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The COP9 signalosome counteracts the accumulation of cullin SCF ubiquitin E3 RING ligases during fungal development

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Summary

Defects in the COP9 signalosome (CSN) impair multicellular development, including embryonic plant or animal death or a block in sexual development of the fungus *Aspergillus nidulans.* CSN deneddylates cullin-RING ligases (CRLs), which are activated by covalent linkage to ubiquitin-like NEDD8. Deneddylation allows CRL disassembly for subsequent reassembly. An attractive hypothesis is a consecutive order of CRLs for development, which demands repeated cycles of neddylation and deneddylation for reassembling CRLs. Interruption of these cycles could explain developmental blocks caused by *csn* mutations. This predicts an accumulation of neddylated CRLs exhibiting developmental functions when CSN is dysfunctional. We tested this hypothesis in *A. nidulans*, which tolerates reduced levels of neddylation for growth. We show that only genes for CRL subunits or neddylation are essential, whereas CSN is primarily required for development. We used functional tagged NEDD8, recruiting all three fungal cullins. Cullins are associated with the CSN1/CsnA subunit when deneddylation is defective. Two CRLs were identified which are specifically involved in differentiation and accumulate during the developmental block. This suggests that an active CSN complex is required to counteract the accumulation of specific CRLs during development.

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Introduction

The proteome of a multicellular organism is highly dynamic due to controlled cycles of synthesis and degradation of proteins. The timely removal of critical proteins is crucial for various cellular processes, including transcriptional regulation and silencing, DNA repair, the cell cycle or differentiation. Protein life span control is required for complex processes as long-term memory, circadian rhythm, appropriate stress response, antigen presentation in the immune system or in combating diseases like cancer or viral infections (Hershko and Ciechanover, 1998; Spataro *et al.*, 1998; Ciechanover *et al.*, 2000).

One essential pathway of targeted protein degradation includes ubiquitination and the 26S proteasome (Tyers and Jorgensen, 2000). Ubiquitin (Ub) is a conserved 76 amino acids protein that is attached covalently to lysine residues of substrate proteins. Ubiquitination can either reduce stability or change activity or localization of a protein. Ub-conjugation requires the sequential action of the E1 Ub-activating enzyme, the E2 Ub-conjugating enzymes and the E3 Ub-ligases as specificity factors for substrates (Hershko and Ciechanover, 1998). The largest known class of ubiquitin ligases comprises the cullin-RING ligases (CRL) (Petroski and Deshaies, 2005). There are three major categories of cullins in fungi (CUL1, CUL3, CUL4), five categories in metazoa (CUL1 through CUL5), and additional CUL7 (Kipreos *et al.*, 1996; Mathias *et al.*, 1996; Dias *et al.*, 2002) and PARC (Parkin-like cytoplasmic protein) (Skaar *et al.*, 2007) in the human genome.

Each cullin forms a distinct class of CRL complexes with substrate recognition subunits, which can be combined with distinct adaptors. CUL1 (in yeast: Cdc53) functions as a stalk-like scaffold, anchoring simultaneously at one site the SKP1/F-box protein heterodimer, and at the other site the RBX1 RING finger protein. SKP1 connects the cullin to the F-box protein. F-box proteins represent

the substrate receptors in the SKP1-CUL1-F-box (SCF) complex. The F-box protein bound substrate is positioned in the proximity of Rbx1 that recruits an E2 Ub-conjugating enzyme. Approximately 70 different proteins with F-box domains were identified in *Aspergillus nidulans* (Busch *et al*., 2007; Draht *et al*., 2007), including one F-box protein, GrrA (Fbx29), which is specifically necessary for sexual development (Krappmann *et al*., 2006). The bound substrate, SCF and the E2 ubiquitin ligase engage in concerted interactions that commit catalysis yielding a covalent linkage of a first ubiquitin. This ubiquitin can be the substrate for further ubiquitination. A poly-ubiquitin chain at K48, one of seven lysine residues of ubiquitin, attached to the protein substrate, is the signature for proteasome-mediated proteolysis (Ohta *et al*., 1999; Tan *et al*., 1999; Chen *et al*., 2000; Wu *et al*., 2000). CUL3 CRL complexes contain RBX1, but differ from other CRL classes in direct binding of the substrate recognition subunit to the N-terminus of CUL3 using a BTB/POZ domain (Furukawa *et al*., 2003). CUL4 CRL complexes contain RBX1 and the adaptor protein DDB1. DDB1 binds to substrate recognition subunits that contain WD-repeats, which mediate interaction with DDB1 (Higa *et al*., 2006).

Despite the cullins diversity, each of the classes of CRL complexes is subjected to similar regulatory mechanisms including the ubiquitin-like protein NEDD8/Rub1. NEDD8 was originally discovered being neuronal-precursor-cell expressed developmentally downregulated in mice (Kumar *et al*., 1992). It encodes a small protein of 81 amino acids, which is 60% identical and 80% homologous to ubiquitin (Kumar *et al*., 1993). The NEDD8 homologue in *Saccharomyces cerevisiae* is Rub1 (related to ubiquitin 1), composed of 77 amino acids and with 59% identity to the human protein (Liakopoulos *et al*., 1998).

The activity of CRL complexes is controlled by the covalent linkage of NEDD8 to a lysine of cullins. This process is called neddylation (rubylation in yeast) and has its own unique set of enzymes to ensure a conjugation pathway distinct from ubiquitin. It requires the Ula1/Uba3 E1 NEDD8-activating and the Ubc12 E2 NEDD8-conjugating enzyme. E3 NEDD8/Rub1p ligase activity has been attributed to the RING finger protein Rbx1 as well as Dcn1 (defective in cullin neddylation-1) in yeast (Morimoto *et al*., 2003; Kurz *et al*., 2005). Dcn1 interacts with NEDD8/Rub1 and cullins and increases the kinetics of the neddylation process (Kurz *et al*., 2005). It is also able to bind to the E2 NEDD8/Rub1-enzyme Ubc12 (Kurz *et al*., 2008). Similar to other ubiquitin-like proteins, NEDD8 is synthesized as a precursor with a C-terminal tail of different length depending on the species (Kerscher *et al*., 2006). Proteases process this tail, resulting in mature NEDD8 which exposes a di-glycine motif covalently linked to the substrate. Deletions of the yeast genes *rub1*, *ubc12*, *ula1* or *uba3*, encoding components of the neddylation machinery, are viable (Hochstrasser, 1998; Lammer *et al*., 1998; Liakopoulos *et al*., 1998). However, the defects of the NEDD8 pathway in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster* and mice, all show a lethal phenotype, demonstrating an essential role for NEDD8 in cell viability (Osaka *et al*., 2000).

The major CRL deneddylase is the constitutive photomorphogenesis complex 9 (COP9), termed COP9 signalosome (CSN), which directly interacts with E3 Ub-ligases (Schwechheimer *et al*., 2001; Suzuki *et al*., 2002). CSN5/JAB1/CsnE is the only subunit conserved in all eukaryotes. It carries an MPN+ domain containing the JAMM motif conferring metalloprotease (deneddylation) activity (Cope *et al*., 2002). The MPN+ domain of CSN controls the activity of CRLs by cleaving the ubiquitin-like protein NEDD8/Rub1 from cullin (Lyapina *et al*., 2001; Yang *et al*., 2002). Neddylated E3 Ub-ligases are the key mediators of post-translational labelling of proteins for the proteasome (Petroski and Deshaies, 2005). In the filamentous fungus *Aspergillus nidulans* defects in the CSN lead to a block in sexual fruit body formation (Busch *et al*., 2003; 2007). Deletion of the gene for the subunit CSN5/CsnE leads to strong changes in the transcriptome, the proteome and the metabolome (Nahlik *et al*., 2010). CSN is linked to several additional developmental functions, including light control of development, the protection against oxidative stress, acting as an internal trigger signal during development, and the control of secondary metabolites which function as pheromones or as protectors against fungivors (Nahlik *et al*., 2010). The relationship between CSN and the fungal-specific velvet complex, which also controls secondary metabolism and development, is yet unknown (Busch *et al*., 2003; 2007; Braus *et al*., 2010; Sarikaya Bayram *et al*., 2010; Bayram and Braus, 2012). A second regulator of proteasomal degradation is the cullin-associated NEDD8-dissociated protein 1 (Cand1). It binds to unneddylated cullins and prevents the formation of SCF complexes (Hwang *et al*., 2003). *A. nidulans* Cand1/CandA is split into two proteins where one polypeptide binds to the SKP1/SkpA adaptor site and recruits the second Cand1/CandA polypeptide interacting with the neddylation site. Both proteins are also necessary for accurate fungal development and secondary metabolism (Helmstaedt *et al*., 2011).

