (0.01 µg – 5 µg) did not cause any detectable cleavage of the Nedd8-GFP adduct compared to the negative control without enzyme (0 µg). Altogether these experiments support the hypothesis that it is not Nedd8 processing that is responsible for the observed *denA* deletion phenotypes in *A. nidulans*.

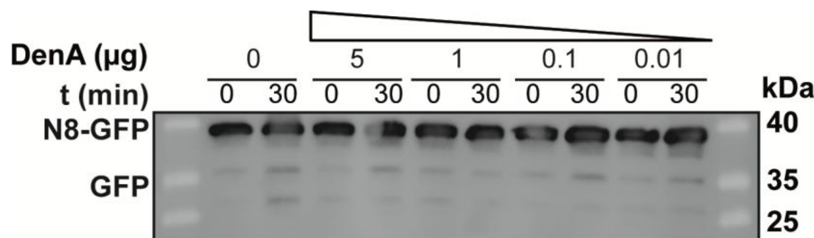

Figure 22: *In vitro* processing activity of *A. nidulans* DenA.

Nedd8-GFP (N8-GFP) substrate was combined with water (0), as a negative control and decreasing amounts of recombinant GST::DenA. One half of each mixture was immediately denatured (0) and the other was incubated for 30 min at 37°C (30). Detection with α-GFP revealed no significant cleavage of the substrate at any amount of GST::DenA added. kDa: kilo Dalton.

3.1.7 DenA exhibits deneddylation function *in vivo* and *in vitro*

The above experiments on the Nedd8 processing activity of *A. nidulans* DenA strongly suggest that this is not the primary function of the protein. It might also be that DenA cleaves Nedd8 off a modified substrate protein and thus functions as a deneddylase showing isopeptidase activity. Similar to the Nedd8 processing activity this opportunity was investigated by different experimental approaches.

Western hybridization experiments were performed with an antibody against fungal Nedd8. Similar amounts of crude extracts from wild type, *denA* deletion (Δ*denA*) and *denA* complementation strains (*comp*^{denA}) were blotted onto nitrocellulose membrane and probed with the Nedd8 antibody. As seen in Figure 23 neddylated proteins accumulated in crude extracts of the *denA* deletion strain. A prominent signal occurred, at the size of 108 kDa, corresponding to neddylated fungal cullin. Furthermore there were additional lower migrating signals that accumulated in Δ*denA* strain which occurred to be more diffuse. Even though the effects were not very strong, they were reproducible and could be reversed by

complementation of the *denA* deletion (Figure 23). This suggests that *denA* is involved in the deneddylation of fungal proteins.

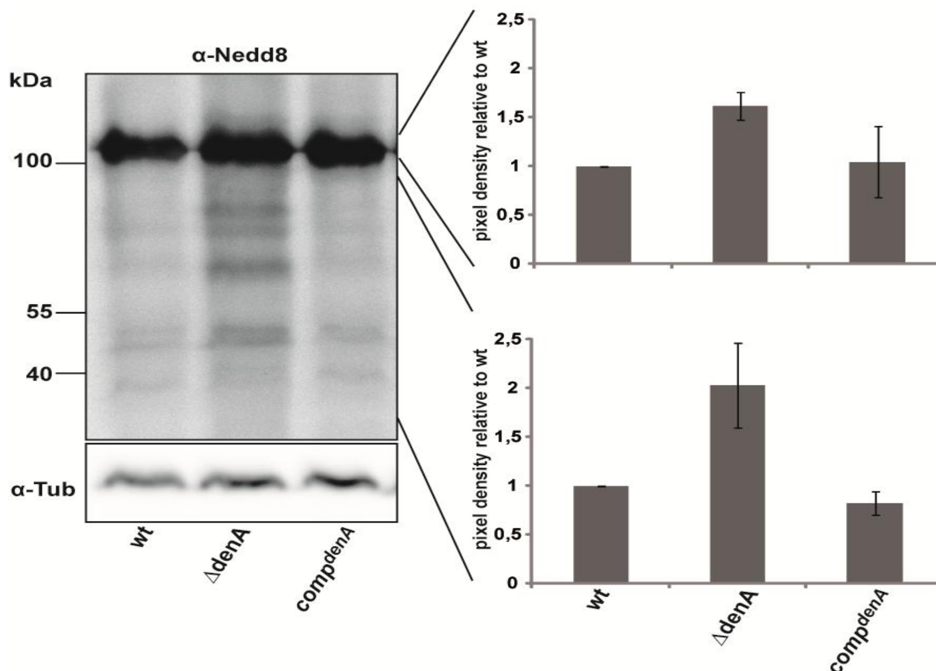

Figure 23: Western hybridization detecting neddylated proteins in the Δ*denA* strain.

Deletion of *denA* (Δ*denA*) increases the number of neddylated proteins at the size of fungal cullins (prominent upper band), as well as faster migrating proteins which do not correspond to any of the fungal cullins. Signals were normalized by reprobing membranes with α-tubulin (α-Tub) and pixel densities obtained with α-Nedd8 were compared to the wild type (wt). comp^{denA}: complementation strain, kDa: kilo Dalton

In a second approach the DenA deneddylation ability was investigated by heterologous expression experiments in yeast. Previous experiments with *A. nidulans culD* expressed in *S. cerevisiae* showed that it can be modified by the yeast Nedd8 homologue Rub1 (data not shown). Expression of *lexA::culD* in *S. cerevisiae* resulted in two high migrating signals in western experiments, when membranes were probed with the Rub1 antibody (Figure 24, lane 1 and 2, red arrows). Two bands were observed because the LexA::CulD fusion might be cleaved unspecifically, generating two species of Rub1 modified *A. niudulans* CulD. Lower migrating signals corresponded to the Rub1 modified forms of yeast cullins. To avoid deneddylation by the intrinsic yeast protease Csn5/Rri1 (Wee et al, 2002) experiments were performed in the corresponding deletion strain. As seen in Figure 24, lanes 3 and 4, expression of *A. nidulans denA* in a yeast strain expressing *lexA::culD*, efficiently removed the signals corresponding to neddylated

LexA::CulD, or CulD alone (red arrows). However, there seems to be a certain species specificity, as the yeast cullins were not really changed in their Rub1 modification by the expression of *A. nidulans denA* (Figure 24, α-Rub1). Expression of DenA in yeast was verified by detection of the V5-tag of a DenA::V5 fusion protein (Figure 24, α-V5), similar to the earlier described yeast experiments on the Nedd8 processing activity. Altogether, the above experiments indicate that DenA is involved in the deneddylation of fungal proteins and can deneddylate *A. nidulans* CulD.

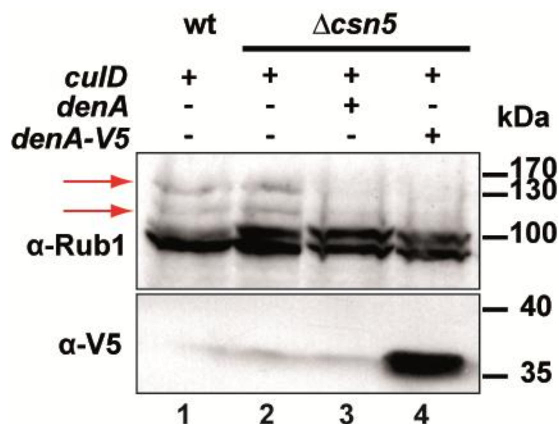

Figure 24: Heterologous expression of *A. nidulans* proteins DenA and CulD in *S. cerevisiae*.

DenA was expressed as native protein or C-terminally fused with a V5/His6 epitope tag. CulD was expressed N-terminally fused with the LexA activation domain under control of the constitutive ADH promoter. *A. nidulans* proteins were expressed in *S. cerevisiae* wild type and Δ*csnE* background. Western hybridization with antibodies against Rub1 (α-Rub1) and the V5 epitope (α-V5) were performed. kDa: kilo Dalton.

The isopeptidase activity of DenA was also tested *in vitro*. Similar to the above experiment on the processing activity, a substrate originating from human proteins was implemented to investigate this issue (kindly provided by Tilo Schmaler, AG Dubiel, Charité Berlin). It is combined of purified His::CUL1 *in vitro* modified with Nedd8. The HIS::CUL1-Nedd8 adduct was added to a reaction mix together with different amounts of recombinant GST::DenA and incubated for 30 minutes at 37°C. As illustrated in Figure 25A CUL1-Nedd8 cleavage becomes visible already at the amount of 0.2 µg GST::DenA added, representing an enzyme:substrate ratio of 1:10. Higher amounts of DenA further increase cleavage efficiency and saturation becomes visible at about 2-4 µg GST::DenA added to the reaction. Several control experiments were performed to prove that the observed

substrate cleavage is really due to DenA mediated isopeptidase activity. A mutant GST::DenA ($DenA^{mut}$) was combined with the CUL1-Nedd8 substrate to see whether the conserved cysteine protease motif is required for cleavage. The DenA mutant had the histidine 123 of the catalytic triad changed to an alanine ($H^{123}A$). It contained a further mutation where an asparagine was changed to a serine ($N^{50}S$). Addition of the purified mutant version of DenA to the reaction mixture with CUL1-Nedd8 did not result in efficient cleavage of the substrate (Figure 25B, $DenA^{mut}$), indicating that DenA activity requires the functional cysteine protease motif. Furthermore, solely purified GST was tested to exclude activity of artificially purified *E. coli* proteases. The corresponding extract showed no significant cleavage of the CUL1-Nedd8 adduct, similar to the negative control without any protein added (Figure 25 B, H_2O and GST).

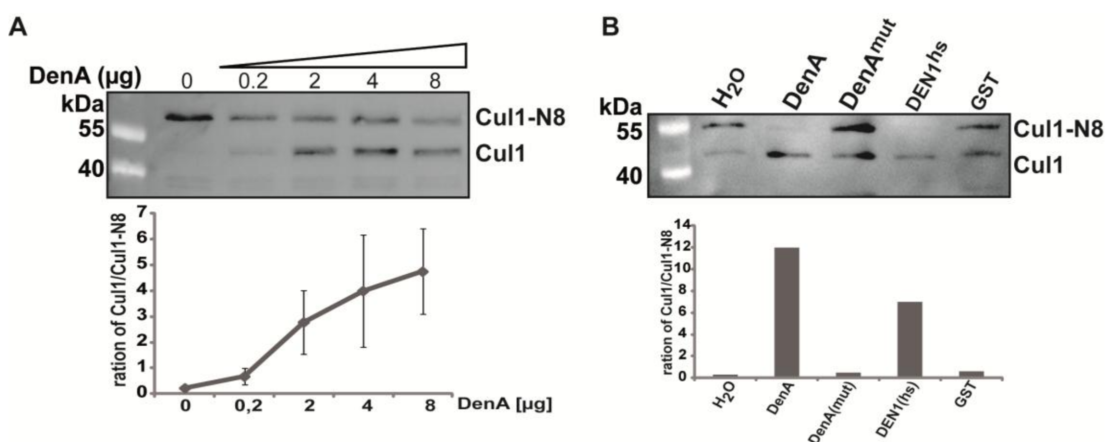

Figure 25: *In vitro* assay testing deneddylase activity of recombinant DenA.

A Recombinant GST-DenA was incubated with neddylated CUL1 (Cul1-N8). **B** Control experiments with water (0), GST-DenA, mutant GST-DenA ($DenA^{mut}$), human DEN1 ($DEN1^{hs}$) and GST-tag only. Cleaved/uncleaved (CUL1/CUL1-N8) ratios were calculated from pixel density values obtained by α-5xHIS western hybridization. kDa: kilo Dalton.

Thus, the *in vitro* activity tests using recombinant GST::DenA purified from *E. coli* and *in vivo* results support the hypothesis that the observed *denA* phenotypes rely on a deneddylase function of DenA in *A. nidulans*. An additional processing activity as reported for the dual functional protease orthologs in mammals (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003) or *S. pombe* (Zhou & Watts, 2005), is, if at all, only of minor or redundant relevance.

3.2 The fungal deneddylases CSN and DenA are interconnected

3.2.1 A *denA/csnE* double knock-out strain is viable and shows an additive phenotype

Deletion of the second major deneddylase in *A. nidulans*, *csnE*, causes a block in sexual development and a pleiotropic phenotype with red colored hyphae, whereas conidiation is hardly affected (Busch et al, 2003). To investigate whether the depletion for both deneddylases CSN and DenA is essential, a *csnE/denA* double knock-out strain was generated.

The corresponding Δ*csnE*/Δ*denA* double deletion strain is viable, but lost almost every developmental potential. Vegetative cultures grew at a poor growth rate in contrast to the wild type strain, reminiscent to the *denA* single deletion (data not shown). Referring to Figure 26 it can be seen that the double deletion strain produced hardly any conidiophores resulting in a reduction of conidiospores to less than 5% that of wild type, reminiscent to the Δ*denA* strain.

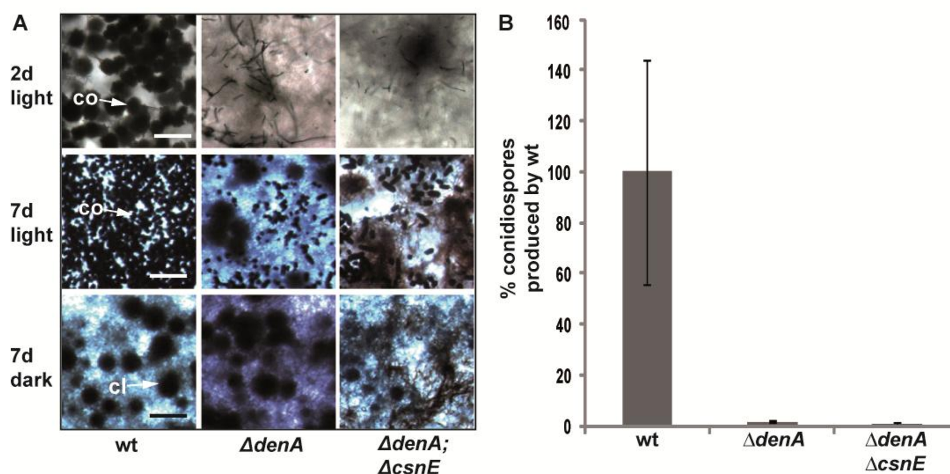

Figure 26: The *denA/csnE* double deletion strain is impaired in the formation of all forms of developmental structures.

A Surface pictures of asexually (light) and sexually (dark) induced plate cultures of wild type (wt), *denA* deletion (Δ*denA*) and *denA/csnE* double deletion (Δ*denA*/Δ*csnE*). Strains were incubated for indicated times at 30°C (scale bar = 50 µm, first row (2d); scale bar = 225 µm, second and third row (7d)). **B** Quantification of conidiospores produced by the mutant strains under normal asexual conditions compared to wild type.

