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The COP9 signalosome mediates transcriptional and metabolic response to hormones, oxidative stress protection and cell wall rearrangement during fungal development

Krystyna Nahlik,[1†] Marc Dumkow,[1] Özgür Bayram,[1] Kerstin Helmstaedt,[1,2] Silke Busch,[1] Oliver Valerius,[1] Jennifer Gerke,[1] Michael Hoppert,[1] Elke Schwier,[1] Lennart Opitz,[3] Mieke Westermann,[4] Stephanie Grond,[4] Kirstin Feussner,[1] Cornelia Goebel,[5] Alexander Kaever,[1] Peter Meinicke,[1] Ivo Feussner[5] and Gerhard H. Braus[1*]

[1]*Institut für Mikrobiologie & Genetik, Georg-August-Universität, D-37077 Göttingen, Germany.*
[2]*DFG Research Center for Molecular Physiology of the Brain (CMPB), Georg-August-Universität, D-37077 Göttingen, Germany.*
[3]*Transkriptom Analyse Labor, Georg-August-Universität, D-37077 Göttingen, Germany.*
[4]*Institut für Organische und Biomolekulare Chemie, Georg-August-Universität, D-37077 Göttingen, Germany.*
[5]*Albrecht-von-Haller Institut für Pflanzenwissenschaften, Georg-August-Universität, D-37077 Göttingen, Germany.*

Summary

The COP9 signalosome complex (CSN) is a crucial regulator of ubiquitin ligases. Defects in CSN result in embryonic impairment and death in higher eukaryotes, whereas the filamentous fungus *Aspergillus nidulans* survives without CSN, but is unable to complete sexual development. We investigated overall impact of CSN activity on *A. nidulans* cells by combined transcriptome, proteome and metabolome analysis. Absence of *csn5*/*csnE* affects transcription of at least 15% of genes during development, including numerous oxidoreductases. *csnE* deletion leads to changes in the fungal proteome indicating impaired redox regulation and hypersensitivity to oxidative stress. CSN promotes the formation of asexual spores by regulating developmental hormones produced by PpoA and PpoC dioxygenases. We identify more than 100 metabolites, including orsellinic acid derivatives, accumulating preferentially in the *csnE* mutant. We also show that CSN is required to activate glucanases and other cell wall recycling enzymes during development. These findings suggest a dual role for CSN during development: it is required early for protection against oxidative stress and hormone regulation and is later essential for control of the secondary metabolism and cell wall rearrangement.

Accepted 27 August, 2010. *For correspondence. E-mail gbraus@gwdg.de; Tel. (+49) 551 393771; Fax (+49) 551 393330. †Present address: Centre for Infection and Immunity, Queen's University, Belfast BT9 7BL UK.

Introduction

The conserved COP9 signalosome (CSN) multiprotein complex is present in higher eukaryotic cells (Wei *et al.*, 1994). CSN controls light regulation, pathogen response, pigment production and seedling survival in plants and is involved in regulation of tumour growth, inflammation, hormone signalling, oxygen homeostasis and early development in mammals. Defects in CSN result in embryonic lethality, presumbably because CSN affects various processes at the cellular level including the ubiquitin–proteasome pathway, DNA damage response, cell cycle control and gene expression (Wei *et al.*, 2008). The main known molecular function of CSN is the control of the activity of cullin–RING E3 ubiquitin ligases (CRLs) conveyed by the metalloprotease-containing Csn5 subunit. CSN is able to regulate CRLs by cleaving the ubiquitin-like peptide Nedd8 from lysine residues of cullins (Wu *et al.*, 2005). In addition, CSN-associated proteins mediate phosphorylation and deubiquitination of substrates, including transcription factors such as plant HY5 (Osterlund *et al.*, 1999) or cJUN (Naumann *et al.*, 1999) in animals affecting their stability and activity (Naumann *et al.*, 1999; Sun *et al.*, 2002). CSN also controls transcriptional networks as a transcriptional corepressor of thyroid hormone receptor (Tenbaum *et al.*, 2003) and as a transcriptional repressor during *Drosophila* development (Oron *et al.*, 2007). Fungi often possess incomplete and non-essential versions of CSN, but the mould *Aspergillus nidulans* carries an eight-subunit CSN holocomplex (Busch *et al.*, 2007). *A. nidulans* CSN is required for fruit body formation but is not essential for filamentous growth or asexual sporulation (Busch *et al.*, 2003).