We addressed here the specific role of deneddylation for development, using the fungus *A. nidulans* as a simple model for a multicellular eukaryote. The neddylation machinery as well as most SCF complex subunits is essential for fungal viability. We found that defects in the CSN deneddylase result in the accumulation of distinct F-box proteins being associated to neddylated cullins as parts of SCF E3 ubiquitin ligase complexes. Two of these F-box proteins are required for precise development, suggesting

that an intact CSN is required to allow accurate fungal differentiation.

Results

The constitutively expressed neddylation and ubiquitination machinery is essential for Aspergillus nidulans *viability*

The proteins for neddylation and ubiquitination, including NEDD8/NeddH, are highly conserved between human and the filamentous fungus *A. nidulans*. The *A. nidulans* (An) NEDD8 encoding gene *neddH* (AN6179.2) exhibits 75% identity to *Homo sapiens* (Hs). NeddH/Nedd8 shares 55% identity with AnUbiquitin (AN2000.2), which has 96% identity to HsUbiquitin (Galagan *et al*., 2005). The human proteins CUL1, SKP1 and RBX1 correspond to AnCulA (AN1019.2) with 46% identity, AnSkpA (AN2302.2) with 58% and AnRbxA (AN8844.2) with 91% identity respectively (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Only SkpA has been previously described as SconC and has been isolated as part of the regulation of sulphur metabolism in *A. nidulans* (Piotrowska *et al*., 2000).

Aspergillus nidulans can grow vegetatively as a filament and has two developmental options (Fig. 1A). Asexual development is favoured in light and results in spores, which are released into the air. In the absence of light, sexual development is supported, resulting in complex, closed fruit bodies (cleistothecia) with sexual spores (Bayram *et al*., 2010; Bayram and Braus, 2012). Comparison of mRNA levels by quantitative RT-PCR (qRT-PCR) for *neddH* or *ubcL* (E2 NeddH-conjugating enzyme) representing the neddylation machinery, *csnA* or *csnE* as parts of the CSN deneddylase, and the transcriptional expression of *culA*, *rbxA* and *skpA*, as parts of the SCF complex, showed that all genes are constitutively expressed during all stages of the fungal life cycle (Fig. S1) with small variations. None of the genes showed a characteristic developmental expression pattern as the control, *esdC*. This gene is specifically induced during sexual development (Vienken and Fischer, 2006). Defects in the gene for Rub1/Nedd8 and the E2 Rub1-conjugating enzyme, *ubc12*, are not essential in budding yeast (Hochstrasser, 1998; Lammer *et al*., 1998; Liakopoulos *et al*., 1998). We analysed the role of *neddH* and *ubcL*, the *rub1/nedd8* and *ubc12* homologues of *A. nidulans*, for the fungal life cycle. Thus, we replaced the *neddH* and the *ubcL* genes by a pyrithiamine (*ptrA*) selection marker in order to obtain deletion mutants for the corresponding genes. Heterokaryons could be obtained, containing two different types of nuclei with one intact copy of the gene and one deleted copy of the same gene (for details see *Experimental procedures*) (Osmani *et al*., 2006). However, the homokaryon, containing only nuclei with the deletion of *neddH* or *ubcL*, could not survive. This suggests that these components of the neddylation pathway are essential for *A. nidulans* (Fig. S2). In mammals, internal NEDD8 lysines were proposed as possible poly-neddylation sites (Ohki *et al*., 2009). However, several partial *neddH* deletions based on the position of the NEDD8 lysines (K11, K22 and K48) did also not result in viable homokaryons, suggesting that the entire *neddH* sequence is necessary for fungal viability (data not shown). A deletion analysis of genes encoding subunits of the SCF complex *culA*, *rbxA* and *skpA* revealed that also these genes are essential in *A. nidulans* (Fig. S2).

In summary, these data suggest that, in contrast to CSN mediated deneddylation (Busch *et al*., 2003; 2007), the neddylation pathway and the SCF complex are essential for fungal viability.

Deletion of the gene for DcnA/Dcn1-neddylase decreases the cellular levels of neddylated proteins but still allows normal fungal development

We analysed whether a subtle reduction of the neddylation machinery, which is essential for fungal growth, has an impact on fungal development. Dcn1 is an auxiliary Rub1/Nedd8 E3 ligase in *Saccharomyces cerevisiae* and higher eukaryotes. It interacts with NEDD8 itself, the RING finger protein Rbx1 and the NEDD8 E2 ligase Ubc12 (Morimoto *et al*., 2003; Kurz *et al*., 2005; 2008). Dcn1 increases the kinetics of the neddylation reaction (Kurz *et al*., 2005) by synergistic E3 ligase activity with the RING protein Rbx1 (Scott *et al*., 2010). The homologue protein in *A. nidulans* DcnA shows 23% amino acid identity to the yeast protein but contains the same domain of unknown function (DUF 298) as it is described for Dcn1 in *S. cerevisiae* (Yang *et al*., 2007; Kurz *et al*., 2008). We verified that DcnA of *A. nidulans* is the Dcn1 homologue by studying its interactions *in vivo* by bimolecular fluorescence complementation (BiFC) (Hu *et al*., 2002). The *dcnA* cDNA was fused to the N-terminal part of eYFP, whereas *neddH/nedd8*, *rbxA/rbx1* (RING) and *ubcL/ubc12* (E2 ligase) cDNAs were fused to the C-terminal part. Only interacting proteins result in an eYFP fluorescent signal. Under promoter inducing growth conditions (nitrate) we observed a fluorescence signal in the cytoplasm as well as a strong signal in the nucleus of the cell (Fig. 1B). We found specific interactions between DcnA/Dcn1 and RbxA/Rbx1 as well as with NeddH/Nedd8 and the NEDD8 E2 ligase UbcL/Ubc12. This corroborates that DcnA/Dcn1 function is similarly connected to the neddylation process as in other organisms.

We deleted the gene for DcnA/Dcn1 to reduce the fungal neddylation machinery. The *dcnA* deletion strain is viable and therefore not essential for *A. nidulans* growth (mature cleistothecia shown in Fig. 1A). We compared the neddylation pattern in mycelia of wild-type and the *dcnA*