Sexual development was no more observed in the double mutant, neither in the light, nor in the dark (Figure 26A). This phenotype was even more pronounced than that of the *csnE* deletion strain which is able to start the sexual cycle, but cannot

proceed beyond primordia formation (Braus et al, 2010). Additionally, the Δ*denA*/Δ*csnE* showed the same intense red color observed for the *csnE* deletion strain (Figure 26A).

To investigate the effect on the molecular level western hybridization experiments were performed. Similar amounts of crude extracts from wild type, Δ*denA*, Δ*csnE*, Δ*csnA* and Δ*denA*/Δ*csnE* were separated by SDS-PAGE, blotted on a nitrocellulose membrane and probed with a Nedd8 antibody. The *csnA* deletion strain was included in this experiment, to show that CSN dependent deneddylation requires complex formation and does not depend on *csnE* only. As shown in Figure 27 neddylated proteins were increased in the double mutant compared to the wild type or the single deletion strains. In particular, the protein signal corresponding to the neddylated form of the fungal cullins drastically increased in the double deletion mutant (Figure 27A graph). Signal intensity was already doubled in the Δ*csnE*, as well as in the Δ*denA* strain compared to the wild type, but increased about 8 fold upon deletion of both deneddylases.

Figure 27 further shows that the *denA/csnE* double knock-out strain accumulated an additional band, of yet unknown origin.

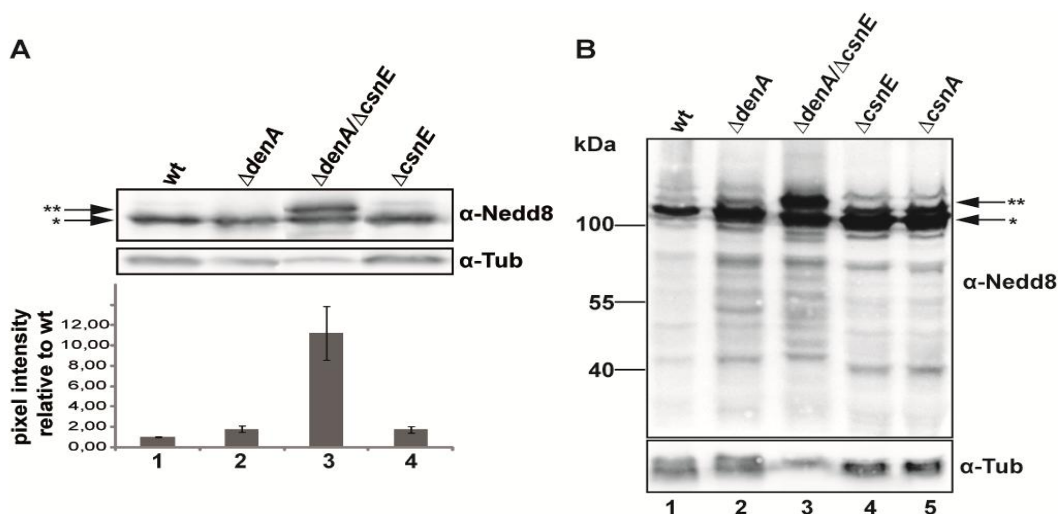

Figure 27: Neddylated proteins are increased in the *denA/csnE* double mutant.

A Signal accumulation at the size of fungal cullins (*) is observed. Additionally a slower migrating band occurs (lane 3, **) which is not observed in the wild type (wt, lane 1) or the single deletion mutants (Δ*denA*, lane 2; Δ*csnE*, lane 4). Signals were normalized by reprobing membranes with α-tubulin (α-Tub) and pixel densities obtained with α-Nedd8 were compared to the wild type (wt). **B** Additional membrane, also displaying the increase in neddylation of proteins smaller than 100 kDa in the Δ*denA*/Δ*csnE* double deletion (3) strain and in the *denA* deletion (2), which is not observed in Δ*csn* strains (4, 5; Δ*csnE*; Δ*csnA*). kDa: kilo Dalton.

It is about 10 kDa bigger than the one described above and was not detectable in wild type or any of the single deletion strains. Additionally the double deletion strain showed the increase of diffuse signals beneath 100 kDa, already observed for the *denA* deletion (Figure 27 B). These genetic and molecular results suggest that both deneddylases might be redundant to a certain extent. However, there seem to be distinct functions for each protein, which cannot be addressed by the other one, explaining the different effects on fungal development in the *denA* and *csn* single deletion mutants.

3.2.2 DenA interacts with the seventh CSN subunit CsnG *in vivo*

The results described in the previous chapter strongly suggest that the COP9-signalosome and DenA in *A. nidulans* are connected. We were wondering if this genetic interaction can be seen on a physical level. Yeast-2-hybrid tests were conducted to investigate whether *denA* recognizes any of the eight single *csn* subunits. All plasmids were transformed into *S. cerevisiae* and protein-protein interactions were determined using leucine prototrophy and ß-galactosidase activity based reporter systems (Golemis et al, 1999). As depicted in Figure 28, both reporter systems indicated a strong interaction of DenA and the seventh CSN subunit CsnG. CsnA, CsnE and CsnF also displayed a weak interaction with DenA in the growth test, but not in the ß-galactosidase activity test.

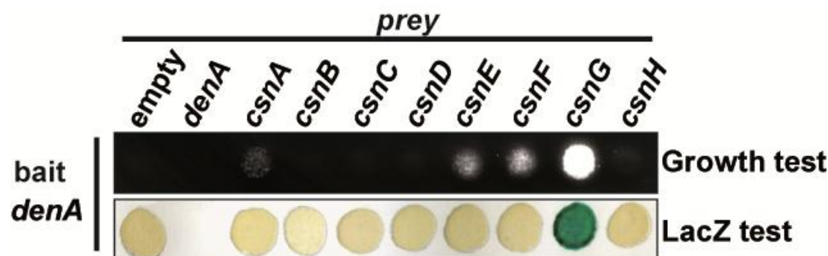

Figure 28: Yeast-2-hybrid interaction trap testing DenA versus single CSN subunits. Growth test and β-galactosidase activity test (LacZ) were performed for all eight CSN subunits vs. DenA. DenA self interaction was tested; the prey vector with the activation domain only served as negative control (empty).

The prominent interaction of DenA and CsnG observed in the yeast-2-hybrid tests was investigated in more detail applying the split-YFP technique in a bi-molecular fluorescence complementation (BiFC) experiment. A plasmid was constructed, containing the N-terminal part of YFP, N-terminally fused to DenA

(nYFP::DenA) and the C-terminal half of YFP fused to the N-terminus of CsnG (cYFP::CsnG). Both fusion constructs were expressed from the bi-directional, inducible nitrate promoter. The plasmid containing the corresponding constructs was ectopically introduced into the *denA* deletion strain. The resulting strain (Δ*denA*; *nyfp::denA/cyfp::csnG*) looked like the wild type, indicating that the ectopically integrated *nyfp::denA* fusion is functional (data not shown). As control for microscopy, an additional BiFC strain was constructed through ectopic integration of a plasmid which is reminiscent to the one above, except that the C-terminal half of YFP is expressed on its own (cYFP) and not fused to any other sequence. Both strains were grown similarly and subjected to fluorescence microscopy. The test strain (Δ*denA*; *nyfp::denA/cyfp::csnG*) expressing both fusion proteins showed an YFP signal in comparison to the control strain (Δ*denA*; *nyfp::denA/cyfp*) (Figure 29B). The YFP signal accumulates at the site of DAPI staining, indicating that the two proteins interact inside the nucleus (Figure 29B). This shows that DenA interacts with CsnG *in vivo* in *A. nidulans* and supports the yeast-2-hybrid results (Figure 28).

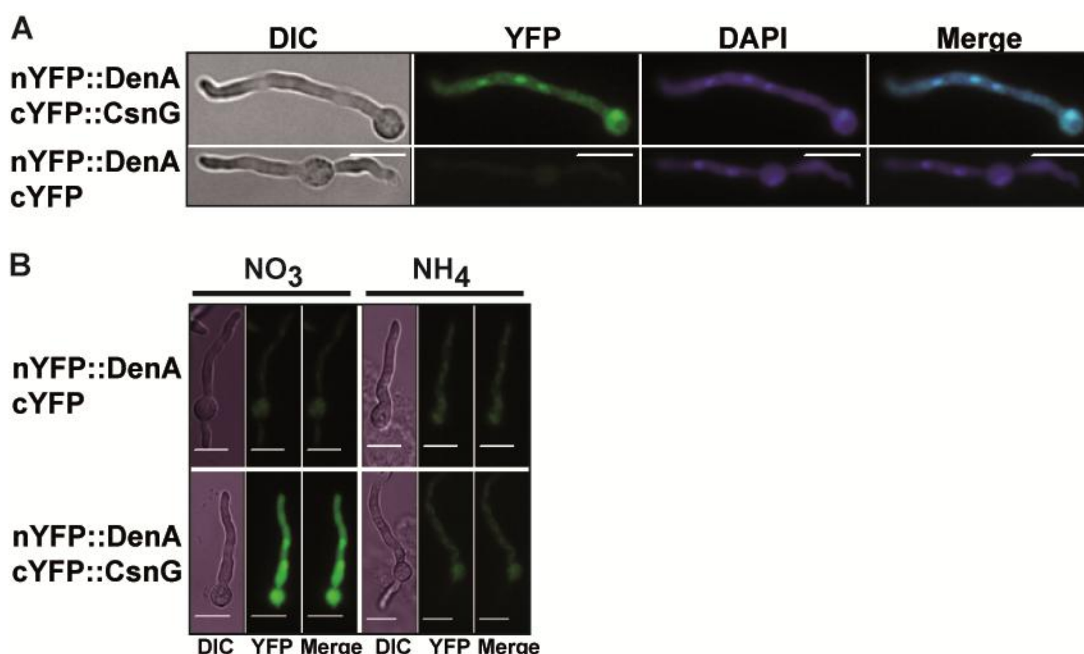

Figure 29: Interaction of DenA and CsnG (nYFP::DenA/cYFP::CsnG) in BiFC experiments.

A The fluorescence signal showed a nuclear localization for the interacting proteins. Nuclei were visualized with DAPI; scale bars = 10µm. The control strain (nYFP::DenA/cYFP) shows no fluorescence signal. **B** Induced (NO_3) and repressed (NH_4) conditions for the control (nYFP::DenA/cYFP) and the test strain (nYFP::DenA/cYFP::CsnG). Scale bar = 10µm.

Figure 29B shows an additional control experiment were both BiFC strains were grown under repressing (NH_4; ammonium-tartrate) or inducing (NO_3; sodium-nitrate) conditions. Neither the control strain (Δ*denA*; *nyfp*::*denA/cyfp*), nor the test strain (Δ*denA*; *nyfp*::*denA/cyfp*::*csnG*) showed any prominent YFP signal under repressing conditions, indicating that the observed signal is due to the expression of both fusion proteins (Figure 29B).

To examine whether the observed interaction with the CSN has any consequences on DenA stability during development, or on sub-cellular localization we created a DenA::GFP strain deleted for *csnA*. The deletion of *csnA* presumably abolishes formation of the CSN complex (Busch et al, 2007) and therefore CSN dependent functions on DenA should be absent in a Δ*csnA* background. Western hybridization experiments along different developmental time points looked similar to control strains, suggesting that *csn* depletion is not affecting DenA protein stability in a detectable way. Also fluorescence microscopy pictures looked similar to DenA::GFP localization in the wild type background (data not shown).

Altogether these data indicate a physical connection of DenA and the CSN in *A. nidulans*. However, the exact binding surfaces, as well as the consequences of this interaction for DenA and the CSN complex are currently speculative and need to be investigated in more detail.

3.2.3 Deletion of *csnG* causes the CSN phenotype

Since CsnG seems to be the main counterpart of DenA for interaction with the fungal COP9-signalosome we were wondering what the corresponding deletion mutant would look like. The *A. nidulans* open reading frame AN3623, encoding *csnG*, was already characterized in 1995 (Lewis & Champe, 1995) named *acoB*, for aconidial gene B in that study. The corresponding deletion strain was thermo-sensitive with regard to asexual and sexual sporulation, which were both abolished at the restrictive temperature of 42°C. This was accompanied by the accumulation of aberrant secondary metabolites (Butnick et al, 1984; Lewis & Champe, 1995).

We generated our own deletion mutant by transformation of AGB152 (Busch et al, 2003) with a cassette combined of the AN3623 flanking regions edging the pyrithiamin resistance cassette (Kubodera et al, 2000). The resulting *csnG* deletion mutant was blocked early in sexual fruit body formation and accumulated a red pigment, indicating an aberrant secondary metabolism (Figure 30), but still produced

conidiospores at 30°C and 37°C (Figure 30). The observed deletion phenotypes were rescued by re-introduction of a genomic copy of *csnG* into the Δ*csnG* strain (data not shown).

This is similar to the observations from previous studies on CsnG/AcoB (AN3623) (Butnick et al, 1984; Lewis & Champe, 1995) and other CSN deficient mutants (Busch et al, 2003; Busch et al, 2007; Nahlik et al, 2010). As seen for other *csn* mutant strains conidiation is hardly affected in the Δ*csnG* strain (Figure 30B) under the investigated circumstances. This suggests, that the *denA* function on asexual spore formation does not require *csnG* and the observed interaction might have other implications for fungal development.

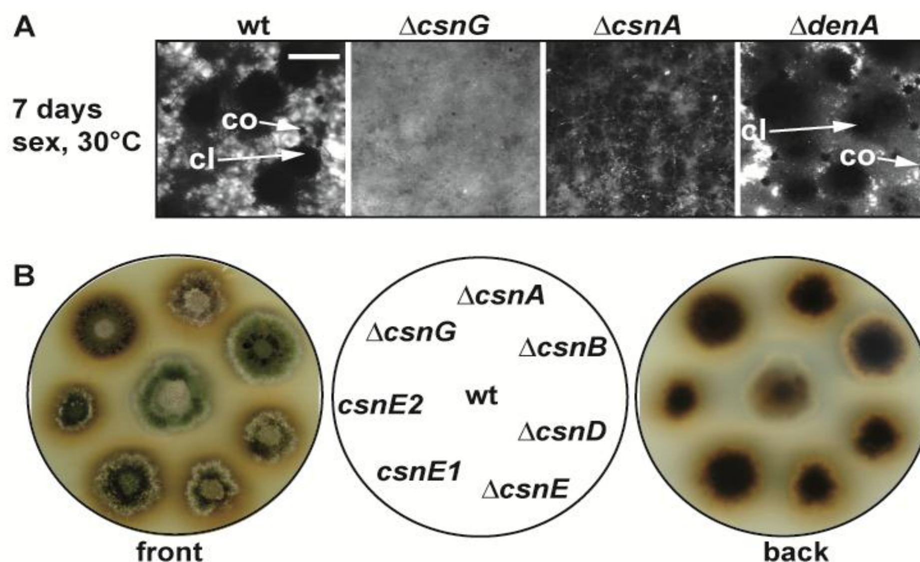

Figure 30: Phenotypic evaluation of a *csnG* deficient *A. nidulans* mutant.