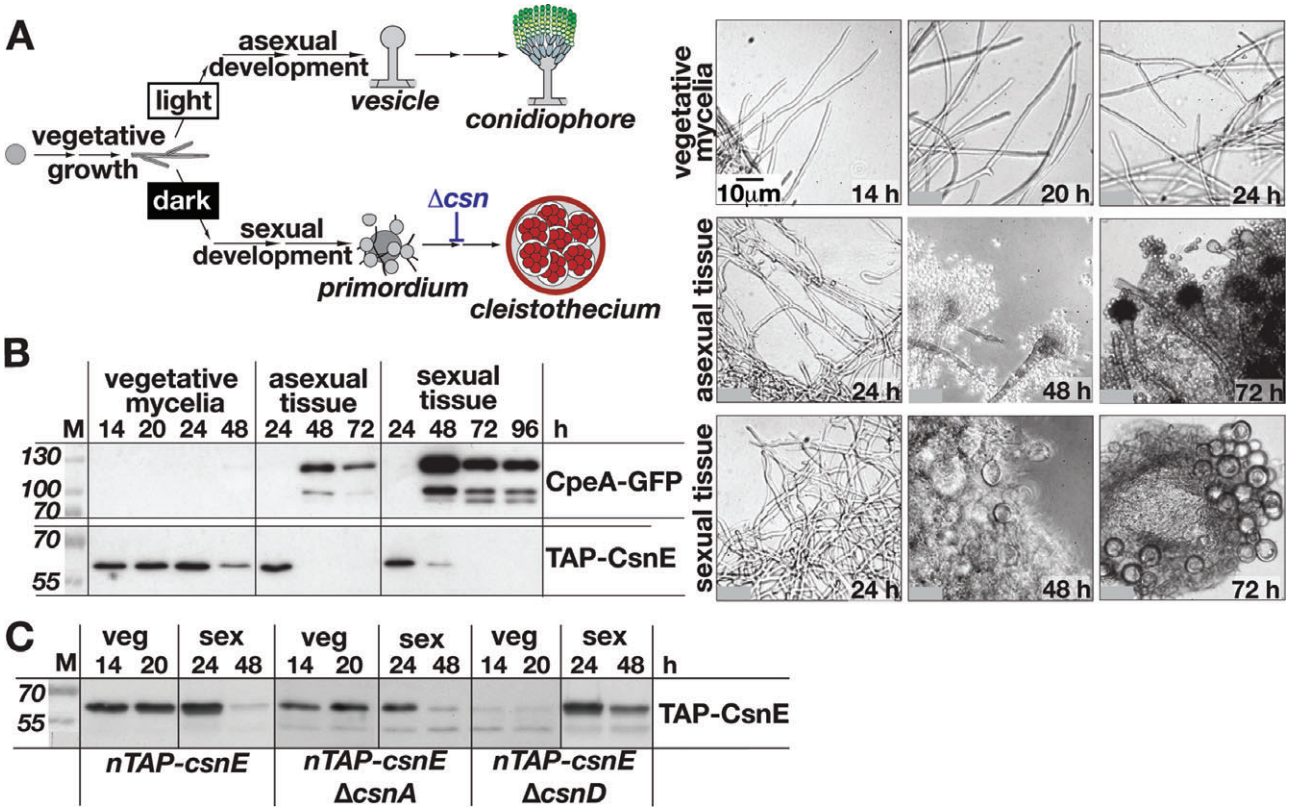


Fig. 1. Fungal CsnE/CSN5 protein expression during development.
A. *A. nidulans* development shown schematically and in light microscopy.
B. Protein expression of TAP-CsnE during development. Crude extracts of strain AGB381 carrying a functional TAP-CsnE and CpeA-sGFP were analysed by Western blot. CpeA-sGFP served as development-specific control.
C. Protein expression of TAP-CsnE in strains defective in other CSN subunits during development. Protein levels of TAP-CsnE were monitored in strain AGB252 (*TAP-csnE*), AGB253 (Δ*csnA*) or AGB254 (Δ*csnD*).