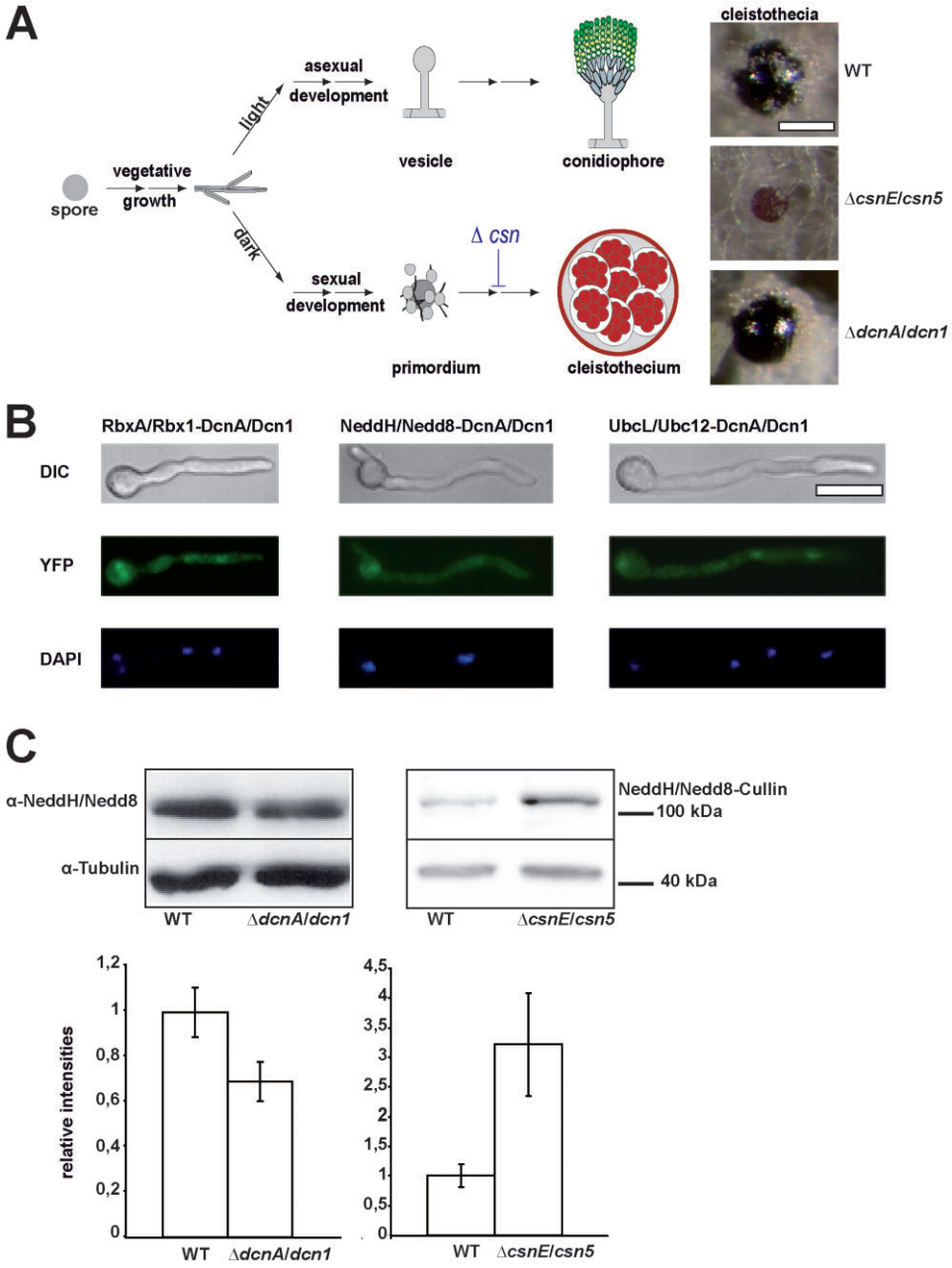


Fig. 1. Fungal development can cope with decreased neddylation in a *dcnA/dcn1* deletion strain.
A. Life cycle of *A. nidulans* wild-type and mutant strains. A spore germinates and develops to a vegetative mycelium. Depending on the environmental conditions, e.g. illumination, *A. nidulans* can form asexual structures, the conidiophores, in the light or sexual fruit bodies, the cleistothecia, in darkness (left). Whereas the WT and the *dcnA*/d*cn1* deletion strain form mature cleistothecia, the sexual fruit body development of the *csnE/csn5* deletion strain stops at the step of primordia (right; bar 100 μm). A total of 1×10^6 spores were plated on minimal medium and grown for 7 days at 37°C under sexual development inducing conditions.
B. Interaction partners of DcnA/Dcn1 visualized by bimolecular fluorescence complementation (BiFC). Indicated fungal strains were grown over night in 500 μl London medium with nitrate for induction of the corresponding promoters. DIC, YFP and DAPI counterstained pictures of the respective hyphae are shown. YFP signals correspond to interactions of the N-terminal eYFP fused to DcnA/Dcn1 (N-eYFP::DcnA) with RbxA/Rbx1-, NeddH/Nedd8- and UbcL/Ubc12 fused to C-terminal eYFP [C-eYFP::NeddH (AGB 501), C-eYFP::RbxA (AGB 502), C-eYFP::UbcL (AGB 503)]. Negative control (AGB 500) showed no YFP signal (data not shown). Bar, 10 μm.
C. Neddylated proteins in various *A. nidulans* strains. Immunoblot analyses using 50 μg protein extract of deletion strains with their corresponding parental strains with NeddH/Nedd8 and tubulin antibody (left: WT and *ΔdcnA/dcn1* (AGB 504); right: WT and *ΔcsnE/csn5* (AGB209). Strains were grown 20 h in liquid culture. Top: Shown is the most intensive signal with a size of around 108 kDa, representing neddylated cullin. Bottom: Quantification of signal intensities using Fusion-SL7 system with Bio1D software (Peqlab). The relative intensity of the deletion strains cullin band was put in relation to the WT, which relative intensity of the cullin band corresponds to 1. The signal of tubulin was used as internal standard to normalize signal intensities. Data are derived from two (for AGB209) or three (for AGB504) independent experiments with four replications each.

deletion strain grown under vegetative conditions for 20 h using an antibody against NeddH/Nedd8. Western hybridizations revealed a corresponding prominent band in a size range of neddylated cullins. NeddH/Nedd8 has a size of 9 kDa in addition to the size of the three cullins CulA/Cul1 (89 kDa), CulC/Cul3 (94 kDa) and CulD/Cul4 (100 kDa). The *dcnA*/*dcn1* deletion strain reduces the signal intensity of neddylated cullins in comparison to wild-type by approximately 30% using α-tubulin as control (Fig. 1C). Analysis of the different phases of the fungal life cycle revealed that the reduction of neddylated cullins, as it is provided by the *dcnA* deletion strain, does not result in a visible developmental defect during sexual (Fig. 1A) or asexual differentiation (data not shown). This suggests that the capacity of the fungal neddylation machinery is sufficiently high to tolerate subtle reductions without an impact on fungal development.

Defects in the COP9 deneddylase result in accumulation of SCF complexes and a developmental block

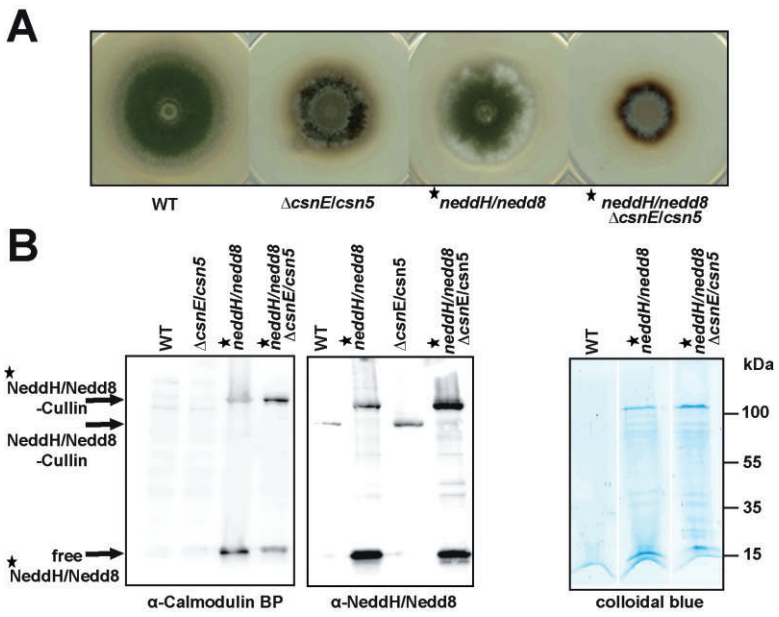
The *dcnA/dcn1* deletion strain reduces neddylated cullins in fungal cells without a developmental phenotype, whereas defects in the CSN deneddylase CsnE/Csn5 result in a block in sexual development (Busch *et al*., 2003; 2007). This corresponds to an approximately threefold increase of neddylated proteins in the *csnE/csn5* deletion strain during vegetative growth in comparison to wild-type (Fig. 1C). We analysed which neddylated complexes have to be deneddylated during development. Therefore, we identified the proteins accumulating in a *csnE/csn5* mutant when the fungus establishes developmental competence. This is the time point after a period of approximately 20 h of growth after germination. *A. nidulans* spores need this time to respond to external signals on a surface (Axelrod *et al*., 1973). For this purpose, we replaced the essential *neddH* gene for a *neddH* fusion to *tap* (tandem affinity purification) tag (**neddH*) (Busch *et al*., 2007; Helmstaedt *et al*., 2008; Bayram *et al*., 2008b). The fusion corresponds to N-terminal TAP-tagged *neddH* derived from the cDNA fragment for the mature protein to avoid interference of the tag with the protease mediated maturation. Correct transformants were verified by Southern hybridization. The **neddH* was also introduced into the CSN-deficient Δ*csnE* strain by crossing. Asexual and sexual development of tagged **neddH* strains and the respective *neddH* wild-type strains were similar (Fig. 2A). Expression of the **neddH* fusion gene was visualized by Western analysis using anti-calmodulin binding protein and anti-NeddH/Nedd8 antisera (Fig. 2B). This demonstrates that the N-terminal TAP-tag at NeddH/Nedd8 is neither interfering to cell viability and development nor to neddylation or deneddylation processes of *A. nidulans*.