A Surface pictures of sexually induced plates of wild type (wt), *csnG* deletion (Δ*csnG*), *csnA* deletion (Δ*csnA*) and *denA* deletion (Δ*denA*) (scale bar = 225 µm; cl: cleistothecium; co: conidiophore). **B** CSN deletion mutants (Δ*csnA*; Δ*csnB*; Δ*csnD*; Δ*csnE*; Δ*csnG*) and enzymatic inactive *csnE* mutants (*csnE1*; *csnE2*) were point inoculated on agar plates (middle picture, distribution on plate) and grown under asexual conditions for 2 days. All produced conidiospores (seen on front, left hand picture), but showed aberrant red color (back, right hand picture) compared to wild type (wt).

3.2.4 DenA/CSN interaction seems to be transient and not stable in co-purification studies under the observed conditions

Physical protein-protein interactions of DenA with other fungal proteins were further investigated by using the tandem affinity purification (TAP)-tag and GFP-Trap® approaches with DenA as bait. DenA was fused with a C-terminal TAP-tag, optimized for *A. nidulans* (Bayram et al, 2008b; Busch et al, 2007; Helmstaedt et al,

2008). The fusion construct under control of the native *denA* promoter was integrated at the endogenous *denA* locus. A scaled-up enrichment was performed for the *denA::tap* strain and the wild type control (FGSC A4). Similar to the TAP, purifications using the GFP-Trap® technique were accomplished. Strains carrying DenA::GFP or GFP::DenA, already used in western experiments and localization studies (chapter 3.1.2 and 3.1.3), were used. The final protein eluates achieved by either purification method were separated on a SDS gradient gel. Protein bands from coomassie stained gels were sliced out and containing proteins were tryptically digested for LC-MS analysis. Samples from *tap::denA* (Table VI), *gfp::denA* or *denA::gfp* (Table VII) strains shared some identified proteins (marked with bold letters in Tables VI and VII), but also contained unique interacting proteins.

Table VI: LC-MS identifications from purifications with C-terminally TAP tagged DenA.

Bold type indicates proteins identified in purifications described by Table VI and VII. Bold red indicates the tagged protein. Names of ortholog proteins from other species are italic. Cellular functions were deduced from the AspGD database (http://www.aspergillusgenome.org).

Acc.	Name/*S.c. homolog*	Cellular function	Mw	No. of unique peptides	Seq. Coverage	Best XCorr	Best P value
AN10352	***NOP56***	**unknown**	**56717,6**	**7**	**7,7**	**3,82**	**$2{,}33x10^{-10}$**
AN10456	**DenA**	**protease**	**29450,3**	**11**	**32,9**	**3,75**	**$8{,}15x10^{-13}$**
AN10557	***DED1***	**putative RNA helicase**	**71195,6**	**18**	**30,0**	**3,64**	**$3{,}11x10^{-08}$**
AN10598	**Anxc4**	**calcium ion binding**	**93951,2**	**11**	**18,5**	**3,36**	**$7{,}84x10^{-11}$**
AN10614	***STM1***	**unknown**	**32600,3**	**5**	**19,6**	**3,24**	**$7{,}06x10^{-11}$**
AN10946	**none**	**putative phosphatase**	**74469,8**	**11**	**21,9**	**3,10**	**$3{,}17x10^{-12}$**
AN2068	***SCP160***	**RNA binding**	**141429,4**	**5**	**4,6**	**2,89**	**$3{,}55x10^{-07}$**
AN2480	*IMH1*	unknown	130268,5	9	8,4	2,89	$9{,}00x10^{-09}$
AN2992	*SU13*	translation initiation	33769,4	7	31,3	2,95	$1{,}23x10^{-09}$
AN4861	*DST1*	translation elongation	33607,2	4	19,4	3,01	$1{,}82x10^{-09}$
AN5186	*UBP3*	ubiquitin thiolesterase	91855,4	7	18,0	2,98	$7{,}25x10^{-10}$
AN5521	AlpA	microtubule binding	96305,5	9	16,8	4,05	$9{,}09x10^{-13}$
AN6060	*TIF4632*	translation initiation; binding	162780,8	17	18,7	3,69	$2{,}36x10^{-09}$
AN6125	NudE	nuclear migration	64601,4	5	11,1	2,72	$5{,}69x10^{-08}$
AN7496	*(NF-H)$_{Rn}$*	unknown	140953,8	7	10,2	4,02	$7{,}68x10^{-07}$
AN8268	*NTF2*	nucleotide binding; transport	55565,6	5	12,0	4,39	$5{,}69x10^{-10}$

$_{Rn}$ *Rattus norvegicus*

Table VII: LC-MS identifications from purifications with GFP tagged DenA.

Bold type indicates proteins identified in purifications described by Table VI and VII. Bold red indicates the tagged protein. Names of ortholog proteins from other species are italic. Cellular functions were deduced from the AspGD database (http://www.aspergillusgenome.org).

Acc.	Name/*S.c. homolog*	Cellular function	Mw	No. of unique peptides	Seq. Coverage	Best XCorr	Best P value
AN0134	RmtC	methyl-transferase	93965,4	16	27,6	4,11	7,87x10-^{11}
AN10352	***NOP56***	**unknown**	**56717,6**	**4**	**5,2**	**4,21**	**4,74x10^{-12}**
AN10456	**DenA**	**protease**	**29450,3**	**8**	**20,2**	**4,21**	**1,79x10^{-13}**
AN10557	***DED1***	**putative RNA helicase**	**71195,6**	**3**	**6,0**	**3,18**	**1,68x10^{-04}**
AN10598	**Anxc4**	**calcium ion binding**	**93951,2**	**5**	**8,9**	**3,10**	**6,14x10^{-10}**
AN10614	***STM1***	**unknown**	**32600,3**	**5**	**26,0**	**3,88**	**1,20x10^{-10}**
AN10946	**none**	**putative phosphatase**	**74469,8**	**3**	**7,2**	**2,95**	**3,15x10^{-08}**
AN2068	***SCP160***	**RNA binding**	**141429,4**	**5**	**5,1**	**3,44**	**2,15x10^{-05}**
AN2968	IppA	diphosphatase; Mg-binding	32341,5	2	18,5	3,10	2,09x10^{-09}
AN4464	PurH	cyclo-hydrolase	65021,6	7	25,7	4,49	2,53x10^{-12}
AN5999	*CPA2*	carbamoyl-phosphate synthase	128786,6	5	9,0	3,38	3,77x10^{-08}
AN7742	*RIM1*	DNA binding	16869,4	4	45,2	4,57	2,04x10^{-11}
AN7894	none	unknown	12555,4	5	35,5	3,58	1,45x10^{-09}
AN7895	CipB	oxidoreductase; zinc binding	37169,2	8	32,8	5,76	1,00x10^{-30}
AN8862	*MYO2*	ATP-binding	178970,6	8	10,6	3,74	9,26x10^{-09}

Proteins which appeared in TAP and GFP Trap® samples in a DenA dependent manner, were often related to translation initiation like AN10557, which is a predicted relative of the yeast DEAD-box RNA helicase DED1 (Noueiry et al, 2000).

Furthermore, the predicted STM1 (Nelson et al, 2000) ortholog AN10614, and AN2068, which is similar to the RNA-binding G-protein effector SCP160 (Wintersberger et al, 1995) belong to this group. AN5186 encodes a putative ubiquitin protease similar to yeast UBP3 (Baker et al, 1992), which is reported to interact with SCP160 (Ossareh-Nazari et al, 2010). Also AN10352, a homolog of budding yeast NOP56 (Gautier et al, 1997), is involved in translation by its predicted role in rRNA processing. Further candidates related to translation were identified only with TAP tagged DenA. Here AN2992 has to be mentioned, whose yeast ortholog SU13 localizes to eIF2 (Laurino et al, 1999), or AN6060, a predicted

ortholog of the yeast translation initiation factor eIF4G (Goyer et al, 1993), and AN4861 which is related to *S. cerevisiae* DST1, a general transcription elongation factor (Ubukata et al, 2003).

AN10598 again showed up in TAP-tag and GFP-TRAP® derived samples encoding a putative ortholog of the *A. fumigatus* annexin-like protein ANXC4 (Khalaj et al, 2004). The same holds true for the putative, fungal specific Ser/Thr phosphatase encoded by AN10946. This protein found with both DenA fusion constructs contains a domain of unknown function (DUF2433; pfam10630) and a metallophos_2 motif (pfam12850) and is not described so far.

Some proteins co-purified either with TAP tagged DenA, or GFP fused DenA are related by their predicted metabolic functions or their putative role for GTP dependent cellular transport. The first group includes AN4464 encoding the ADE17 ortholog PurH (Sato et al, 2009), a putative bi-functional enzyme involved in purine metabolism, the CPA2 (Lusty et al, 1983) ortholog AN5999, which has a predicted enzymatic function during arginine or pyrimidine metabolism, the putative oxidoreductase CipB (AN7895) (Malavazi et al, 2006) and the predicted ortholog of yeast inorganic pyrophosphatase IPP1 (Kolakowski et al, 1988), IppA, which is encoded by AN2968 (Pusztahelyi et al, 2011).

The latter group contains AN8862 which encodes a predicted myosin (Harris et al, 2009), found in GFP-Trap®, the ortholog of *S. cerevisiae* IMH1 (AN2480) a protein involved in vesicular transport in yeast (Setty et al, 2003) and AN8268 whose yeast ortholog NTF2 is required for coordinated nucleocytoplasmic transport (Corbett & Silver, 1996), found in TAP-tag samples only. Also the nuclear distribution deficient gene E (*nudE*) product of AN6125 (Efimov & Morris, 2000) and the microtubule stabilizing, plus end-binding protein AlpA (AN5521) (Enke et al, 2007), as well as AN7496 encoding a protein that revealed similarities to the neurofilament triplet H protein (NF-H) of rats in BlastP search, are found in TAP samples.

Another interesting candidate for DenA interaction was identified in the GFP-Trap® samples, which was the histone arginine methyltransferase RmtC (AN0134) (Trojer et al, 2004). Further candidates obtained by GFP-Trap® purification were AN7742 encoding an ortholog of the yeast single-stranded DNA binding protein RIM1 (Li et al, 1998) and the small fungal specific protein AN7894, which is not characterized so far.

The only candidate somehow directly related to ubiquitin or ubiquitin-like modifications, co-purified along with DenA, was the UBP3 ortholog AN5186. The predicted protein contains a conserved UCH (ubiquitin C-terminal hydrolase) motif and the human ortholog USP10 regulates stability and localization of p53 (Yuan et al, 2010). Interestingly, none of the above proteins or any of their orthologs was described to be modified by Nedd8 or another ubiquitin-like protein. A peptide pattern search against the *A. nidulans* genome database (Galagan et al, 2005) using the PatMatch tool on AspGD (http://www.aspergillusgenome.org/cgi-bin/PATMATCH/nph-patmatch) with a neddylation consensus sequence "[ILHP][VITS][EQSVR][ISHT][MLV]K[MASEQ][RHEK]" obtained from previous studies (Mikus & Zundel, 2005) revealed about 20 hits, including all three fungal cullins, but none of the above proteins, co-purified with DenA (data not shown).

Unexpectedly, no CSN subunit was co-purified with DenA. Also CsnG which interacted in yeast-2-hybrid and *in vivo* in *A. nidulans*, as shown with BiFC, was not identified by MS. This suggests that the interaction with CSN subunits and Nedd8 observed in yeast-2-hybrid and BiFC occurs as transient connection in the cell. It is presumably not corresponding to the formation of stable complexes between the interaction partners or the investigated conditions were not that where this complex is formed. The DenA interacting proteins identified by co-immunoprecipitation and subsequent MS analysis might therefore be parts of higher ordered complexes containing also the CSN, CRL or neddylated proteins. They might also be neddylated proteins with an unusual neddylation site directly targeted by DenA. Furthermore identified proteins might be regulators of DenA or are co-precipitated with translating DenA still associated with parts of the translation machinery. Further studies are required to investigate reasons and consequences of these interactions.

3.3 Dynamics of CSN complex formation in *A. nidulans*

3.3.1 The CSN holo-complex is required for deneddylase activity

The filamentous ascomycete *A. nidulans* possesses a COP9-signalosome composed of eight heterologous subunits, reminiscent to the situation in mammals and plants (Braus et al, 2010; Busch et al, 2007; Chamovitz, 2009). CSN function is required for fungal sexual development (Busch et al, 2003; Busch et al, 2007) and

despite that, is involved in a variety of cellular functions, e.g. secondary metabolism, hormone signaling and cell wall integrity in *A. nidulans* (Nahlik et al, 2010). The deneddylase activity harbored within the fifth subunit was shown to be crucial in fungal CSN function (Busch et al, 2007). Deletion of different subunits causes similar pleiotropic phenotypes combined with the inability to produce mature sexual fruit bodies (Busch et al, 2003; Busch et al, 2007). Not only a strain deleted for the catalytic active subunit five, but also those missing other CSN subunits accumulated neddylated protein at the size of neddylated cullins in western experiments (Figure 31).

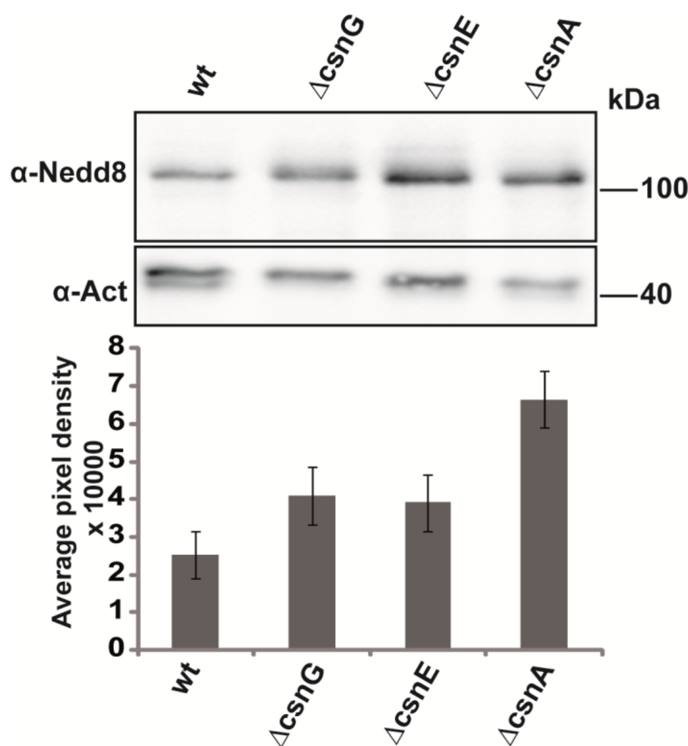

Figure 31: Immunoblot detecting Nedd8 modified proteins in *csn* deficient *A. nidulans* strains.