In contrast to simple modular vegetative hyphae, the development of sexual fruit bodies results in a complex three-dimensional structure and requires formation of specialized cells and tissues. *A. nidulans* fruit body formation can be induced in the dark by oxygen limitation and is inhibited by light, which causes primarily airborne asexual spores to be formed (Adams *et al.*, 1998; Bayram *et al.*, 2008a; Purschwitz *et al.*, 2008). CSN is essential for this light-dependent control and its defects result in 'blind' organisms constitutively forming initial sexual tissues. Mature *A. nidulans* fruit bodies are closed cleistothecia, developing from primordia emerging from nests (Fig. 1A). Within these fruit bodies, meiotic cells are enclosed by maternal envelope tissue and surrounded by nursing Hülle cells. CSN is required for the light-dependent inhibition of fruit body formation, but also for its maturation, because defective CSN results in a block in fruit body formation at the state of primordia (Busch *et al.*, 2003).

In filamentous fungi, reactive oxygen species (ROS) produced by NADPH oxidases provide a specific trigger for sexual development (Lara-Ortiz *et al.*, 2003; Aguirre *et al.*, 2005). Endogenous lipogenic signal molecules called psi (precocious sexual inducer) factors, derived from linoleic acid, also play a role in regulation of spore development; the proportion of different psi factors controls the ratio of asexual to sexual spore production (Tsitsigiannis *et al.*, 2004a). The reorganization of vegetative hyphae to form the specialized cells and tissues and ultimately the structure of the fruit body is accompanied by massive cell proliferation. This process requires mobilization of energy and building materials, as well as protection of the fruit body, co-ordinated by internal signalling. The energy accumulated during growth in cell wall glucans has to be mobilized by degrading enzymes during development, mostly α-1,3-glucanases such as MutA, expressed during sexual development in Hülle cells (Wei *et al.*, 2001). Protecting the fruit body involves developmental synchronization of the synthesis of specific secondary metabolites including bioactive molecules with the potential to repel fungivores (Rohlfs *et al.*, 2007; Bayram *et al.*, 2008b).

We took advantage of an organism possessing a complete non-essential CSN to investigate the molecular mechanisms of CSN and its role in *A. nidulans* develop-

ment by a first combined transcriptome, proteome and metabolome analysis of this fungus. We found that vegetative CSN functions necessary to handle oxidative stress are linked to development. CSN is required to set up the stage for the light-mediated decision between asexual and sexual development by ensuring the appropriate ratio of psi factor hormone-like molecules. CSN also appears to protect the fungus during the internal ROS signalling events triggering development. Furthermore, in absence of CSN we found dramatic changes in secondary metabolism and reduced expression of glucanases necessary to mobilize sugars stored in cell wall during development.

Results

Fungal CSNE deneddylase is expressed during vegetative growth and early development

Aspergillus nidulans CSN expression was estimated by monitoring the appearance of the CsnE/CSN5 deneddylase subunit during different developmental stages (Fig. 1B). A functional N-terminal fusion of *csnE* to TAP tag (TAP-CsnE) was highly expressed in vegetative mycelia and expression ceased by 48 h in developmentally induced tissues. Reduction of CsnE protein levels coincided with the time point of the developmental block in Δ*csn* strains. Developmental markers such as the catalase–peroxidase (CpeA-sGFP), expressed in Hülle cells (Scherer *et al*., 2002), only appeared at this time point (Fig. 1B), confirming that CSN expression ceases after development is initiated. The pattern of CsnE expression implies that CSN activity is restricted to vegetative mycelia and early development, prior to the appearance of the Δ*csn* phenotypes.