The functional *NeddH/Nedd8 was used to enrich NeddH/Nedd8-containing complexes from fungal protein crude extracts. The fungus was cultivated for 30 h vegetatively until developmental competence has been acquired. Afterwards, it was further incubated for additional 48 h on solid medium under conditions directing towards sexual fruit body formation (darkness). Under these conditions there were no phenotypical differences between wild-type and *csnE/csn5* mutant strains (Nahlik *et al*., 2010). Samples were subjected to gradient (8–20%) SDS-PAGE analysis and the proteins were visualized using Colloidal blue staining (Fig. 2B). Primary bands were excised and identified by mass spectrometry (Fig. 2C). Among the identified proteins we detected all three cullins (CulA, CulC and CulD), which are present in the fungal genome (Galagan *et al*., 2005) in wild-type as well as in the *csnE/csn5* deletion strain. *NeddH/Nedd8 was found at the same molecular weight as all three cullins during vegetative growth and sexual development, suggesting the conjugation to *NeddH/Nedd8 in wild-type. The E1 NeddH/Nedd8-activating enzymes UlaA and UbaC could be identified in wild-type as well as in the *csnE/csn5* mutant. In contrast, the E2 NEDD8-conjugating enzyme UbcL was exclusively found in wild-type. This supports that in the absence of an active CSN deneddylase, when neddylated proteins are accumulated, the neddylation machinery is only partially recruited to neddylated cullins.

Furthermore, a protein with unknown function (AN4491) and the 60S ribosomal protein L11 (AN4475) were identified in wild-type and mutant strain. Ribosomal Protein L11 was previously identified as target for the NEDD8 pathway (Xirodimas *et al*., 2008). A possible transcription factor Zn(II)(2)Cys(6)-domain containing protein (AN9050) was found exclusively in wild-type. Two other proteins with unknown function (AN4149 and AN8462) were only found in the *csnE/csn5* mutant. These five proteins were not further investigated, because NeddH/Nedd8 could not be co-identified with these proteins and their molecular weights also suggest that they are non-neddylated proteins, which might interact with the neddylation machinery or CRLs.

The F-box proteins of accumulated SCF complexes in the CSN mutant are required for fungal development

Several striking differences turned out between the TAP-tagged NeddH/Nedd8 (*NeddH/Nedd8) enriched proteins, which we identified from the deneddylase-deficient *csnE/csn5* mutant strain and the wild-type *A. nidulans* (Fig. 2C). Only in the absence of *csnE/csn5* we identified CsnA/Csn1, which has an essential role in CSN complex assembly (Wang *et al*., 2002). This might reflect a specific role of CsnA/Csn1 in the interaction of the CSN to neddylated Cullins.



C

AN number	Protein	WT	ΔcsnE
AN6179.2	NeddH/Nedd8	■	■
AN1019.2	CulA/Cul1	■	■
AN3939.2	CulC/Cul3	■	■
AN10008.3	CulD/Cul4 (An0037.2)	■	■
AN2302.2	SkpA /Skp1	■	■
AN2416.2	UbaC /Uba3	■	■
AN2441.2	UlaA/Ula1	■	■
AN10324.3	UbcL/Ubc12	■	
AN8844.2	HrtA/E3 ligase RbxA/Rbx1	■	■
AN1491.2	CsnA /Csn1		■
AN6086.2	F-Box1		■
AN6816.2	F-Box2		■
AN2505.2	F-Box15		■
AN5593.2	F-Box23		■
AN10516.3	GrrA/Grr1 (F-Box29, An4200.2)	■	■
AN0596.2	UV-damaged DNA binding protein (DdbA)	■	
AN10137.3	WD40 domain protein	■	
AN8282.2	WD40 domain, G-beta repeat protein	■	
AN9050.2	Fungal Zn(2)-Cys(6) binuclear domain	■	
AN4149.2	Protein of unknown function		■
AN4491.2	Protein of unknown function	■	■
AN8462.2	Protein of unknown function		■
AN4475.2	60S ribosomal protin L11	■	■

Fig. 2. Neddylated proteins in wild-type and *csnE/csn5 A. nidulans* mutant strains.
A. Growth of *A. nidulans* wild-type (TNO2a3) and the mutant strains *ΔcsnE/csn5* (AGB209), **neddH/nedd8* (AGB457) and **neddH/nedd8* *ΔcsnE/csn5* (AGB460). A total of 1×10^6 spores were point inoculated on minimal medium and grown for 3–4 days at 37°C.
B. Left: Neddylated proteins detected by NeddH/Nedd8 immunoblot analysis of the wild-type (TNO2a3), *ΔcsnE/csn5* (AGB209), **neddH/nedd8* (AGB457) and **neddH/nedd8* *ΔcsnE/csn5* (AGB460) with calmodulin binding protein and neddH/nedd8 antibodies. Strains were grown 20 h in liquid culture. Free NeddH/Nedd8 without TAP tag cannot be visualized on the blot because of its small size. Right: Colloidal blue staining of an SDS Gel loaded with tandem affinity purified crude extracts of a WT (TNO2a3), **neddH/nedd8* (AGB457) and **neddH/nedd8* *ΔcsnE/csn5* (AGB460) strain.
C. Neddylated and NeddH/Nedd8 associated proteins identified by Nano-LC-EST-MS2 after tandem affinity purifications with *NeddH/Nedd8 in wild-type (WT) and *ΔcsnE/csn5* background. Left column: *Aspergillus nidulans* (AN) number; middle column: protein name; right column: strain background. **neddH* = *neddH* TAP tagged. Grey squares indicate in which genetic background the corresponding protein was identified.

Cullin 4 is known to link to DDB1, ROC1/RBX1 and substrate-specific adaptors WD40 repeat containing proteins (Higa *et al.*, 2006). In addition to CulD/Cul4 we identified the protein DdbA/Ddb1 and two different WD40 repeat containing proteins (AN10134 and AN8282) in wild-type but not in the *csnE/csn5* mutant strain. The RING protein RbxA/Rbx1/Hrt1 we found in both strains. The assembly of these proteins with CulD/Cul4 might therefore be coordinated or controlled by a functional CSN-deneddylase complex.

The *A. nidulans* genome comprises approximately 70 F-box protein encoding genes which are normally bridged by SkpA (Skp1-homologue) to cullin-1 (CulA) (Galagan *et al.*, 2005; Draht *et al.*, 2007). There were hardly any

F-box proteins identified as substrate recognition units for degradation by the Ub-proteasome system in wild-type fungi, due to the intact CSN-deneddylase, destabilizing SCF E3-Ub ligase complexes. These data go along with the increase of *NeddH/Nedd8-Cullin complexes in the CsnE/Csn5 defective strain (Fig. 2B). In this strain the transient and dynamic SCF E3-Ub ligase complexes are stabilized. SkpA, the protein that bridges the F-box proteins to CulA, was present in wild-type as well as in the *csnE/csn5* mutant. Once the neddylation process is active, SkpA is recruited to the neddylated SCF complex. The only F-box protein, which was found in wild-type and the mutant was the GrrA protein. It was shown that it is involved in ascosporogenesis during sexual development (Krappmann *et al.*, 2006). The Δ*grrA* strain cannot undergo meiosis and therefore displays a sterile phenotype. Several F-box proteins were exclusively recruited by *NeddH/Nedd8 in the *csnE/csn5* strain but not in wild-type. This includes F-Box1 (AN6086), F-box2 (AN6816), F-box15 (AN2505) and F-box23 (AN5593) (Fig. 2C).

We examined whether we find factors among these F-box proteins, which play a role in fungal development. Four of the identified *fbx* genes were deleted (Fig. 3A). The deletion of *fbx1* and *fbx2* resulted in wild-type-like asexual and sexual development. In contrast, deletion of *fbx15* resulted in a fungal strain drastically reduced in both asexual and sexual development. Deletion of *fbx23* resulted in a strain, which in contrast to wild-type, is not repressed in sexual fruit body formation by light. It performs the sexual life cycle under conditions when the asexual development normally is preferred. These data suggest that the CSN is required to prevent the accumulation of neddylated SCF Ub-ligases. They include novel SCFs relevant for development, like SCF-Fbx15, or required for developmental control in response to environmental signals (light), as SCF-Fbx23. This SCF accumulation is linked to the observed block in sexual fruit body formation phenotype of a *csnE/csn5* mutant strain.