Deletion strains of different *csn* subunits (Δ*csnG*, Δ*csnE*, Δ*csnA*) showed a similar increase of neddylated protein. Signals were normalized by reprobing the membranes with α-actin (α-Act) and the average pixel density two independent experiments were compared to the wild type (graph). kDa: kilo Dalton.

Deletion of any of the investigated CSN subunits *csnA, csnE* or *csnG* caused signal intensification in western hybridization. These data support previous observations that not only an intact JAMM motif within the fifth subunit is required for CSN function, but also the proper formation of the CSN holo-complex (Braus et al, 2010; Busch et al, 2003; Busch et al, 2007; Nahlik et al, 2010).

3.3.2 N-terminal TAP tagged CsnA is functional and recruits six additional subunits

A lot is already known about the deneddylase function of the CSN in fungi and higher eukaryotes. Also the role and interaction of CSN associated proteins, like kinases, cullins, or de-ubiquitinating proteins is investigated in a variety of studies (refer to chapter 1.5.3 for details). But still the process of complex formation and its implications for CSN function are poorly understood.

We investigated the dynamics of COP9-signalosome assembly and the role of specific subunits for the formation of sub-complexes and the CSN holo-complex. A previous study conducted in our lab revealed that CSN subunits can recognize one another in yeast-2-hybrid and that the catalytic active, but rather small subunit CsnE can recruit all other subunits by tandem affinity purification (TAP) (Busch et al, 2007). However this is only possible in a wild type background, as soon as another subunit is missing only the tagged CsnE is recruited in TAP (Busch et al, 2007). These data lead to the hypothesis that CsnE might be the last subunit to be integrated into the CSN complex and deletion of another subunit is preventing holo-complex formation.

To test this the fungal TAP tag (Bayram et al, 2008b; Busch et al, 2007; Helmstaedt et al, 2008) was fused to the N-terminus of the second largest CSN subunit CsnA and introduced at the endogenous locus. The resulting *tap::csnA* strain is phenotypically indistinguishable from the wild type (Figure 32) indicating that the fusion protein is functional. It neither showed a block in sexual fruit body development (Figure 32A), nor did it display the pleiotropic phenotype accompanied by the characteristic red pigmentation of hyphae and medium (Figure 32B) observed for Δ*csnA* and other *csn* deficient strains of *A. nidulans* (Busch et al, 2003; Busch et al, 2007; Nahlik et al, 2010).

Western hybridization on crude extracts from *tap::csnA* showed that the fusion construct is present during vegetative growth, but disappears with the onset of development. However, TAP::CsnA seems to reappear later in sexual development, but not during asexual progression of the fungus (Figure 32C). This supports the idea of a function of the CSN complex for sexual development deduced from the

observed block in fruit body formation displayed by the corresponding deletion mutants (Braus et al, 2010; Busch et al, 2007).

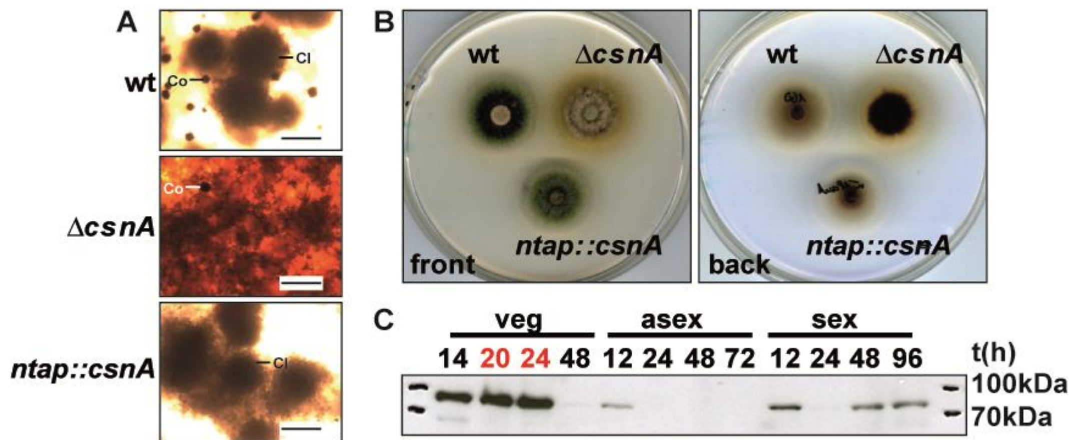

Figure 32: The upstream TAP tag fusion construct (*ntap::csnA*) of *csnA* is functional.

A Surface pictures of sexually grown plates of wild type (wt), *csnA* deletion (Δ*csnA*) and the strain restored with *ntap::csnA* (scale bar = 200 µm; Co: conidiophore; Cl: cleistothecium). **B** Front and backside pictures of plates with point inoculations of the mentioned *csnA* strains. **C** Western hybridization with α-calmodulin on similar amounts of crude extracts from the *ntap::csnA* strain grown under the indicated conditions (veg: vegetative; asex: asexual; sex: sexual).

Samples for TAP were all harvested after 22 hours of vegetative growth. In samples of three independent purifications CsnA itself (AN1491), CsnB (AN4783), CsnD (AN1539) and CsnF (AN2233) could always be identified by LC-MS. CsnG (AN3623) was detected only twice, whereas CsnC (AN5798) and CsnH (AN10208) where identified only once along the CsnA purifications. However, the fifth subunit CsnE (AN2129) was not at all recruited by the TAP-tag CsnA fusion protein (Table VIII). This supports the initial idea that all other CSN subunits had to be assembled to recruit CsnE to the complex.

Table VIII: LC-MS identifications from purifications with N-terminally TAP tagged CsnA.

Acc.	Name	Mw	No. of unique peptides	Seq. Coverage	Best XCorr	Best P value
AN1491	CsnA	55629,8	18	42,2	5,60	$6{,}03x10^{-12}$
AN4783	CsnB	58077,0	13	37,6	5,48	$4{,}44x10^{-15}$
AN5798	CsnC	55140,7	17	47,0	6,07	$2{,}02x10^{-12}$
AN1539	CsnD	44848,4	18	53,4	6,71	$2{,}08x10^{-13}$
AN2129	CsnE	-	-	-	-	-
AN2233	CsnF	42062,5	9	35,2	4,59	$8{,}34x10^{-10}$
AN3623	CsnG	35310,8	8	34,9	6,60	$6{,}78x10^{-11}$
AN10208	CsnH	23898,2	8	35,9	5,68	$5{,}22x10^{-10}$

3.3.3 Overexpressed CsnD::GFP recruits the complete set of eight subunits in GFP Trap® purification

As a further line of evidence in investigating CSN complex formation we used the previously described *alcA*^{P}*::csnD::gfp* strain AGB197 (Busch et al, 2003) for GFP mediated affinity purification (GFP-Trap®, CHROMOTEK). The *csnD::gfp* fusion is expressed from the very strong *alcA* promoter (Waring et al, 1989) leading to excess amounts of protein in the cell. The strain was grown under inducing conditions for 22 hours in vegetative culture and then subjected to the purification procedure, similar to the above strain grown for TAP. For control purposes the complete procedure was also accomplished with a strain expressing *gfp* alone (Ö. Bayram, personal communication), from the strong, constitutive *gpdA* promoter (Punt et al, 1991).

GFP alone was not capable to recruit any CSN subunit during GFP-Trap® purifications (data not shown). Contrary, the overexpressed CsnD::GFP fusion protein efficiently co-purified all other seven CSN subunits, including CsnE in two independent experiments (Table IX). This shows that a single CSN subunit is able to recruit the holo-complex. The only prominent protein aside of the CSN subunits, identified along with CsnD::GFP was AN2068, encoding the putative ortholog of *S. cerevisiae* SCP160.

Table IX: LC-MS identifications from purifications with overexpressed C-terminally GFP tagged CsnD.

Acc.	Name	Mw	No. of unique peptides	Seq. Coverage	Best Xcorr	Best P value
AN1491	CsnA	55629,8	23	52,2	4,98	$3{,}40 \times 10^{-12}$
AN4783	CsnB	58077,0	17	47,2	5,61	$1{,}00 \times 10^{-30}$
AN5798	CsnC	55140,7	19	48,6	4,52	$5{,}88 \times 10^{-14}$
AN1539	CsnD	44848,4	21	64,7	5,99	$2{,}00 \times 10^{-14}$
AN2129	CsnE	37783,3	11	35,5	4,64	$4{,}23 \times 10^{-10}$
AN2233	CsnF	42062,5	12	42,0	4,95	$5{,}93 \times 10^{-12}$
AN3623	CsnG	35310,8	9	41,9	5,29	$1{,}17 \times 10^{-12}$
AN10208	CsnH	23898,2	8	33,5	4,98	$1{,}47 \times 10^{-13}$
AN2068	*(SCP160)*	141429,4	16	15,1	4,60	$4{,}08 \times 10^{-10}$
ΔcsnG						
AN1539	CsnD	44848,4	29	73	6,52	$1{,}00 \times 10^{-30}$

AN2068 could also be purified along with DenA (Table III and IV), representing a possible link between DenA and the CSN inside of the fungal cell. Deletion of *csnG*, however lead to a complete loss of the ability of CsnD::GFP to recruit the remaining subunits in that procedure. Also the SCP160 ortholog AN2068 was not anymore detectable in MS, suggesting that it might be connected to the CSN complex, but not directly to CsnD (Table IX).

4 Discussion

In vitro studies by several groups, investigating human DEN1/NEDP1, generated evidence that the protease has two functions concerning the ubiquitin-like protein Nedd8. At first, the biochemical data obtained with purified components revealed that the protease cleaves Nedd8 adducts with much higher affinity than those with ubiquitin or SUMO, indicating deneddylase activity. Secondly, it was shown that substrates, mimicking the precursor form of the Nedd8 protein were efficiently cleaved by the Ulp1-family protease, alluding to a further processing activity of DEN1/NEDP1 towards Nedd8 (Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003). The latter function is also dedicated to the UCH-L3 protein in humans (Wada et al, 1998), or the related protein Yuh1 in yeast (Linghu et al, 2002). All results obtained during this study suggest that the fungal DEN1 homolog DenA functions predominantly as a deneddylase and Nedd8 processing activity is, if at all only of minor importance.

4.1 *A. nidulans* DenA is a deneddylase

4.1.1 *denA* deletion results in accumulation of neddylated proteins

The results obtained during this work show that the *A. nidulans* DEN1/NEP1 ortholog DenA displays isopeptidase activity and can deneddylate CulD (Figure 24). The protein displays the ability to remove Nedd8 from cullin *in vivo*, as well as *in vitro* (Figure 25). Accordingly, the *denA* deletion strain accumulated neddylated proteins in western experiments (Figure 23). Some correspond in size to Nedd8 modified cullins. In addition there are further signals that increase in the Δ*denA* strain, which are presumably due to other neddylated proteins not corresponding to any of the fungal cullins.

Similar observations have been made in the fruit fly *Drosophila melanogaster*. The corresponding *DEN1* deletion mutant accumulates a number of proteins detectable with a Nedd8 antibody, which do not correspond to cullins. Neddylation of cullins in contrast is hardly affected by *DEN1* depletion in the fly, even though *in vitro* experiments indicate the ability of the protease to remove Nedd8

from Cul1 and Cul3 (Chan et al, 2008). However, DenA was not able to recruit any of the three fungal cullins in co-purification experiments. Also none of the proteins identified along with DenA contained a conserved neddylation consensus sequence (Table VI and VII). One explanation might be that adjacent to the cullins only few proteins are described to be neddylated and already this differ significantly in the consensus sequence from that found in cullins (Mikus & Zundel, 2005). The only real prerequisite for neddylation is an accessible lysine residue within the recipient protein and this is given in almost all proteins. It might very well be that some of the identified proteins are targets of neddylation, but bear an unusual consensus sequence. On the other hand, it is possible that DenA does not get in direct or strong contact with the substrate of deneddylation and the interaction is therefore not detectable in co-purification experiments. However, DenA can recognize both Nedd8 variants in yeast-2-hybrid interaction tests (Figure 15), suggesting that it might recognize a neddylated target by Nedd8 and not by direct contact sites within the substrate protein. Furthermore DenA might be incorporated into some kind of higher ordered complexes mediating between substrate and deneddylase. The de-ubiquitinating enzyme USP15 in mammals for example is reported to associate with the CSN to stabilize the APC-(adenomatous polyposis coli) complex (Huang et al, 2009). Furthermore, also CRLs are known to be recruited to the COP9- signalosome complex what promotes their *in vivo* activity and protects them from cellular depletion (Wee et al, 2005; Wu et al, 2006).

4.1.2 DenA deneddylates CulD in a yeast model

The deneddylase activity of *A. nidulans* DenA was also demonstrated by heterologous co-expression in yeast (Figure 24). In this experiment neddylation of the fungal CulD was efficiently reversed by the expression of DenA. However, the results indicate that some differences among species exist, as the yeast cullins were hardly affected in their Nedd8 modification by expression of *A. nidulans* DenA. This might be due to the fact, that *S. cerevisiae* does not contain any ortholog of DenA (DEN1/NEDP1) and thus relies only on the CSN5 ortholog Rri1 for deneddylation (Wee et al, 2002). One assumption is that the required mechanism cannot adapt a deneddylase of the kind of DenA for deneddylation of endogenous yeast proteins.

4.1.3 DenA deneddylates CUL1 *in vitro*

Another line of evidence for DenA deneddylase activity is given by *in vitro* experiments clearly resulting in cleavage of an artificial CUL1-Nedd8 adduct, by recombinant GST-DenA (Figure 25). The *in vitro* studies further indicate that the observed deneddylase activity is really due to cysteine protease activity. A mutant version, with the histidine of the catalytic triad exchanged for an arginine ($H^{123}A$), occurred to be inactive (Figure 25B). The imidazole ring of histidine is known to be a potent proton exchange factor in active sites of enzymes and it is likely that the change to a different residue sufficiently changes the properties of the active site to abolish protein activity.