CsnE is able to assemble the CSN holocomplex only when all other subunits are present (Busch *et al*., 2007), but the levels of CsnE do not depend on the presence of other subunits. Expression of CsnE was monitored in strains lacking CsnA/CSN1 or CsnD/CSN4, which showed the same developmental block as Δ*csnE* (Fig. 1C). We observed the same pattern of TAP-CsnE expression independent on the presence of the complex. Whereas TAP-CsnE ran as a single band in the presence of all other *csn* genes, additional lower molecular weight bands appeared in strains lacking CsnA or CsnE. This suggests that an intact CSN complex might prevent degradation of the CsnE subunit.

CSN affects transcription of 15% of the fungal genome during development

Microarrays of *csn* mutants in *D. melanogaster* revealed an achronic gene expression pattern, suggesting that CSN acts as a transcriptional corepressor during development (Oron *et al*., 2007). We compared fungal transcriptomes of wild-type and Δ*csnE* mutant strains during development to monitor the effects of CSN on gene expression at different developmental stages. Four time points were compared pairwise between both strains (Fig. S1): after 14 and 20 h of vegetative growth in liquid culture (V14 and V20, respectively, before and after achieving developmental competence) and after 48 h of growth on solid medium, induced to develop either asexually in light (A48) or sexually in darkness (S48). At these time points mutant and wild-type morphologies were similar (Fig. 2A and B).

The microarray data derived from two biological and four technical replicates revealed that the expression levels of 15.4% of 10 546 annotated *A. nidulans* genes were strongly ($\log_2$ ratio ≥ 3.0 and adjusted $P \leq 0.01$) or moderately ($\log_2$ ratio ≥ 2 and adjusted $P \leq 0.01$) altered in the mutant throughout development (Table S1). The expression of 1256 genes, representing 11.9% of the genome, was moderately altered, and expression of 369 genes, representing 3.5% of the genes on the array, was strongly altered at least at one developmental stage. The transcript levels of the remaining seven CSN subunits were similar in wild-type and Δ*csnE* (data not shown). Only the genes showing strongly altered expression were analysed further.

Differentially expressed genes were assigned to 11 functional categories based on available annotation or similarities to characterized proteins from other organisms (Fig. 2C). During vegetative growth, the most changes in the Δ*csnE* mutant were observed among genes involved in secondary metabolism and redox reactions. In developing cultures, CSN repressed many genes playing a role in protein degradation, stress response and defence, cell wall biosynthesis as well as redox reactions, which were mainly upregulated in the mutant (Table S2). At the same time, CSN activated numerous genes involved in secondary metabolism and cell wall degradation, accordingly downregulated in the mutant.

During vegetative growth CSN acts mainly as a transcriptional activator, with two to three times (at V20 and V14 respectively) as many genes downregulated than upregulated in the Δ*csnE* mutant. Under development-inducing conditions, the number of up- and downregulated genes was similar (Table S1). More than half of the genes differentially expressed during vegetative growth showed parallel regulation at both time points; notably, twice as many genes were downregulated in Δ*csnE* at V14 than at V20. Thus, the transcriptome underwent significant changes in the absence of the COP9 signalosome even before achieving developmental competence by the fungus. These data do not support an exclusive transcriptional corepressor function of CSN, but suggests its involvement in control of both transcriptional activation