CSN stabilizes the F-box protein Fbx15

Several developmentally relevant F-box proteins were enriched by the TAP-tagged *Nedd8/NeddH in the CSN-deficient *csnE*/*csn5* deletion strain. We examined whether a developmental phenotype is typical for F-box encoding genes but could not find a single developmental phenotype when a sample of 20 F-box protein encoding genes was deleted (data not shown). This suggests that the applied biochemical approach specifically enriched developmental F-box proteins. Further analysis addressed whether CSN affects the stability of Fbx15, as the deletion of the corresponding gene causes the most severe developmental phenotype in *A. nidulans.* A gene for GFP-tagged Fbx15 under the control of the constitutively active *gpdA* promoter was ectopically expressed in the wild-type and *csn5*/*csnE* deletion strains respectively. Transformants were verified by Southern hybridization (data not shown). Two strains, which showed similar expressions of the Fbx15 encoding gene in quantitative real-time PCR, were cultivated. Proteins were extracted from vegetative cultures and GFP-Fbx15 levels were compared by western hybridization using anti-GFP and as control anti-actin antisera (Fig. 3C). The wild-type and *csn5*/*csnE* deletion strains show significant differences in the overall Fbx15 protein levels, suggesting that CSN is required to stabilize the Fbx15 protein pool. Protein synthesis was stopped by cycloheximide and the Fbx15 protein levels were monitored at different time points. These data further corroborated that the Fbx15-GFP fusion protein was more stable in the wild-type strain than in the *csn5*/*csnE* deletion strain. This suggests that CSN is required for stabilizing the pool of Fbx15. Presumably, the subpopulation of the protein, which is arrested at the neddylated SCFs during development, was biochemically enriched in the *csn*E/*csn*5 mutant. The data suggest that without an active CSN deneddylase the neddylation/deneddylation cycles are interrupted and the overall pool of Fbx15 is destabilized.

Discussion

Dynamic and transient cycles of neddylation and deneddylation have been proposed to regulate the activity of CRL E3 ubiquitin-ligases catalysing the final step in ubiquitination. This labelling controls stability, activity or localization of target proteins (Wee *et al.*, 2002; Bayram *et al.*, 2010; Helmstaedt *et al.*, 2011). We used the fungus *A. nidulans* as multicellular model system, which does not only produce filaments but specialized tissue to form fungal fruit bodies. Neddylation and deneddylation are not equally important in fungal life, because only neddylation is required for fungal growth as a modular filament. Deneddylation is only required when the fungus is forming the more differentiated cell types (Busch *et al.*, 2003; 2007). We analysed the developmental block caused by defects in deneddylation biochemically and could identify developmentally relevant fungal CRLs.

The highly conserved eukaryotic NEDD8 is expressed in most tissues of plants, slime moulds, fungi and animals (Kumar *et al.*, 1993; Rao-Naik *et al.*, 1998; Burroughs *et al.*, 2007; Ande *et al.*, 2009). Similar to *A. nidulans*, proteins involved in the neddylation process are required for cell viability in *S. pombe* (Osaka *et al.*, 2000). Studies in other organisms revealed that defects in this process also cause drastic flaws in survival due to development instability in *D. melanogaster* (Ou *et al.*, 2002), or early defects in embryogenesis in *C. elegans* and mice (Jones and Candido, 2000; Tateishi *et al.*, 2001). All enzymes responsible for neddylation might act in a complex in

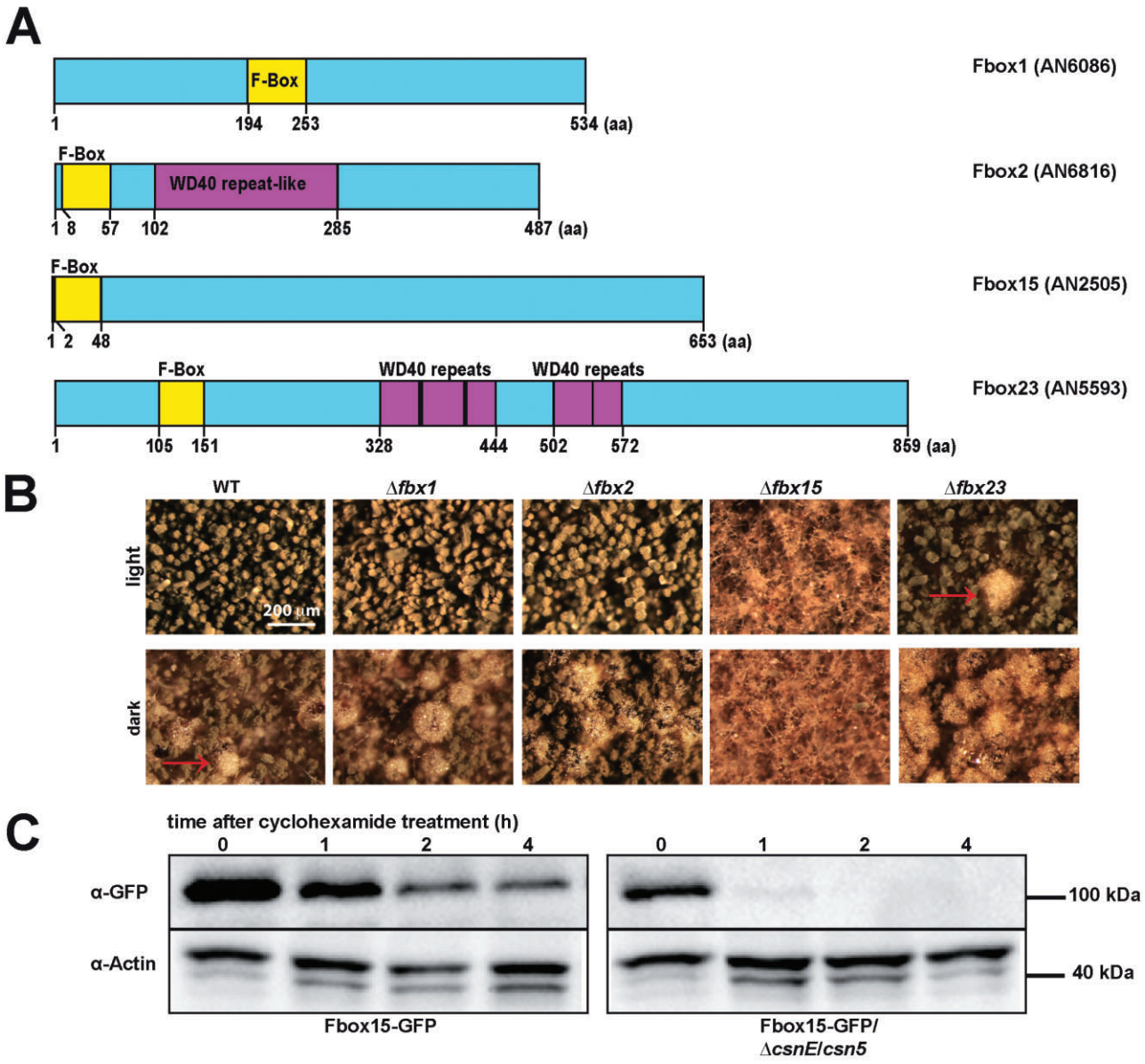


Fig. 3. Analysis of F-box proteins co-purified with neddylated proteins in deneddylase-deficient *csnE/*csn*5 A. nidulans* strain.
A. Conserved domains in identified F-box proteins. Conserved domains were identified using InterProScan (http://www.ebi.ac.uk). Fbx1 and 15 show a F-box domain (IPR022364; SSF81383 for Fbx1 and IPR001810; PS50181 for Fbx15). Fbx2 and Fbx23 show a second conserved motif (WD40-like domain IPR015943; G3DSA:2.130.10.10 for Fbx2 and WD40 repeats IPR019782; PS50082 for Fbx23) beside the F-Box domain (IPR001810; PS0181).
B. Phenotypes of the *fbx* deletion strains. A total of 1×10^4 spores were point inoculated on minimal medium plates and incubated 4 days for asexual (in the light) and 6 days for sexual development (in the darkness). Strains were pictured under stereo microscope.
C. Immunoblot of Fbx15-GFP ectopically expressed in wild-type or the *csnE/csn5* deletion strain using GFP antibody and actin antibody as control. Crude extracts were prepared from 20 h vegetatively grown mycelium (0 h) and mycelium shifted to 25 μg ml^{-1} cycloheximide containing medium for 1, 2 and 4 h.