4.1.4 DenA is no efficient processing enzyme for linear Nedd8 adducts

DEN1/NEDP1 from humans, DEN1 from the fly, as well as NEP1 and NEP2, the two ortholog isoforms of DEN1 in *S. pombe*, all display a processing function for the Nedd8 precursor, additionally to their deneddylase function (Chan et al, 2008; Gan-Erdene et al, 2003; Mendoza et al, 2003; Wu et al, 2003; Zhou & Watts, 2005). The experiments aiming to show this dual functionality for *A. nidulans* DenA did not corroborate these findings for the fungal protein, neither *in vivo* nor *in vitro*. DenA cannot rescue the deletion of the processing enzyme *yuh1* in yeast (Linghu et al, 2002), when expressed in the corresponding mutant background (Figure 21). The reason for this is most probably the poor relationship between the two proteins, which both belong to different clans within the family of cysteine peptidases (Barrett & Rawlings, 2001). As illustrated in Figure 33 this results in miscellaneous secondary structures and different organization of the catalytic center. Therefore it can be explained why one protein cannot fulfill the function of the other.

But also experiments in *A. nidulans* revealed no evidence for a processing function. The *denA* deletion mutant was combined with a strain containing a Nedd8 variant that is already translated without the C-terminal extension and thus did not require processing. However, this was not sufficient to even partially rescue the *denA* deletion phenotype (Figure 20), clearly indicating, that Nedd8 processing is no, or at least not the predominant function of DenA *in vivo*.

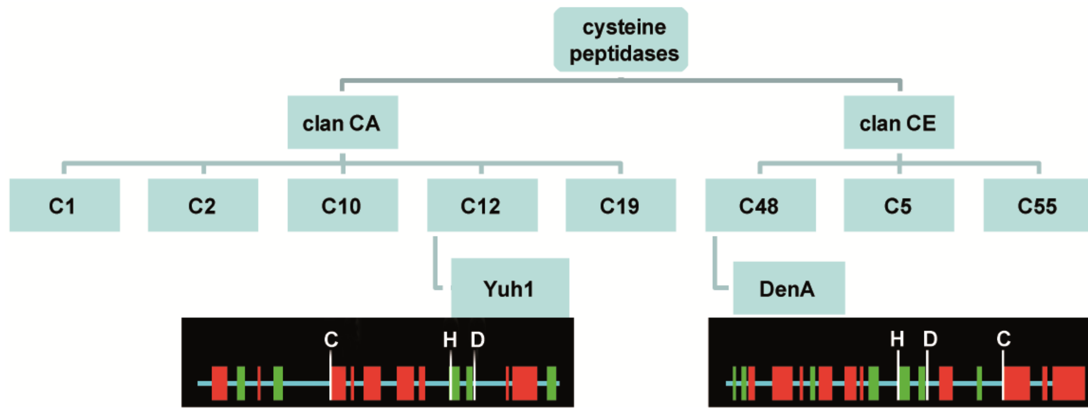

Figure 33: Internal relationships of cysteine proteases (Rawlings et al, 2006).

The family of cysteine proteases contains 7 clans, of which the two relevant ones are depicted in the scheme. Secondary structures and organization of the active site residues cysteine (C), histidine (H) and asparagine (D) are shown (N- to C-terminal end). α-helices are shown in red, β-sheets are green and coiled-coil regions are blue.

In vitro experiments further support these findings, as recombinant DenA, which displayed efficient deneddylase activity (Figure 25), was unable to cleave a linear substrate composed of Nedd8 and GFP, mimicking unprocessed Nedd8 (Figure22). It is, even though unlikely, possible that the processing activity requires some co-factor that needs to be identified. Furthermore the C-terminal extension of premature Nedd8 differs between organisms, what could account for the inability of DenA to recognize and process the yeast ortholog or the *in vitro* substrate, which originated from human Nedd8 (Figure 34).

Figure 34: Sequence alignment of Nedd8 peptides containing the C-terminal extension.

Amino acid sequences from *Homo sapiens* (hs, NP_006147), *Mus musculus* (ms, NP_032709), *A. nidulans* (an, XM_658691), *A. fumigatus* (af, XM_750058) and *S. cerevisiae* (sc, NP_010423) were aligned. Red arrows indicate conserved internal lysine (K) residues. The different C-terminal amino acid extensions of the precursor forms of Nedd8 are indicated by a black box. High consensus residues (>90%) are highlighted in red and low consensus (>50%) in blue.

However, since expression of an artificially mature Nedd8 in *A. nidulans* was not able to rescue the *denA* deletion phenotype, it can be assumed that other enzymes are the main Nedd8 processing proteins in *A. nidulans*. Orthologs of UCH-L3 (Yuh1) are existing in the fungal genome (Galagan et al, 2005), but are poorly investigated

yet. Furthermore DenA interacted with the UBP3/USP10 ortholog of *A. nidulans* in TAP experiments (Table VI), which also belongs to the family of UCHs. Preliminary experiments on the fungal ortholog of the de-ubiquitinating protein USP15, UspO, indicate that this might also be involved in the regulation of fungal development (J. Schinke, unpublished data). Furthermore, the fungal genome (Galagan et al, 2005) encodes at least two additional members of the C48 clan of cysteine proteases. UlpA and UlpB are supposed to be specific for the fungal ortholog of SUMO, acting as isopeptidases on SumO modified proteins and/or as processing enzymes for the precursor form of fungal SumO. Preliminary results indicate that DenA and UlpA might have redundant functions, as *denA* overexpression is able to, at least partially, rescue the defects of a *ulpA* deletion mutant (R. Harting, unpublished data). This suggests that DenA might have a role in SumO modification of fungal proteins as well, or vice versa, the SumO isopeptidases are involved in Nedd8 modification. Altogether the results obtained in this study strongly indicate that DenA functions mainly as deneddylase and has only minor or redundant functions regarding Nedd8 processing (Figure 35). Whether a distinct Nedd8 processing enzyme exists in *A. nidulans* or if this is a function distributed along several redundant proteases has to be shown in future studies.

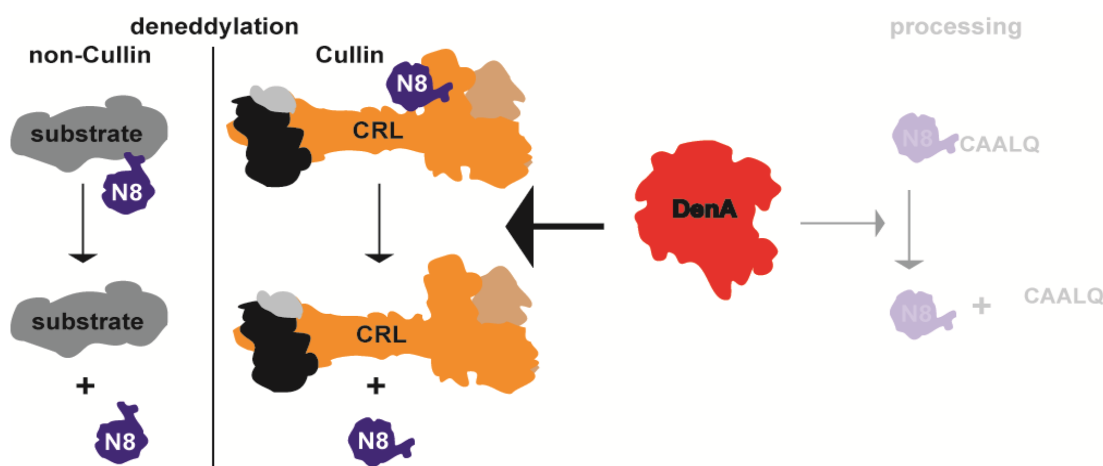

Figure 35: *A. nidulans* DenA is a deneddylase, rather than a processing enzyme for Nedd8 (N8).

The data suggest that DenA can deneddylate CRLs as well as non-cullin substrate proteins (left hand side) but did not support a Nedd8 processing function of DenA (right hand side). Silhouettes originate from structural data by PDB entry 1LDK (SCF components) and 1XT9 (Nedd8 and DEN1).

4.2 Nedd8 interaction and DenA stability require the DenA N-terminus

Yeast-2-hybrid data obtained in this work show that Nedd8 and DenA can recognize each other (Figure 15), and further indicate an important role of the DenA N-terminus for this interaction (Figure 16). This connection is already described and proven on the structural level in mammals. Hence, a couple of residues at the N-terminus of DEN1 are required to form a surface that stabilizes Nedd8 interaction (Reverter et al, 2005; Shen et al, 2005). Even though the truncated yeast-2-hybrid versions of fungal DenA only covered parts of this residues it seems to be sufficient to reduce the interaction surface to an extend that disables Nedd8 binding (Figure 36). The truncation of DenA might as well cause drastic changes to the overall structure of the protein, thereby disrupting the interaction surface required for Nedd8 association or even the overall shape of DenA preventing proper folding.

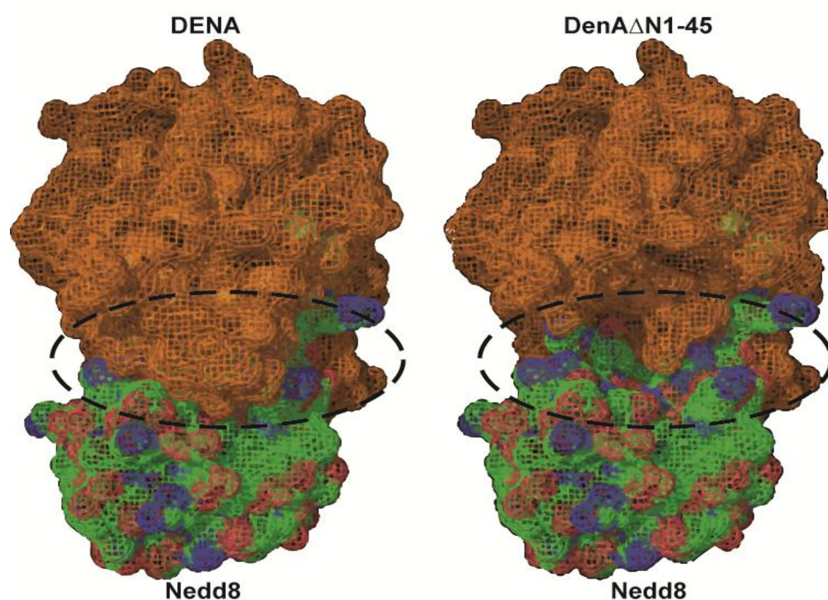

Figure 36: Structural representation for the N-terminal truncation of DEN1.

The structural model (orange) is based on PDB 1XT9. Deletion of the N-terminal 21 amino acid residues (right picture), which are shared among species (corresponds to DenAΔN1-45) reduces the interaction surface for Nedd8 (green). Charged surface regions at Nedd8 are shown in red (+) or blue (-).

Interestingly, the N-terminus of DenA additionally seems to be required for stability of the protein. Accordingly a DenA fusion protein carrying a GFP tag at the N-terminus is significantly decreased in stability compared to a C-terminal fusion construct (Figure 17). Recent studies with mammalian DEN1 support this finding and revealed a connection to the observed Nedd8 interaction. Co-transfection

experiments showed that the N-terminal Nedd8 interaction stabilizes DEN1 in cell culture. However, *in vitro* activity assays showed that this stabilization is accompanied by an inactivation of the protease (W. Dubiel, T. Schmaler, personal communication). The above results clearly indicate a function of the N-terminus for DenA stability. It is likely that this is connected with Nedd8 interaction, which occurs in this region of the protease, like it is shown in mammalian cells. A GFP molecule attached to the N-terminus of DenA might therefore interfere with Nedd8 interaction and thereby reduce stability of the protein.

However, there are additional possibilities, aside Nedd8 interaction, to control DenA stability, or even activity, which can be localized to the N-terminus. Prediction of conserved phosphorylation sites (http://www.hprd.org/ PhosphoMotif_Finder) reveals a conserved motif within the N-terminus (residues 27-33; LSYHDIR; compare also Figure 2A) for serine phosphorylation by the pyruvate dehydrogenase kinase. So far phosphorylation of DEN1 was not reported in any organism, but it cannot either be excluded yet. Therefore it is possible that phosphorylation in this place, if it occurs, affects Nedd8 interaction, thereby regulating stability and activity of the protease, but this needs to be elucidated in future studies. Consistent with the hypothesis of a possible DenA phospho-regulation the protein interacted with a putative fungal specific Ser/Thr phosphatase (AN10946) in co-purification experiments (Table VI and VII).

4.3 Deneddylation is required for coordinated fungal development

The *A. nidulans denA* deletion mutant displays dramatically reduced asexual sporulation (Figure 18), accompanied by constitutive induction of the sexual pathway, independent of illumination (Figure 19). The latter observation was also made for several fungal *csn* deletion mutants, among which is the gene of the catalytic active subunit *csnE* (CSN5). The corresponding deletion strains are shifted towards sexual development, even in the presence of light, but they are blocked at the primordial stage of fruit body formation under any conditions. Asexual sporulation is instead hardly affected in *csn* deficient *A. nidulans* strains (Braus et al, 2010; Busch et al, 2003; Busch et al, 2007).

A. nidulans development requires a switch from unicellular proliferation, to the differentiation of specialized cells and tissues. This process requires massive

reorganization of the cell on the molecular level including tight regulation of a couple of transcription factors (Adams et al, 1998; Braus, 2002; Dyer & O'Gorman, 2011). Targeted protein degradation through the ubiquitin-proteasome pathway can be assumed to be crucial mechanism involved in these processes. Proteins are either affected directly or indirectly, by modulating the stability of upstream effectors (Hershko, 1991). The crucial components in this pathway, mediating substrate specificity, are the ubiquitin E3-ligases that mark the appropriate protein for breakdown (Kerscher et al, 2006). The biggest groups are the CRLs which are regulated by cycles of neddylation and deneddylation (Figure 8). Thus deneddylation, accomplished by DenA or the CSN is a crucial process for protein degradation and the regulation of subsequent processes like development (Figure 37).