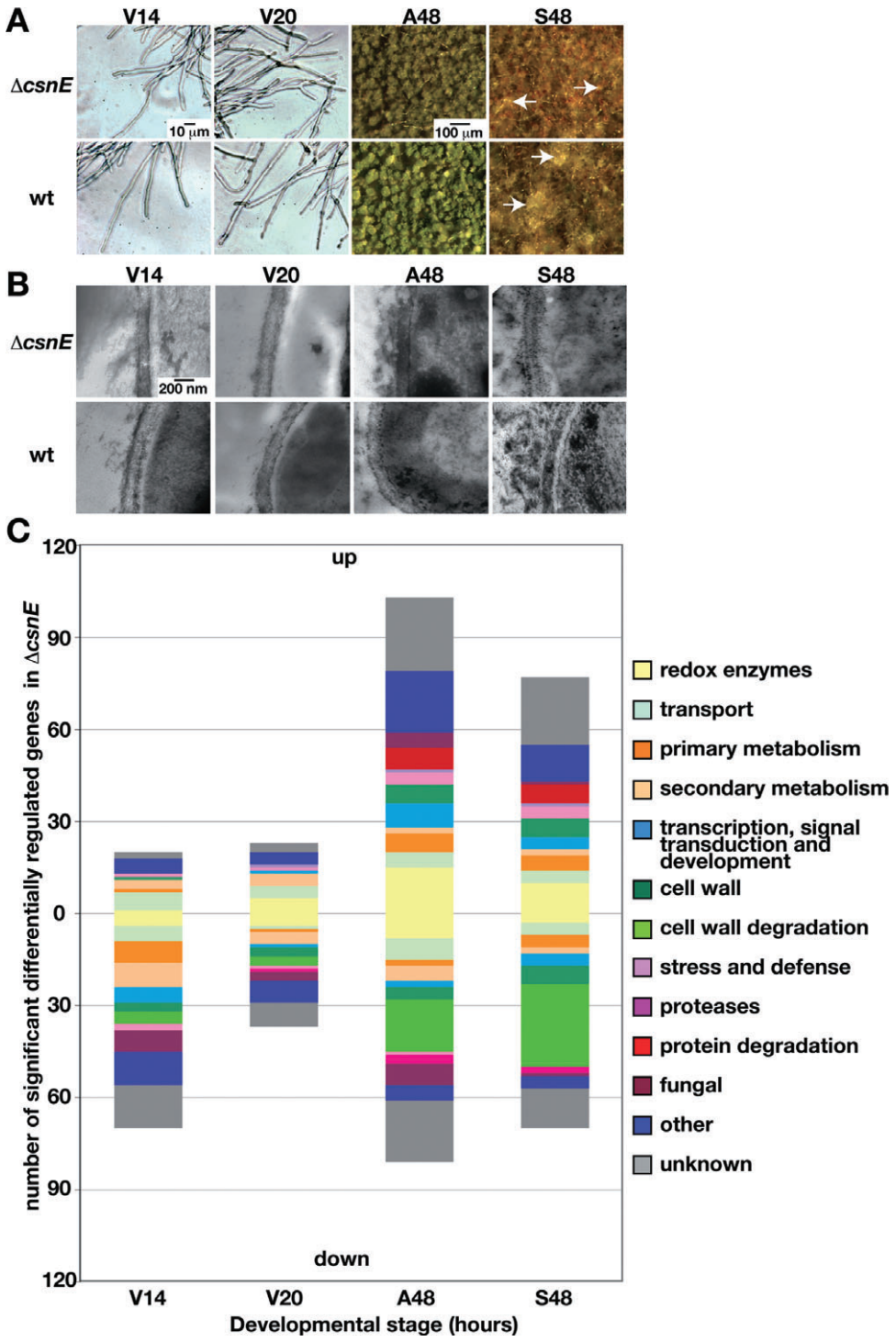
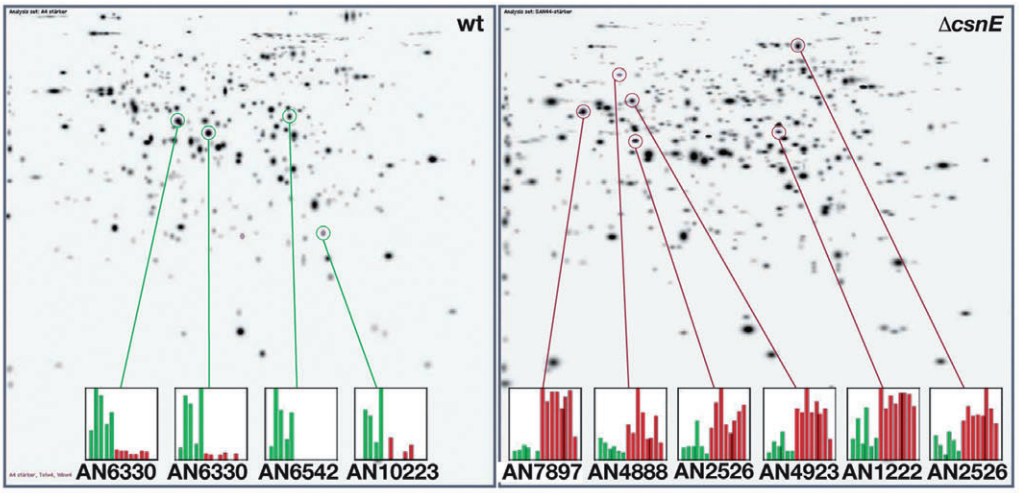