A. nidulans because we could co-enrich them by tagged NEDD8 after the fungus has established the competence for development. DcnA/Dcn1 interacts *in vivo* with the neddylation machinery, but the interaction might be more transient because we could not co-enrich DcnA/Dcn1 with tagged NEDD8. The amount of neddylated cullins is reduced in the *dcnA/dcn1* deletion strain, supporting that it enhances neddylation *in vivo*, presumably by forming an E3 NeddH/Nedd8-ligase complex with the RING protein. All three fungal cullins seem to be targets of the neddylation machinery during vegetative growth (data not shown) as well as sexual development.

The deneddylase activity of the CSN is required for the detachment of Nedd8 from cullins and therefore the

antagonistic process to neddylation (Schwechheimer and Deng, 2001; Suzuki *et al*., 2002). CSN is required for the control of hormone signalling and tumour growth by regulation of c-Jun and p53 protein levels in mammalian cells (Li *et al*., 2000; Pollmann *et al*., 2001). In insects and plants, CSN malfunction results in post-embryonic lethality (Wei *et al*., 1994; Freilich *et al*., 1999). It remains yet unclear how deregulation of CSN leads to tumour formation, but a variety of studies concerning human cancers show the overexpression of especially subunit CSN5 in these tissues (Kato and Yoneda-Kato, 2009). Various fungal CSN complexes known to date are involved in cellular processes, like circadian clock regulation, cell cycle progression, and pheromone response (Mundt *et al*., 1999; Maytal-Kivity *et al*., 2003; He *et al*., 2005).

The *A. nidulans* CSN deneddylase controls fungal development by the correct balance of fungal hormones (Nahlik *et al*., 2010). CSN complex formation as well as a functional JAMM-deneddylase motif has been shown to be critical in fungal development (Busch *et al*., 2003; 2007). We found here several consequences on a molecular level which are caused by defects in the CSN deneddylase. (i) CsnA/Csn1, which is the second largest protein of the CSN complex, is recruited to neddylated cullins. CsnA/Csn1 has been found in a CSN subcomplex together with the CSN subunits 2, 3 and 8. A second subcomplex includes CsnE/Csn5 and both subcomplexes have been proposed to be linked by CSN1 and CSN6 (Fu *et al*., 2001; Sharon *et al*., 2006; 2009; Dessau *et al*., 2008). Thus, the absence of a functional CSN-deneddylation subcomplex (CSN4,5,6,7) might stabilize the Csn1/CsnA interaction to neddylated CRL-complexes. (ii) The deneddylase activity seems to be further important for the assembly of CulD/Cul4 to DdbA/Ddb1, RbxA/Hrt1/Rbx1 and WD40 proteins, which are not found in the absence of a functional deneddylase. (iii) In contrast, CulA/Cul1 can interact with SCF compounds in the absence of the CSN-deneddylase process. (iv) Neither in the presence nor in the absence of an intact CSN interactors of CulC/Cul3 were identified under the tested conditions. Currently, we do not know, whether this might reflect that the formation of CulC/Cul3 complexes is more transient.

(v) The major finding is that TAP tagged NeddH/Nedd8 recruited several developmental relevant F-box proteins in the *csnE*/*csn5* deletion strain. Our data show that overexpression of *fbx15* leads to high and stable levels of this protein in a wild-type fungus, where CSN is intact and cycles of neddylation and deneddylation are possible. In contrast, a deneddylase-deficient *csnE*/*csn5* deletion strain destabilizes Fbx15. This is consistent with human cell lines, where the protein levels of F-box proteins are decreased upon down regulation of CSN5 caused by autoubiquitination (Cope and Deshaies, 2006). A possible explanation might be that the absence of a functional CSN complex arrests a small subpopulation of specific F-box proteins in SCF complexes. This can be enriched biochemically, whereas the free pool of this F-box protein is instable. The corresponding interaction in wild-type might be more transient and more dynamic and might result in an overall protection of the F-box protein. This suggests that the wild-type fungus with an intact deneddylation machinery stabilizes Fbx15 which might be due to neddylation and deneddylation cycles preventing autoubiquitination. CSN controls the disassembly of F-box proteins from SCF complexes, whereas the SkpA/Skp1 binding to CulA/Cul1 seems to be independent of the CSN-deneddylase activity. Fungal development requires one major permanently assembled SCF, which is GrrA/Fbx29 corresponding to Grr1 in yeast. GrrA in *A. nidulans* is required for the production of mature sexual ascospores (Krappmann *et al*., 2006) and the SCF-GrrA can be as well found in the presence or absence of the CSN. The process of sexual reproduction in *A. nidulans* is linked to amino acid starvation (Eckert *et al*., 1999) and a specific expression of the putative hexose transporter HxtA was observed (Wei *et al*., 2004). Defects in Grr1 can also result in developmental defects in yeast (Butler *et al*., 2006), which is presumably a consequence of the physiological role of Grr1. SCF-Grr1 complexes in yeast are important regulators for adapting to different nutrient conditions. In the presence of glucose, Grr1 mediates the degradation of Mth1, which is a negative regulator for the expression of hexose transporter encoding genes (Flick *et al*., 2003; Spielewoy *et al*., 2004). Furthermore, SCF-Grr1-mediated protein degradation has a role in the activation of genes encoding amino acid permeases (Iraqui *et al*., 1999; Bernard and Andre, 2001).

From approximately 70 possible SCF complexes only a small subpopulation accumulates during fungal development when the COP9 deneddylase is not functional. This links the accumulation of these SCF complexes on a molecular level to the different phenotypes of the *csnE*/*csn5* mutant strain. This mutant is not only blocked in sexual development but also insensitive against the environmental signal 'light' with defects in hormone sensing and secondary metabolism (Busch *et al*., 2007; Nahlik *et al*., 2010). SCF complexes with different F-box proteins might have different target proteins for ubiquitination. The role of the F-box proteins Fbx1 and Fbx2, which associate in the absence of the CSN-deneddylase is not yet elucidated. Fbx15 and Fbx23 seem to have distinct and at least partially antagonistic functions during development. Fbx15 is required for asexual as well as for sexual development. Fbx23 is required for the developmental decision during illumination when asexual development is promoted and simultaneously sexual development is reduced. Similar to GrrA/Fbx29, Fbx23 seems to be required for ascospore maturation and to develop fully grown sexual fruit bodies. In contrast, Δ*fbx15* shows almost no cleistothecia. In

A. nidulans, one important regulator of secondary metabolism and development is the velvet complex composed of VelB/VeA/LaeA. Deletions in the genes encoding VeA and VelB result in a loss of cleistothecia formation (Bayram *et al*., 2008b), whereas the deletion of the gene encoding the methyltransferase LaeA leads to constitutive sexual development and a reduction in fruit body size (Sarikaya Bayram *et al*., 2010). The molecular connection between the described F-box proteins and the regulators of the velvet complex is still elusive.

Our data suggest that development of a complicated multicellular structure, as a fungal fruit body, requires an accurate sequence of SCF activities. Defects in the deneddylase required for SCF E3-Ub ligase complex disassembly disturbs development presumably by the accumulation of specific SCF activities. It will be interesting to see whether an accumulation of developmentally relevant SCF complexes can be also observed during embryonic development of plants or mammals with impaired deneddylase activity.

Experimental procedures

Strains, media and growth conditions

The *A. nidulans* strains used in this study are listed in Table S1. The media used were minimal media (MM; 1% glucose, original high-nitrate salts, trace elements, 2% agar, pH 6.5) with or without 1.2 M sorbitol (SORB) and supplemented with 0.1% pyridoxine-HCl (PYRO), 5 mM each uridine and uracil (UU). Trace elements, vitamins and nitrate salts were included as described by Käfer (1977). BiFC media nitrate promoter control: London medium consists of 1% glucose, 2% salt solution [26 g l^{-1} KCl, 26 g l^{-1} $MgSO_4$, 76 g l^{-1} KH_2PO_4, 5% (v/v) trace elements] pH 6.5 plus 10 mM $NaNO_3$ or 5 mM NH_4-tartrate. Standard genetic techniques for *A. nidulans* were used for all strain construction (Käfer, 1977). *Escherichia coli* strain DH5α was employed for the preparation of plasmid DNA and grown in Luria–Bertani (LB) medium (1% tryptophan, 0.5% yeast extract, 1% NaCl) in the presence of 100 μg ml^{-1} ampicillin.