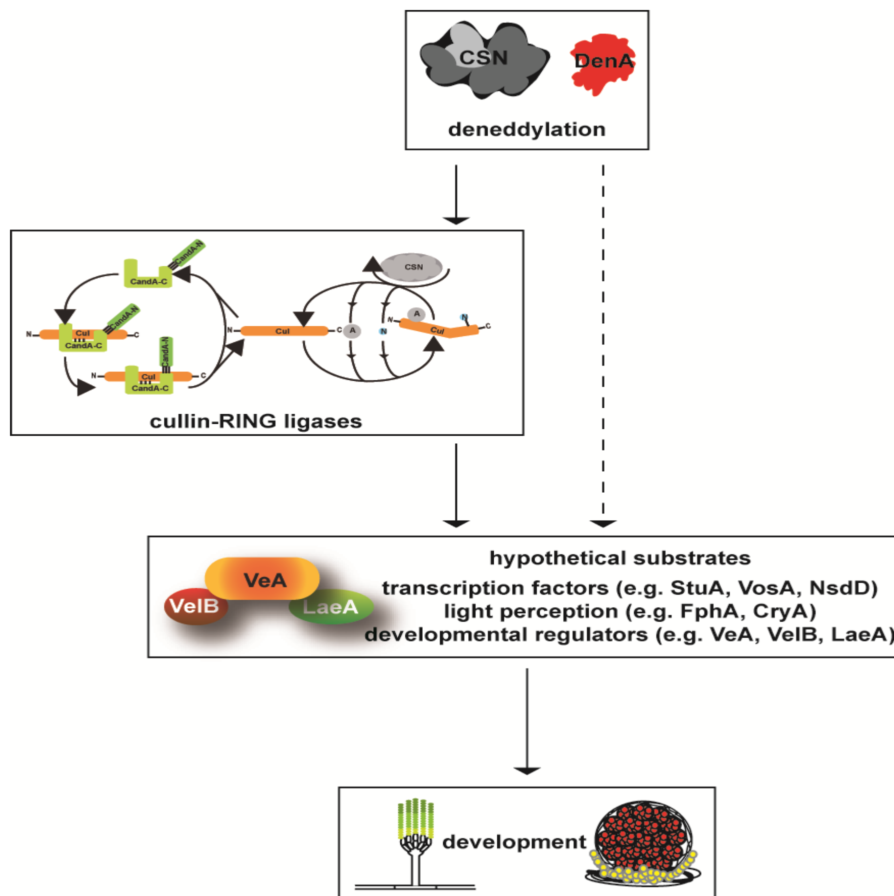

Figure 37: Hypothetic model on the influence of deneddylation on the regulation of fungal development.

DenA and CSN deneddylase activity is required to maintain CRL activity affecting stability of substrate proteins regulated by ubiquitin-dependent proteasomal degradation. Presumably (dashed arrow) there are also substrates which are directly affected by deneddylation. Misregulated stability (activity, localization) of regulatory proteins, directly or indirectly, results in the observed consequences for fungal development.

Regarding the phenotypes of deneddylation deficient mutants observed in this study and by others (Busch et al, 2003; Busch et al, 2007; Nahlik et al, 2010) the velvet complex, or an up- or downstream effector of it, is most probably affected.

Apparent resemblance is seen for the phenotypes of the *denA* deletion and that of a *laeA* deletion strain in the presence of intact VeA (Sarikaya Bayram et al, 2010). Both deletion strains are drastically reduced in conidiation and constitutively form cleistothecia, even in the presence of light. It has been shown in *N. crassa* that the regulation of methyltransferases is affected by CRL (Xu et al, 2010). Thus deneddylases might affect methyltransferase dependent processes indirectly, or directly. DenA for example could recruit the methyltransferase RmtC in co-purification experiments presented in this study (Table VII). However it is not clear whether RmtC is neddylated and therefore a substrate for DenA dependent deneddylation, or if the connection has different reasons.

Also *veA* phenotypes resemble those of deneddylation deficient mutants. Overexpression of *veA* leads to constitutive sexual development uncoupled from illumination and a reduction of conidiophores (Kim et al, 2002) reminiscent to what is seen for the *denA* deletion (Figure 18 and 19). One possible option would be that deneddylation is required to allow degradation of the cytoplasmic VeA fraction in the presence of light promoting the asexual pathway and inhibiting sexual differentiation.

Contrary, a *veA* deletion mutant is blocked in sexual development, shows an altered secondary metabolism, that manifests in red color within the hyphae, but is hardly affected in conidiation (Kim et al, 2002), what reminds one of the *csn* mutants (Braus et al, 2010; Busch et al, 2003; Busch et al, 2007; Nahlik et al, 2010). The corresponding idea would be that CSN deneddylase activity is required to control the nuclear *velvet* complex to overcome the stage of primordia formation during sexual development. One hypothesis is that the CSN mediated deneddylation activity controls the balance between the dimeric (VeA-VelB) and the trimeric (VeA-VelB-LaeA) *velvet* complex. A high ratio of trimeric/dimeric would presumably increase secondary metabolism and Hülle cell formation, but inhibit further steps in sexual development. Therefore the ratio needs to be reduced with proceeding sexual development to allow fruit body formation. Thus, when the CSN deneddylase is inactive fruit body formation is blocked and secondary metabolism is constitutively increased.

The absence of any deneddylation activity might destabilize the VeA protein or repress expression of *veA*, leading to reduced amounts of the regulatory protein. It might also prevent the translocation of VeA, e.g. by destabilizing the KapA protein that is required to import VeA into the nucleus in the dark. This would abolish the interaction with VelB and LaeA and the subsequent formation of the *velvet* complex. This complex is thought to promote sexual development and secondary metabolism in the dark (Bayram et al, 2008b).

Based on recent models (Bayram & Braus, 2011), it is conceivable that sexual development is inhibited because VeA cannot enter the nucleus and form the *velvet* complex. At the same time LaeA is transported into the nucleus independently of VeA, driving secondary metabolism, but in an abnormal way. The corresponding strain would be incapable to form any developmental structures and produce red color like the *denA/csnE* double deletion strain (Figure 26).

Additionally it is reported that the oxylipin balance is affected in deneddylase deficient mutants. It was shown that expression of the oxylipin regulator *ppoA* is controlled by mechanisms including VeA and CSN (Tsitsigiannis et al, 2004). Furthermore the CSN is reported to promote asexual sporulation, regulating oxylipin production by influencing the dioxygenases PpoA and PpoC (Nahlik et al, 2010). Thus it is likely that an imbalance of cellular deneddylation activity will cause subsequent alterations of the oxylipin level misleading fungal development as observed for both deneddylase deficient mutant strains.

4.4 Deneddylation is a prerequisite for development, but not for growth

The *denA/csnE* double deletion shows a combined phenotype of the two single deletion strains. Similar to the Δ*denA* strain, the *denA*/*csnE* double deletion strain produces hardly any conidiospores and reminiscent to the Δ*csnE* phenotype there is red color accumulating in the hyphae and sexual development is abolished (Figure 26) (Busch et al, 2003; Busch et al, 2007; Nahlik et al, 2010). Sexual development is even more severely affected in the Δ*denA*/Δ*csnE* as the strain is not only blocked at the stage of primordia, but fails already in the initiation of sexual development. This suggests that the two deneddylases have not only distinct but also common developmental functions. Thus deneddylation of the CSN seems to be specifically required after the initiation of sexual development, as depletion for *csnE*

only, causes a block of fruit body formation at the stage of primordia. However, there seems to be an earlier step in the developmental decision process requiring deneddylase activity. Here both enzymes might be equally well suited. Therefore the double deletion strain can no more enter the sexual cycle, while the *csnE* single deletion can, possibly due to DenA activity, but is than blocked at the primordial stage (Figure 38).

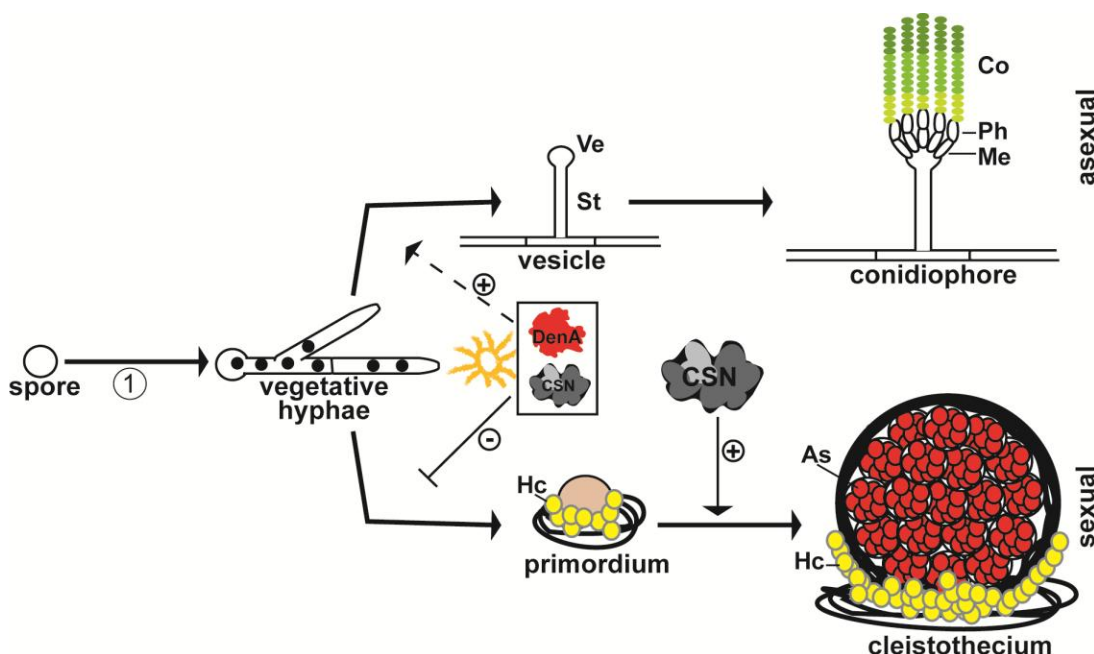

Figure 38: DenA and CSN function in fungal development.

Deneddylation plays only a minor role for vegetative growth. Deletion of *denA* results in decreased conidiation, suggesting that DenA activity is required for proper asexual spore formation in the light (+). Deletion of *csn*, as well as *denA* leads to sexual development in the presence of light, indicating a repressive function of both deneddylases on sexual development in light (-). CSN activity is further required during sexual development in the dark to proceed beyond the stage of primordia (+). Hülle cells (Hc; yellow); asci (As); metulae (Me); phialides (Ph); conidiospores (Co, green).

According to the idea that the two deneddylases are redundant to some extent, both single deletion mutants accumulate neddylated cullins in western hybridization experiments (Figure 23 and 27). Additionally the Δ*denA* strain accumulated other proteins that can be detected with a Nedd8 specific antibody, which did not correspond in size to any of the fungal cullins. This was also observed for *DEN1* mutants in the fly (Chan et al, 2008) and is maintained in the *denA*/*csnE* double deletion strain in *A. nidulans*. As non-cullin target for DEN1 deneddylation in *Drosophila* the MDM2 protein has been reported (Chan et al, 2008). In humans neddylated MDM2 is stabilized towards degradation by the UPS. DEN1 removes the

Nedd8 and accelerates MDM2 degradation concomitant with the activation of the tumor suppressor p53 (Watson et al, 2010).

The Δ*denA*/Δ*csnE* strain accumulates a further band that is not observed in any of the single deletion mutants (Figure 27), potentially due to the modification of a cullin with more than one Nedd8 molecule. Whether this corresponds to multi-neddylation or Nedd8 chain formation is not clear yet. Cullins contain more than one lysine residue and Nedd8, similar to ubiquitin, contains some conserved lysine residues that might function in Nedd8 chain formation (Figure 32, red arrows). Furthermore, the observed signal might also correspond to additional ubiquitination or sumoylation together with neddylation or formation of mixed chains of ubiquitin and/or ubiquitin-like proteins. In mammals SUMO can be attached to proteins prior to their ubiquitination for proteasomal degradation (Geoffroy & Hay, 2009). Nevertheless, the observed signal might as well correspond to some non-cullin protein which is not yet identified.

The results clearly indicate a function for DenA, as well as for CSN in cullin deneddylation and according to this in the regulation of CRLs. This is supported by the finding, that the phenotype which was recently described for CandA resembles that of the *csnE/denA* double deletion (Helmstaedt et al, 2011). CandA is the fungal homolog of CAND1 (cullin-associated Nedd8-dissociated protein 1) (Aoki et al, 1999; Siergiejuk et al, 2009; Zheng et al, 2002a). Similar to the human CAND1, CandA interaction only occurs to non-neddylated cullins and requires deneddylation as prerequisite for binding to cullin (Hwang et al, 2003; Liu et al, 2002; Zheng et al, 2002a). Absence of deneddylation activity stabilizes CRL complexes, thereby altering stability, localization or activity of downstream substrates (Wu et al, 2005).

Given that CsnE and DenA, like their orthologs in other organisms, affect various cullin-dependent and independent targets (Bosu & Kipreos, 2008; Chan et al, 2008) this might result in the observed developmental phenotypes. Just like deneddylation, association of CandA is required to facilitate adaptor exchange in cullin complexes like the SCFs. In *S. pombe* defects in CAND1 can lead to increased stability of certain types of SCF ligase complexes while the formation of others is abolished (Dubiel, 2009; Schmidt et al, 2009). This would at least partially explain why the defects in sexual differentiation and secondary metabolism of a *csnE* deletion mutant (Busch et al, 2003; Busch et al, 2007), as well as the asexual phenotype of the *denA* deletion strain (Figure 18) appear not only in the

corresponding double deletion mutant (Figure 26), but also in the fungal *candA* mutants (Helmstaedt et al, 2011). It is also a further indication that both, the CSN complex and DenA, act in a similar pathway.

4.5 DenA and the CSN are connected in *A. nidulans*

The observed genetic connection converges in a physical interaction of the two deneddylases observed in yeast-2-hybrid (Figure 28) and verified by BiFC experiments (Figure 29). DenA interacted strongly with CsnG in yeast-2-hybrid experiments. Interaction with CsnA, CsnE and CsnF where only seen in the growth tests of the yeast-2-hybrid assay, but not in the β-galactosidase tests (Figure 28). Interaction with CsnG was also verified by BiFC and seems to occur predominantly in the nucleus (Figure 29). This is consistent with results from this work and other studies that DenA (Figure 14) and CsnD (Busch et al, 2003) both localize to the nucleus. CSN-DenA interaction seems to be conserved among species even though the preferential CSN subunit for DenA/DEN1 interaction seems to have changed during evolution. Recent biochemical data in mammals report CSN1 as the main subunit interacting to DEN1 (W. Dubiel, T. Schmaler, personal communication). Yeast-2-hybrid data dedicate this role to CSN2 in *S. pombe* (C. Gordon, personal communication). All three subunits have a PCI domain in common. However, the mammalian experiments showed that the N-terminal part of CSN1 interacts with DEN1, what excludes the PCI domain as the binding motif. This is supported by bioinformatic data which describe the PCI domain to be required for CSN complex formation, rather than for the interaction with associated proteins (Scheel & Hofmann, 2005). There are segments of low complexity in the N-terminal regions of the three proteins, which might be responsible for protein-protein interactions. The exact binding interfaces, however, have to be determined in the future. Overexpression experiments in mammalian cells indicate that the observed physical interaction corresponds to a function of the CSN in mediating DEN1 degradation. In the corresponding experiments binding of the CSN to DEN1 accelerates the degradation of DEN1 by the proteasome system. This was shown by overexpression of transfected DEN1 in cells possessing low levels of CSN complexes (siCSN1 cells) as compared to control cells. Down regulation of the CSN results in high steady state concentrations of DEN1 and reduced degradation of the protein. Since the

degradation can be reduced by MG132, the process seems to be proteasome-dependent (W. Dubiel, T. Schmaler, personal communication). It has been shown in other studies that the CSN targets p53 for proteasome-dependent degradation *via* phosphorylation by the CSN associated kinase CK2 (Bech-Otschir et al, 2001). In addition, $p27^{Kip}$ is phosphorylated by the CSN which accelerates its degradation by the UPS (Huang et al, 2006). Corresponding experiments did not show DEN1 phosphorylation by purified CSN. CSN binding alone seemed to destabilize the protein (W. Dubiel, T. Schmaler, personal communication). Similar observations were made with a number of other UPS substrates (Kato & Yoneda-Kato, 2009; Wei et al, 2008). Similar effects were not observed for endogenous DEN1, what is consistent with experiments in *A. nidulans* presented in this work where deletion of *csnA* had no detectable effect on the stability of DenA::GFP or its localization within the hyphae under the investigated conditions (chapter 3.2.2). Therefore CSN mediated control of DEN1/DenA stability is presumably required to cope with unphysiological amounts of the protein and maintain a certain balance between DenA and the CSN.