Fig. 2. CSN-dependent transcriptome during fungal development.
A. Light microscopy. Hyphal morphologies of wild-type and *ΔcsnE A. nidulans* grown vegetatively (V14 and V20) are similar. Sexual development (S48) leads to formation of nests (white arrows) in both strains; only the mutant will later be blocked at the primordia stage. During asexual development (A48), mutant and wild-type look similar, but only the mutant will later develop sexual nests, which are inhibited in wild-type.
B. Transmission electron microscopy images of wild-type and *csnE* mutant. *A. nidulans ΔcsnE* mutant and wild-type cell wall thickness is similar at the time points of transcriptome analysis.
C. Transcriptome analysis summary. Genes with $\log_2$ ratio ≥ 3.0 were used for categorization. The 'fungal' category denotes significantly conserved or unique fungal genes with no assigned function; 'other' denotes genes with assigned function of other categories.

A



B

AN#	X_{corr}/pep	% amino acid identities						proposed function
		H. sapiens		*A. thaliana*		*S. cerevisiae*		
AN10223.3	3.03 / 06	46.7	PRDX6	42.6	ATPER1	47.7	Prx1p	peroxiredoxin
AN6330.3	5.29 / 12	66.7	EF-2	62.3	EF-2	78.0	Eft2p	translation elongation factor 2
AN6542.3	5.48 / 09	90.1	ACTG1	83.3	ACT1	92.0	Act1p	actin
AN4888.3	5.22 / 11	---		31.9	PDC4	46.6	Pdc6p	pyruvate decarboxylase
AN2526.3	6.30 / 12	---		22.9	Ilv-like	69.5	Ilv5p	ketol-acid reductoisomerase
AN4923.3	4.38 / 09	43.2	HMGCS1	44.4	HMGsc-like	56.7	Erg13p	hydroxymethylglutaryl (HMG)-CoA synthase
AN7897.3	5.34 / 11	---		---		---		monooxygenase-like
AN1222.3	4.59 / 06	66.6	MAT2A	58.7	Sam-like	74.0	Sam2p	S-adenosylmethionine (SAM) synthetase

Fig. 3. CSN-dependent fungal proteome during acquisition of developmental competence.
A. Crude extracts from *A. nidulans* wild-type (wt) or Δ*csnE* strains grown vegetatively for 20 h (V20) were separated by 2D gel electrophoresis.
B. *A. nidulans* proteins from spots in (A) identified by mass spectrometry. Best cross-correlation coefficients (X_{corr}), number of different peptides (pep), comparison to known proteins and their proposed function are given.

and repression. Although CSN is expressed only during the early stages of development (until 24 h), the largest changes in the transcriptome were observed later in development (at 48 h), indicating delayed downstream effects on gene expression.