Transformation procedures

Escherichia coli cells were transformed as described previously (Inoue *et al*., 1990). *A. nidulans* was transformed by polyethylene glycol-mediated fusion of protoplasts as described previously (Punt & van den Hondel, 1992).

PCR-mediated construction of neddH*,* culA*,* skpA*,* rbxA*,* dcnA *and* ubcL *deletion cassettes and Heterokaryon Rescue Technique*

The *neddH*, *culA*, *skpA* and *ubcL* knockout cassettes were generated by applying the *ptrA* resistance marker from *A. oryzae* (Kubodera *et al*., 2000), and *rbxA* knockout cassette was generated by applying the *pyrG* auxotrophic marker from *A. fumigatus*. From each gene a 2000 bp 5′- and 3′-flanking fragment was amplified via PCR using *A. nidulans* TNO2a3 (Nayak *et al*., 2006) gDNA as a template. Fusion PCR was applied according to Szewczyk *et al*. (2006). Briefly, the PCR primers were synthesized with '20 bp tails' such that the gene flanking region fragments anneal to the marker during fusion PCR. Fusion PCR has created a cassette containing the marker surrounded by the genes flanking sequences and transformation with this fragment has led to replacement of the gene with the marker. The polymerase used to amplify the fragments as well the fusion PCR was Platinum® *Taq* DNA polymerase High Fidelity (Invitrogen) or Phusion High Fidelity polymerase (Finnzymes). The sequence of the primers used in this assay is listed in Table S3 and the deletion cassettes were constructed with the following primers: *neddH* – MK003, MK025, MK026, MK027, MK028 and MK007; *culA* – MK042, MK043, MK044, MK045, MK046 and MK047; *rbxA* – RH043, RH044, RH045, RH046, RH047 and RH048; *skpA* – MK206, MK207, MK208, MK209, MK210 and MK211; *dcnA* – RH011, RH012, RH013, RH014, RH015 and RH016; and *ubcL* – MK089, MK090, MK091, MK092, MK093 and MK094. The fusion PCR-mediated cassettes were transformed into the strain TNO2a3. In case of at least three transformations that were not successful, the corresponding deletion strain was tested with the Heterokaryon Rescue Technique according to Osmani *et al*. (2006). The fusion PCR-generated deletion cassettes were transformed in the TNO2a3 strain and recovered by selection with 100 ng ml^{-1} pyrithiamine (*ptrA* selective marker) or 1.2 g l^{-1} uridine and uracil (UU-Afu *pyrG* auxotrophic marker) in the culture media. Spores of primary transformants were replica streaked onto new agar plates with and without the addition of the selection (pyrithiamine or UU) and incubated for 48 h at 30°C. During asexual spore formation, heterokarya carrying both *marker* (–) *geneX* (+) parental nuclei and deleted *marker* (+) *geneX* (–) nuclei produced uninucleate spores with either type of nuclei. When these mixed spores were inoculated on non-selective media, the *marker* (–) *geneX* (+) spores have germinated and have grown into colonies. The *marker* (+) *geneX* (–) spores have germinated but arrested growth due to lack of *geneX* function. However, the same mixed spores from the heterokaryon streaked on selective media are unable to form colonies because the *marker* (–) *geneX* (+) spores cannot grow in the presence of pyrithiamine (100 ng ml^{-1}) and the *marker* (+) *geneX* (–) spores cannot form colonies without *geneX* function. Homologous integration of each cassette at the locus of the gene of interest was monitored by PCR using primers with homology for the region direct after or before the gene in the 5′- or 3′-flanking region and other primers upstream or downstream of the 2000 bp 5′- or 3′-flanking region. The following primers were used and the sequences are listed in Table S3: *neddH* – MK026 and MK041, MK032 and MK041; *culA* – MK138 and MK139; *rbxA* – RH066 and RH067, RH066 and RH046; *skpA* – MK212 and MK213; and *ubcL* – MK190 and MK193. The *dcn*A deletion strain (AGB504) was confirmed by Southern hybridization using the 3′-flanking region as probe (amplified with RH015 and RH016).

Construction of Fbox deletion strains

Deletion cassettes were generated using fusion PCR as described before (Szewczyk *et al*., 2006). The auxotrophic

marker was amplified from plasmid pCDA21 (Chaveroche *et al*., 2000), and is referred to as *zeo-pyrG* cassette, because the amplified fragment contains the zeocin-resistance gene and the *A. fumigatus pyrG* gene. The marker was fused to the 5′- and 3′-flanking regions of the F-box genes and the cassettes were transformed into TNO2A3 (Nayak *et al*., 2006). The following primers were used and the sequences are listed in Table S3: *fbx1*-01-1, 01-2, 01-3, 01-4, 01-zeo, 01-pyr, *fbx2*-02-1, 02-2, 02-3, 02-4, 02-zeo, 02-pyr, *fbx15*-15-1, 15-2, 15-3, 15-4, 15-zeo, 15-pyr, *fbx23*-23-1, 23-2, 23-3, 23-4, 23-zeo, 23-pyr.

Plasmid and strain construction for bimolecular fluorescence complementation (BiFC) and fluorescence microscopy

Plasmids used in this study are listed in Table S2. The cDNA of *dcnA* (primers RH017 and RH018) and *rbxA* (primers MK097 and MK098) were amplified from an *A. nidulans* cDNA library derived from RNA of vegetative grown wild-type mycelia and ligated into the pJET 1.2/ blunt vector (Fermentas) resulting in the plasmids pME3671 and pME3672.

The cDNA of *dcnA* was amplified from pME3672 (primers RH097 and RH098) and fused to the N-Terminal part of eYFP amplified from pME3012 (primers RH099 and OLKM91) (Blumenstein *et al*., 2005) by fusion PCR (Szewczyk *et al*., 2006). The construct was cloned into plasmid pME3160 (Bayram *et al*., 2008b) containing a bidirectional *niiA*/*niaD* promoter using the PmeI site resulting in plasmid pME3673. cDNAs of *rbxA* (primers RH101 and MK030), *neddH* (primers MK98 and RH100) and *ubcL* (primers MK96 and RH102) were amplified from the plasmids pME3671, pME3005 and pME3678 and fused to the C-terminal part of eYFP amplified from pME3013 (primers OLKM86 and OLKM87) by fusion PCR. Fusion constructs were ligated into pME3673 using the SwaI restriction site resulting in the plasmids pME3674 (*eyfp::rbxA*), pME3675 (*eyfp::neddH*) and pME3676 (*eyfp::ubcL*). As control the C-terminal part of eYFP alone was ligated into pME3673 using the SwaI restriction site resulting in pME3677. The plasmids were transformed into the *A. nidulans* strain AGB152, resulting in the strains AGB500, AGB501, AGB502 or AGB503 respectively. Ectopical integration was confirmed by Southern hybridization with a nitrate promoter probe amplified from genomic DNA of the wild-type strain A4 (primers OLKM67 and OLKM68). The strains were incubated in London media supplemented with nitrate to induce the promoter, or with ammonium to repress the promoter. Microscopy was performed as described (Helmstaedt *et al*., 2008).