A further hypothesis on the consequence of DenA-CSN interaction is that the CSN is associated with DenA and the appropriate target protein to mediate deneddylation. A similar mechanism is supposed for the de-ubiquitinating enzyme USP15 which associates with the CSN in mammals to stabilize the APC (adenomatous polyposis coli) protein (Huang et al, 2009). Different options exist for that case, whereof some are listed consecutively and are illustrated in Figure 39. (A) DenA is associated to the complete eight subunit complex as an additional deneddylase adjacent to CsnE. (B) DenA is an alternative deneddylase incorporated into a CSN complex lacking CsnE. (C) DenA is involved in another complex which associates to the CSN to form a higher structured molecule. (D) DenA and CsnG interact independently of the CSN complex (Figure 39). This dimer might also be involved in the formation of higher ordered complexes, other than the CSN.

However, these are only a few possibilities of many conceivable prospects and it might turn out that none of them is true, but this requires further investigations.

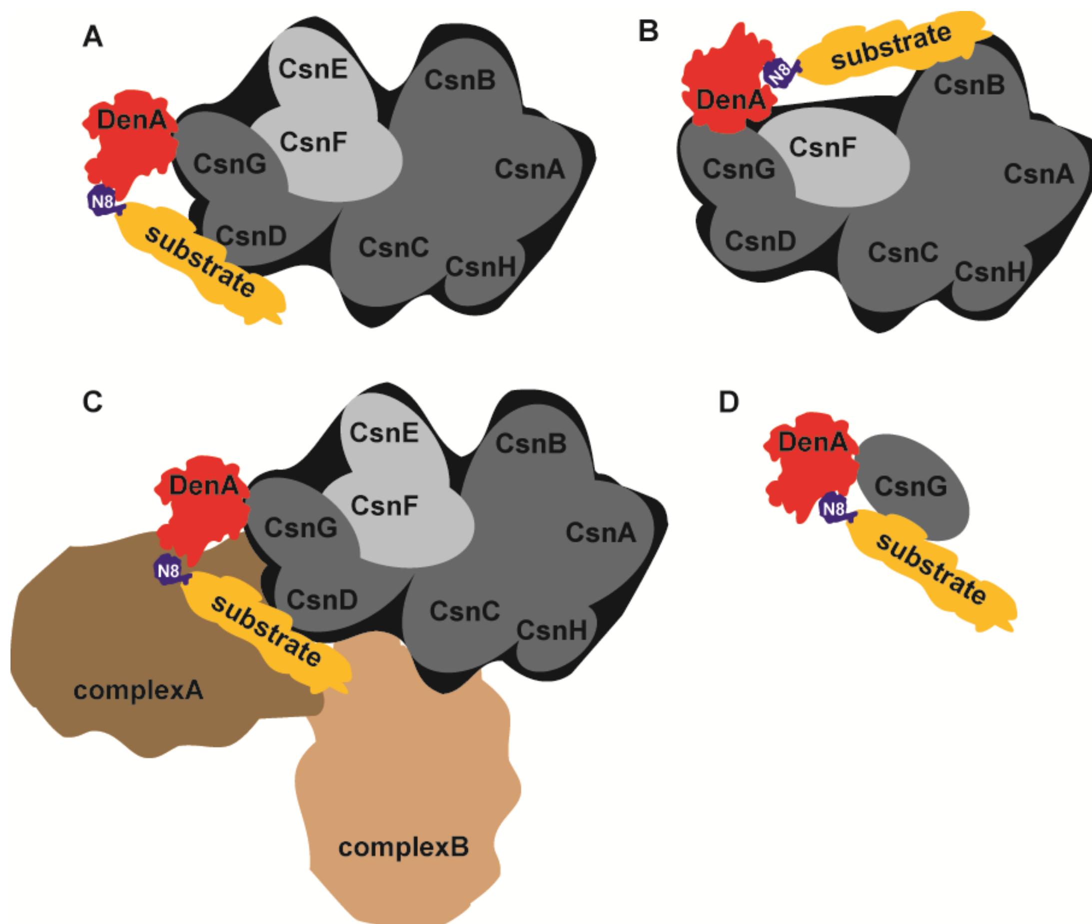

Figure 39: Hypothetical models for possible options of DenA-CSN interaction.

A DenA is associated to the holo-complex as an additional deneddylase. **B** DenA is an alternative deneddylase for CsnE. **C** DenA and CSN are part of a higher structured super-complex. **D** DenA and CsnG form a dimer independent of the CSN complex.

4.6 DenA interacting proteins

Yeast-2-hybrid interaction tests made in this study indicate that DenA is capable of interacting with Nedd8, either in the processed or the unprocessed form (Figure 15). Additionally yeast-2-hybrid data supported by further BiFC results showed an interaction between DenA and the seventh subunit (CsnG) of the fungal COP9-signalosome (Figure 28 and 29).

The latter bears a possible explanation why the fungal cullins showed no direct interaction to DenA in yeast-2-hybrid tests. In other organisms the CSN was shown to interact with cullins of CRLs via CSN1, CSN2 and CSN6 possibly acting as a platform for ubiquitin ligase assembly (Lyapina et al, 2001; Schwechheimer & Deng, 2001; Yang et al, 2002). Thus, one can hypothesize that the CSN might associate with both, CRL and DenA to mediate the deneddylation of cullins. The interaction with the CSN occurs mainly with the seventh subunit CsnG, as could be

verified in BiFC experiments. This is a subunit which would not be occupied by the CRL at the time. Interestingly this subunit is also target of phosphorylation (Kapelari et al, 2000) introducing a possible regulative element (discussed later).

Co-purification experiments with *A. nidulans* Nedd8 recruited components of the neddylation pathway and CRLs suggesting that these are the predominant targets of neddylation in the fungus. But there were also other proteins identified along with Nedd8. It is yet speculative whether they are Nedd8 modified or are targets of CRLs (Marcia von Zeska Kress, unpublished data).

However, co-purification experiments applying different tagged versions of DenA did not pull down Nedd8, CSN subunits or any components of the neddylation pathway or CRLs. One explanation is that the observed interactions are due to transient connections and not to the formation of stable complexes. Many of those proteins that were co-purified with DenA function in translational control, some are found to be involved in cellular trafficking, and others are connected to metabolic functions (Table VI and VII). All these processes need to be tightly regulated and it is possible that some of the proteins are targets for neddylation. Anyway, none of them contained a described neddylation consensus sequence (Mikus & Zundel, 2005), even though all of them contain at least one lysine residue, which is accessible for modification. The only protein that was found to interact to DenA as well as to the CSN complex was AN2068, the ortholog of *S. cerevisiae* SCP160 (Table VI, VII and IX). SCP160 in yeast is a RNA-binding G protein effector involved in translational control at the ribosome. It is an essential component of the mating response pathway in yeast (Guo et al, 2003). It interacts with a couple of yeast proteins (Baum et al, 2004) including the WD40 protein ASC1, the ubiquitin protease UBP3 and RPN5 and RPN11, which are components of the *S. cerevisiae* proteasome and CSN-like complex. The UBP3 ortholog AN5186 was also identified along with DenA (Table VI and VII). The CSN in other organisms is shown to associate with other regulatory complexes. In mammalian cells CSN can associate with CRLs and the beta-catenin destruction complex forming a super-complex that promotes beta-catenin degradation (Huang et al, 2009). Therefore it can be speculated that the CSN is involved in super-complexes with components of other regulatory pathways like the translation machinery, which also contain DenA.

Another interesting DenA interaction partner found is AN10598, which encodes a putative ortholog of the *Aspergillus fumigatus* annexin ANXC4 (Table VI

and VII). Annexins can bind to calcium and phospholipids in higher eukaryotes and are reported to bind to membranes or form aggregates in the cell and are involved in functions like membrane fusion or exocytosis (Khalaj et al, 2004). This partially coincides with the localization of DenA at ball shaped structures near the fungal septum (Figure 14).

Similarly, the RmtC histone arginine methyltransferase (AN0134) also co-purified with DenA, might account for this DenA localization pattern (Table VII). The yeast ortholog of RmtC, HSL7 localizes to the bud neck in a sentrin and HSL1 dependent manner in *S. cerevisiae* (Shulewitz et al, 1999). *A. nidulans rmtC* null mutants additionally show decreased conidiation (Trojer et al, 2004), a phenotype also observed for the *denA* deletion (Figure 18). Additionally, in *Neurospora crassa* Cul4 based CRLs are required for the regulation of methyltransferase dependent DNA methylation (Xu et al, 2010), linking these proteins to the Nedd8 pathway and neddylation/deneddylation to gene regulation.

Another interesting candidate is the putative, fungal specific Ser/Thr phosphatase AN10946, which was also co-purified with DenA (Table VI and VII). This result is a hint corresponding to the idea that DenA might be phosphorylated, but it might also be a product of DenA-CsnG interaction (Figure 28 and 29). CsnG, or CSN7 in mammals and plants is known to be phosphorylated (Kapelari et al, 2000), what implicates that it requires a phosphatase to remove the modification. De-phosphorylation might become necessary when another protein is recruited to the subunit. In contrast, it is possible that phosphorylation is a prerequisite for interaction and the phosphatase removes the modification and thereby the associated protein. The putative phosphatase (AN10946) might also be a direct interacting partner of the above mentioned RmtC, since HSL7 in yeast can be a target of phosphorylation as well (Shulewitz et al, 1999).

AN8268 encodes a putative protein, found along with DenA (Table VI), whose yeast ortholog NTF2 is involved in nucleocytoplasmic transport (Corbett & Silver, 1996; Quimby et al, 2001). Therefore the *A. nidulans* protein might be as well required for the transport of proteins from the cytoplasm to the nucleus, such as the translocation of VeA and VelB (Bayram et al, 2008b; Stinnett et al, 2007) or the localization of DenA to the nucleus.

It is not clear from these results, whether the proteins enriched with DenA are really neddylation targets. One can assume that some of them are, but others might

interact with DenA for different reasons or are interacting partners of another protein which interacts with DenA. The identified proteins involved in translation might be even artificially co-purified by partially translated DenA still connected to the translation machinery. The details of these interactions and the subsequent consequences for protein functions have to be investigated in future studies.

4.7 Deletion of single subunits abolishes CSN complex formation

Another part of this work, initially separated from the DenA project, focused on the dynamics of CSN assembly in *A. nidulans*. A CsnA fusion protein linked with a TAP tag at the N-terminus was applied to perform co-purification experiments (Figure 32). The results of different rounds of purification and subsequent MS analysis revealed that the fusion construct recruits six additional subunits, but one was always missing (Table VIII). CsnE was not obtained in any experiment. Also CsnC and CsnH were not identified in all experiments, suggesting that CsnA, CsnB, CsnD, CsnF and CsnG are more tightly connected to each other than with the remaining three. This is supported by yeast-2-hybrid data that indicate vice-versa protein-protein interactions between CsnA, CsnB, CsnD and CsnG and of CsnG with CsnF (Busch et al, 2007). The recent theory postulated by two publications, that the CSN consists of two sub-complexes (Enchev et al, 2010; Sharon et al, 2009) is also, in parts, consistent with these results. As illustrated in Figure 40, CsnA and CsnF are those subunits that facilitate the connection and CsnB interacts to CsnA, whereas CsnD is in direct contact with CsnF. If one rotates the model released by Sharon et al. (2009) the dynamics observed in the TAP::CsnA purification experiments become even more plausible (Table VIII). Thus CsnA, CsnB, CsnD, CsnF and CsnG form some kind of core to which CsnC, CsnE and CsnH are recruited. This model would result in at least two subunit-subunit interactions for all, except CsnE, what might be the reason for a more loose connection. This supports the hypothesis, that CsnE might be the last subunit to be integrated into the already assembled seven subunit complex.

Earlier studies support this idea. Purifications with CsnE fused to the TAP tag in *A. nidulans* and subsequent MS analysis revealed that CsnE recruits the complete eight subunit COP9-signalosome complex. Interestingly, deletion of either *csnA* or

csnD caused a complete loss of complex formation and only the tagged CsnE was obtained by purification (Busch et al, 2007).

Mammalian CSN5 is known to exist and fulfill certain functions independent of the CSN complex, however, CSN deneddylase activity requires incorporation of CSN5 into the holo-complex (Wei et al, 2008). It can also be speculated whether it is possible that other proteins, such as DenA, can be incorporated into the CSN complex, instead of CsnE.

GFP-Trap® purification was performed to change the point of view and an existing GFP fusion of CsnD was applied for this experiments. The fusion construct was initially developed to study CSN localization and is expressed by the strong inducible *alcA* promoter (Busch et al, 2003; Waring et al, 1989). In the wild type background CsnD::GFP recruited all other CSN subunits in two independent purifications. Deletion of *csnG* lead to a complete loss of all other CSN subunits in the purification and only CsnD was maintained in MS identifications (Table IX). A result that is reminiscent to what is observed in CsnE purifications (Busch et al, 2007).