*Construction of a mature TAP-tagged neddH cDNA (*NeddH) cassette and introduction into* A. nidulans *wild-type and Δ*csnE *strain*

The DNA-cassette containing the mature *neddH* cDNA sequence (without the C-terminal processed part) fused at its 5′ end with the TAP-tag (*S. aureus* protein A, TEV, calmodulin binding domains) sequence was constructed using fusion PCR strategy (Szewczyk *et al*., 2006) and ligation reactions. The construction into the vector pGEM®-5Zf (+)/nTAP (Busch *et al*., 2007) was performed in two steps. First, the ligation of the fusion PCR-mediated cassette *neddH* cDNA*::neddH*-terminator (411 bp)::*pyroA* (*A. fumigatus* auxotrophic marker)::*neddH* 3′-flanking region (2000 bp) into the SacI and MluI restriction sites of the vector pGEM®-5Zf(+)/nTAP. The PCR-amplified *neddH* 5′-flanking region (2000 bp) was ligated in the SphI and ApaI restriction sites of the previous vector, resulting in the plasmid pME3612. The template and primers used to amplify the fragments were: (i) pME3005 to amplify the mature *neddH* (MK029 and MK030); (ii) genomic DNA of TNO2a3 strain to amplify 2000 bp *neddH* 5′-flanking region (MK003 and MK004), 411 bp terminator (MK031 and MK005R) and 2000 bp *neddH* 3′-flanking region (MK006F and MK007); and (iii) genomic DNA of *Aspergillus fumigatus* to amplify *pyroA* auxotrophic marker (MK005F and MK006R). The primer sequences used for amplification are listed in the Table S3. The PCR reaction, as well as the fusion PCR, was performed with Platinum® *Taq* DNA polymerase High Fidelity (Invitrogen) or Phusion High Fidelity Polymerase (Finnzymes) respectively. The cassette was removed from the vector by digestion with BamHI and introduced by transformation in *A. nidulans* TNO2a3 strain and integrated into the *neddH* locus. The transformants were recovered onto selective culture medium (MM + UU + SORB). The integration of TAP-tag::*neddH* (**neddH*) cassette into the locus of *neddH* was confirmed by Southern hybridization and also, the DNA sequence region was verified by sequencing (data not shown). The TAP-tag::*neddH* cDNA Δ*csnE A. nidulans* strain is the result of a genetic crossing of TAP-tag::*neddH* cDNA strain with the strain AGB209.

Plasmid and strain construction for Fbx15-GFP overexpression

The *fbx15::gfp* fusion was generated using fusion PCR (Szewczyk *et al*., 2006). *fbx15* was amplified from genomic DNA of *A. nidulans* using the primers OZGFBX15-Start and OZGFBX15-GFP. *sgfp* was amplified from pME3185 (Bayram *et al*., 2008a) using the primers OZG207 and OZG208. The two fragments were fused in equimolar amounts with the primers OZG15-Start and OZG208. The *fbx15–sgfp* fusion was cloned into the PmeI restriction site of pME3856, resulting in plasmid pME3950. The plasmid was ectopically introduced into AGB152 and AGB209. The resulting strains AGB678 and AGB679 were verified by Southern hybridization and RNA expression levels were confirmed by qRT-PCR using the primers JS110 and JS111.

Tandem affinity purification (TAP)-tag purification

TAP was performed by the modified method of Busch *et al*. (2007). Briefly, the total protein of TAP-tag:: *neddH* cDNA strain and its parental strain (TNO2a3), as well as the TAP-tag::*neddH* cDNA *ΔcsnE* were extracted by incubation of the grinded mycelia or sexual structures in liquid nitrogen with mortar and pestle with BufferB* [300 mM NaCl, 100 mM Tris-HCl pH 7.2, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol (DTT), and protease inhibitors cocktail – Complete, EDTA-free; Roche]. The purification was followed by incubation of the crude extract with IgG-agarose (Amersham

Biosciences/GE Healthcare) for 3 h on a rotating platform at 4°C. The suspension was poured into a Bio-Rad PolyPrep column (Bio-Rad Laboratories), in which beads were washed twice with IPP300 (25 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.1% NP-40, 2 mM DTT), once with IPP150 (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40, 2 mM DTT), and once with 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). In the sealed column, beads were incubated with 1 ml of TEV cleavage buffer containing 300 U TEV protease on a rotating platform for 16 h at 4°C. Proteins cleaved from the beads were eluted into a reaction tube, and elution was repeated with 1 ml of TEV cleavage buffer. After addition of 6 ml 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 β-mercaptoethanol) and 6 µl of 1 M $CaCl_2$, the solution was incubated with calmodulin beads (Stratagene) on a rotating platform for 4 h at 4°C. The calmodulin beads were equilibrated with 5 ml of CBB prior to incubation and after incubation were washed twice with 1 ml of CBB containing 0.1% NP-40 and with 1 ml of CBB containing 0.02% NP-40. Proteins were eluted with 3 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 β-mercaptoethanol). The final eluate was precipitated by trichloroacetic acid (TCA), including the adjustment of aliquots to 25% TCA, incubation 16 h. Centrifugation at 4°C, 10 000 *g* for 1 h, and two washes with cold acetone. After being dried in a speed vacuum, the pellet was resuspended in protein loading dye. After separation on a SDS-PAGE gradient gel electrophoresis (8–20%) protein bands were stained with Colloidal blue staining and cut out of the gel.

Protein identification by tandem mass spectrometry

Peptides of in-gel trypsinated proteins (Shevchenko *et al*., 1996) 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, Amsterdam, the Netherlands) prior to mass analyses with a LCQ DecaXP mass spectrometer (Thermo Scientific, San Jose, USA). 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 analysed 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.

Vegetative, sexual and asexual development induction

The conidia from *A. nidulans* strains were grown in a reciprocal shaker at 30°C for 16–24 h in liquid MM medium (plus supplementation if necessary). Mycelia were aseptically transferred to fresh MM agar medium and incubated at 30–37°C to induce the asexual (presence of light and normal aeration) and sexual (absence of light and sealed plates) development. Each sample was harvested by filtration through Miracloth sheet, washed thoroughly with washing solution (0.98% NaCl, 1 mM phenylmethylsulphonyl fluoride), 1% dimethylsulphoxide) and quickly frozen in liquid nitrogen. The time-points collected were: vegetative 24 h (v24), asexual development 12, 24, 48 h (a12, a24, a48), and sexual development 24, 48, 72 h (s24, s48, s72).

Protein stability assay using cycloheximide

Strains were grown under vegetative conditions for 20 h in liquid minimal medium at 37°C. The mycelium was harvested by filtration through a sterile miracloth sheet, washed with 0.98% NaCl solution and further incubated in liquid MM containing 25 µg ml^{-1} cycloheximide for 1, 2 and 4 h at 37°C.

Protein assays and Western hybridization analysis

The total protein was extracted by grinding the mycelia and incubating it at 4°C with extraction buffer (300 mM NaCl, 100 mM Tris-HCl pH 7.2, 10% glycerol, 0.1% NP-40, 1 mM DTT, and protease inhibitors cocktail – Complete, EDTA-free; Roche). The protein concentration was determined by use of a modified Bradford assay (Bio-Rad), and samples bands were fractionated in SDS-PAGE gradient (8–20%) or 12% non-gradient gel. After protein separation, the gel was blotted onto a pure nitrocellulose membrane (0.2 µm; Bio-Rad), and after being blocked in 5% dried milk TBS/T buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20), the membrane was probed with anti-Calmodulin binding protein antibody (Upstate/Millipore), GFP antibody (Santa Cruz), actin antibody (Sigma), tubulin antibody (Sigma) or anti-NeddH/Nedd8 antibody (Genescript) respectively. As secondary antibody horseradish peroxidase-coupled goat anti-rabbit IgG (Invitrogen) or rabbit anti-mouse (Jackson ImmunoResearch) was used. The blot was developed by enhanced chemiluminescence method (Tesfaigzi *et al*., 1994). The signals intensity was quantified using Fusion-SL7 system with Bio1D software (Peqlab). As control strains the corresponding parental strains TNO2a3 for the *dcnA* deletion strain and AGB152 for the *csnE* deletion strain were used.

mRNA isolation

Aspergillus nidulans mycelia was disrupted by grinding with liquid nitrogen, and total RNA was extracted with RNeasy plant Kit (Qiagen). To verify RNA integrity, 20 µg of RNA from each sample was fractionated in 2.2 M formaldehyde, 1.2% agarose gel, stained with ethidium bromide, and visualized with UV light. The presence of intact 28S and 18S ribosomal RNA bands was used as the criteria to determine if the RNA was degraded.

RT-PCR and quantitative PCR

DNase digestion and subsequent cDNA synthesis was carried out in duplicates for each sample using 0.8 µg of 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 *A. nidulans* primers (Table S3). 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). The amount of gene of interest relative to histone H2A RNA was quantified using the ΔCT method with efficiency (Pfaffl et al., 2002). All qRT-PCR experiments were performed at least in triplicate.

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