Recent studies on structure and dynamics of complex assembly of mammalian CSN postulate the existence of sub-complexes composed of up to four subunits. These split the CSN complex more or less in two distinguishable parts, which are mainly connected via subunits CSN1 and CSN6 (Enchev et al, 2010; Sharon et al, 2009). These studies, however, deal with purified or *in vitro* reconstituted CSN complexes. Therefore the observed sub-complexes might very well exist under laboratory conditions separated from any degradation mechanism. *In vivo* the dynamics of CSN complex formation might prevent the enrichment of the holo-complex under certain conditions. It is as well possible that complexes with less than eight subunits become highly instable and therefore are not detectable by co-purification. One option for a model is that CsnD is involved in a sub-complex with CsnE, CsnF and CsnG. That is connected via CsnF to CsnA in the second sub-complex that additionally contains CsnB, CsnC and CsnH (Enchev et al, 2010; Sharon et al, 2009) (Figure 40). The results of this study and the earlier experiments with TAP::CsnE strongly suggest that CSN sub-complexes in *A. nidulans* are either not existing or are rapidly degraded. However, only a few options to combine the subunit to be purified with another subunit being deleted were tested so far. It might

be that future studies will find a combination that allows the purification of CSN sub-complexes directly from a living cell.

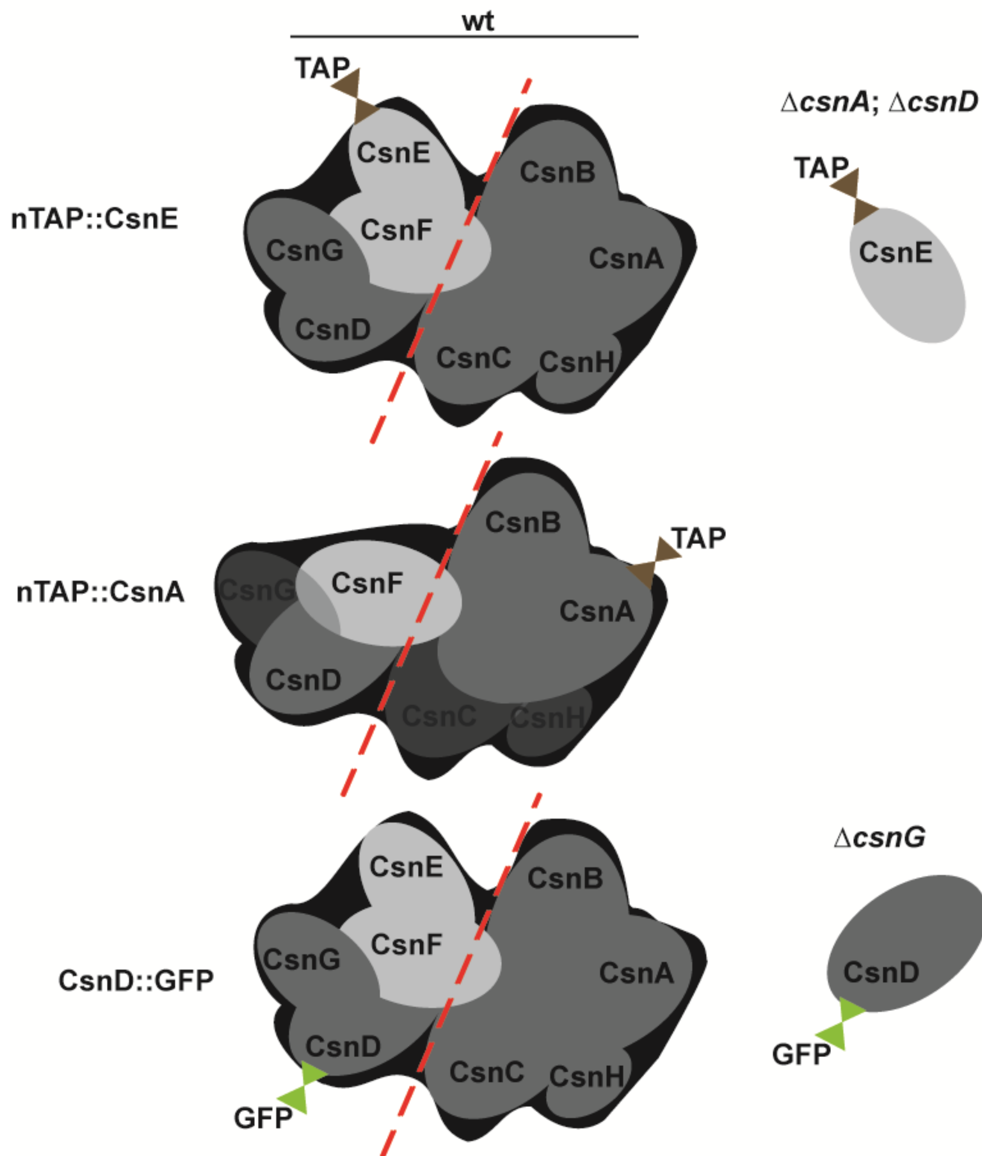

Figure 40: Schematic representations of the CSN complexes obtained in co-purification experiments.

CsnE (nTAP::CsnE) recruits all other subunits in a wild type background (wt), but interactions are completely lost in *csnA* (Δ*csnA*) or *csnD* (Δ*csnD*) deletion mutants (Busch et al, 2007). CsnA (nTAP::CsnA) co-purifies with all subunits except CsnE. Purifications were only done in wild type background, so far. GFP-Trap® purification with CsnD (CsnD::GFP) recruits the complete CSN in wild type background, but interactions are lost completely upon deletion of *csnG*, similar to the observations with CsnE. Graphical illustration derived from the electron density map by (Kapelari et al, 2000). The dashed, red line represents the proposed axis between the two sub-complexes suggested by Enchev et al. (2010) and Sharon at al. (2009). Subunits with an MPN domain are light grey and PCI subunits are dark grey in both complexes.

4.8 Outlook

The results obtained in this work imply that DenA has isopeptidase activity and acts as a deneddylase in *A. nidulans*. It is required for the deneddylation of proteins involved in the light dependent coordination of development, such as the cullin scaffolds of CRLs. DenA activity is required for the stimulation of asexual development, as well as the repression of sexual development in the light. That differs from COP9-signalosome mediated deneddylation which is most notably required for sexual development. Whether the affected protein is one of the three *A. nidulans* cullins or a non-cullin protein is still speculative.

It would be one approach to investigate the stability of potential downstream targets of deneddylation, such as the velvet complex components VeA, VelB and LaeA in the corresponding DenA or CSN deletion strains by western hybridization experiments. Furthermore, inactive point mutations of DenA, such as the $H^{123}A$ mutant would be a versatile instrument to further dissect which aspects of the deletion phenotype are due to the isopeptidase activity. Additionally those might aid future efforts to identify neddylated substrates, as the potentially transient interaction of a Nedd8 adduct with DenA might be “frozen” when the enzyme is inactive.

Nevertheless, there are already some interesting candidates that where identified together with DenA in co-purification experiments accomplished during this study and it would be worthwhile to study those in more detail, such as the putative annexin Anxc4, the methyltransferase RmtC or the fungal specific phosphatase. For all of these proteins one could verify the interaction by BiFC, what would additionally reveal information on the site of interaction in the cell. Yeast-2-hybrid experiments would be another instrument to validate protein-protein interactions. Those have the additional potential to identify contact surfaces by testing truncated versions of the investigated proteins. Furthermore it would be very attractive to follow the idea of higher structured complexes containing DenA suggested by the co-purified orthologs of yeast SCP160 and UBP3 which are described to be connected in yeast. Regarding this some more elaborated experiments are required, which might start with making use of the SCP160 ortholog as bait for co-purification. But also co-immunoprecipitation experiments applying a recently developed DenA antibody, which is currently in production, would be

applicable to identify DenA interaction partners, as well as potential substrates for deneddylation.

DenA was initially supposed to be a Nedd8 processing enzyme, auxiliary to its isopeptidase function. During the course of this study it became obvious, that this processing activity is not an important function of DenA. Therefore, the question remains, which protein is responsible for Nedd8 processing in *A. nidulans*. There are several candidates, such as the USP15 ortholog UspO or the Sumo isopeptidases UlpB. Also an ortholog of the yeast hydrolase Yuh1 can be found in the fungal genome. It will be interesting to see whether Nedd8 processing can be assigned to a distinct protein or is the result of different promiscuous enzymes.

Similarly it would be interesting to follow the preliminary results suggesting that DenA might also be able to act as a SumO isopeptidase, mentioned in the discussion section. Vice versa it will be interesting to see whether SumO isopeptidases are able to cleave Nedd8 adducts. Purified proteins could be investigated by *in vitro* experiments with corresponding substrates.

The deneddylation of proteins seems to be of major importance for the mechanisms underlying coordinated fungal development. Both deneddylases, DenA and CSN are involved in the light dependent repression of sexual fruit body formation. Without the two deneddylases the fungus lost its developmental potential and can only grow as a filament. In addition to the aggravation of the developmental phenotype the *denA/csnE* double deletion strain also accumulated high amounts of neddylated proteins in contrast to the rather small differences observed between a wild type and the *denA* or *csnE* single deletion strain. Furthermore, an additional band which might be due to the modification of a cullin with multiple Nedd8 molecules was observed only in the Δ*denA*/Δ*csnE* strain. The observed CSN-DenA connection converges in physical interaction that is supposed to regulate the balance between the two deneddylases to allow regular coordination of development.

This interesting and unexpected connection of the two main deneddylases in *A. nidulans* raises a couple of questions to answer in the future. The functional consequences of this interaction need to be unveiled to see whether DenA can be an alternative deneddylase for the CSN complex, the CSN affects DenA stability or DenA and the CSN are parts of higher ordered complexes. Therefore it might be interesting to investigate DenA protein stability in *csn* deficient strains by western experiments with an antibody which is currently in production, but was not available

during the course of this study. Similarly, antibodies for fungal CSN subunits will be available soon, which allow to look for CSN stability also. All antibodies can also be applied to co-immunoprecipitation experiments to specify the observed DenA-CSN interaction, as well as to address the question of other proteins or protein complexes to interact with DenA and/or the CSN. Another approach would be to express a tagged DenA version applicable for co-purification, in a *csnE* deletion strain to see whether DenA can be integrated into the CSN complex instead of CsnE. In a wild type background DenA did not recruit CSN components, but this might be changed in the *csnE* deletion strain. Additionally the above mentioned co-purification experiment employing the SCP160 ortholog as bait might lead to interesting results as this was also found in purifications with CsnD. Therefore it might be capable of identifying complexes containing CSN subunits, as well as DenA. Furthermore overexpression of *denA* in *csn* mutants and vice versa might yield a notion of how important the balance between the two deneddylases really is for coordinated regulation of development.

Similarly to the DenA and CSN antibodies, the production of antibodies recognizing the three fungal cullins CulA, CulC, CulD was initiated at the end of this study. These have the potential to identify certain specificities in the deneddylation activity of DenA and the CSN on a distinct cullin in western experiments comparing wild type with Δ*denA*, Δ*csnE*, and Δ*denA*/Δ*csnE*.

Fungal CSN holo-complexes can be purified from whole cell extracts of *A. nidulans* by overexpressed *csnD::gfp*. However, sub-complexes could not be found *in vivo* by experiments accomplished during this study. It can be assumed that there are interactions of different strength between distinct CSN subunits, but in the cases investigated so far, deletion of one CSN subunit is accompanied by the complete loss of the CSN complex.

The ability to purify adequate amounts of the CSN holo-complex from fungal cells in a single experiment allows further experiments to study structure and dynamics of the complex. Cryo electron microscopy can be applied to study the structure of the complex, after cross-link of the subunits and additional purification of the extract by size exclusion chromatography. This would also be applicable to sub-complexes if in future studies conditions are found which allow purification of those.

The orbitrap mass spectrometer would be another powerful instrument to investigate the composition of purified CSN complexes. It can identify the size of a native particle with high accuracy, which is not only useful in terms of the CSN complex composition, but may additionally aid to identify interacting proteins or posttranslational modifications. Also for the investigation of other proteins this instrument is highly interesting, since it allows MS identification without prior trypsin digestion of the sample. This may preserve modifications and interactions which are lost in other protocols.

Future studies, which maybe imply some of the experimental approaches suggested above, will be able to reveal specific targets of DenA and/or CSN mediated deneddylation. Together with a more defined knowledge on structure and dynamics of the COP9 signalosome complex and associated proteins this will contribute to an improved understanding of the molecular mechanism, by which protein stability control affects cell fate and development.

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Abbreviations

A	alanine (Ala)
AIDS	aquired immuno deficiency syndrome
alc	alcohol dehydrogenase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BiFC	bi-molecular fluorescence complementation
BLAST	basic local alignment search tool
bp	base pair
C	cysteine (Cys)
cDNA	complementary DNA
COP	constitutive photomorphogenic
CRL	cullin-RING ligase
CSN	COP9-signalosome
C-terminus	carboxy terminus
DAPI	4',6-diamidino-2-phenylindole
DMF	*N*,*N*-dimethylformamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DUF	domain of unknown function
EDTA	2,2',2'',2'''-(Ethane-1,2-diyldinitrilo) tetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ERAD	endoplasmatic reticulum associated protein degradation
GFP	green-fluorescent protein
GST	glutathione S-transferase
H	histidine (His)
h	hour(s)
HECT	homologous to the E6-AP carboxyl terminus
HRP	horseradish peroxidase
IPTG	isopropyl β-D-1-thiogalactopyranoside
JAMM	Jab1/MPN domain metalloenzyme
K	lysine (Lys)

kb	kilobase(s)
kDA	kilo Dalton
LacZ	β-galactosidase
LB	lysogeny broth (Luria-Bertani medium)
LC-MS	liquid chromatography-mass spectrometry
leu	leucine
MAT	mating type
met	methionine
min	minute(s)
mM	milli-molar
mRFP	monomeric red fluorescent protein
mRNA	messanger RNA
ms	milli-second(s)
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	poly-alcrylamide gelelectrophoresis
PCR	polymerase chain reaction
PDB	protein data bank (RCSB)
pfam	protein family
PIM	protease inhibitor mix
PMSF	phenylmethanesulfonyl fluoride
PPi	diphosphate
ptrA	pyridthyamin
Q	glutamine (Gln)
qRT	qantitative real-time
RACE	rapid amplification of cDNA ends
rcf	relative centrifugal force
RNA	ribonucleic acid
RT	room temperature
S	sedimentation coefficient
SC	synthetic complex
SDS	sodium dodecyl sulfate
sec	second(s)

t	time
TAP	tandem affinity purification
TCA	trichloroacetic acid
TEV	tabacco etch virus
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
UbF	ubiquitin family
ura	uracile
UTR	untranslated region
UV	ultra-violett
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
YFP	yellow fluorescent protein

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"42"

Douglas N Adams, "The Hitchhikers Guide to the Galaxy"

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2001 – 2007 Study of biology at the Georg-August Universität in Göttingen. Subjects: Microbiology, Anthropology and Human Genetics.

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