csnE *deletion leads to changes in protein production reflecting impaired redox regulation*

CSN-mediated deneddylation regulates the activity of cullin-based ubiquitin ligases, which should be reflected by changes in the stability of proteins directed for degradation in the proteasome. To examine this post-transcriptional role of CSN, we examined the changes in the proteome of the *ΔcsnE* strain during vegetative growth in the time window when developmental competence is acquired (Fig. 3). After 2D gel electrophoresis of soluble protein extracts, 10 spots representing eight unique proteins with significant differences in their intensities between the two strains were identified by mass spectrometry (Table S3).

Deletion of *csnE* significantly increased the levels of five proteins (Fig. 3). They include four metabolic enzymes involved in carbon and amino acid metabolism. The branched-chain amino acid biosynthetic enzyme ketol-acid reductoisomerase and carbon metabolism enzyme pyruvate decarboxylase were described as oxidative stress targets in fungi (Cabiscol *et al.*, 2000; Reverter-Branchat *et al.*, 2004; Yoo and Regnier, 2004). *S*-adenosylmethionine (SAM) synthetase is required for biosynthesis of cysteine, which is a precursor of the anti-oxidant glutathione. HMG-CoA synthase is a key enzyme in the mevalonate pathway required for synthesis of many primary and secondary metabolites. The putative monooxygenase is thought to be involved in detoxification reactions or complex metabolite modifications (Harayama *et al.*, 1992).

The levels of three proteins are decreased in the *csnE* deletion mutant. The first two, actin and elongation factor 2 (EF-2), have also been described as targets of oxidative stress (Cabiscol *et al.*, 2000; Reverter-Branchat *et al.*,

2004; Yoo and Regnier, 2004). The third protein is a peroxiredoxin, mediating protection against oxidative stress; peroxiredoxins are important cellular antioxidants identified in a wide range of organisms (Rhee *et al*., 2005).

This proteome comparison reveals an effect of CSN on the levels of cytoplasmatic proteins sharing a connection to oxidative stress. Although putative stress-related genes were also affected by CSN at the transcriptional level, there was almost no correlation between differentially expressed genes and proteins during vegetative growth. This is not surprising as the changes in the proteome are likely due to a direct impact of CSN on protein stability, whereas the transcriptional regulation probably reflects a role of CSN in regulating the corresponding transcription factors. A notable exception is the putative monooxygenase, which is less abundant at both transcript and protein level in the *ΔcsnE* strain and might be worth investigating further.

CSN controls stress and defence genes making the ΔcsnE *strain sensitive to redox imbalance*

Comparative analysis of the vegetative transcriptome and proteome suggested a significant impact of CSN on stress and defence-related processes. The largest group of genes affected in the *ΔcsnE* strain encode putative oxidoreductases of primary or secondary metabolism (Fig. 2C, Table S4). Genes encoding proteins of known enzymatic function were mostly assigned to the primary or secondary metabolism functional categories. Therefore, the 'redox enzymes' category comprises mostly oxidoreductases where the molecular function remains to be determined. The transcriptome data suggest that CSN affects some of these genes during vegetative growth and others primarily during development.

Northern hybridization of four oxidoreductase transcripts verified this phase-specific transcriptional regulation (Fig. 4). CSN induces developmental transcription of *catD* gene, encoding catalase D required for oxidative stress response and expressed in Hülle cells (Aguirre *et al*., 2005). It also activates transcription of a putative defensin gene primarily during vegetative growth. On the other hand, CSN represses a tyrosinase gene involved in protective melanin production during vegetative growth and a gene encoding a putative secondary metabolism oxygenase throughout the life cycle (Fig. 4, Table S4).

These changes in gene expression translate to marked differences in response to oxidative stress between wild-type and Δ*csnE* strains (Fig. 5A). Δ*csnE* is more sensitive to menadione (which increases cellular levels of ROS) and to the thiol-oxidizing drug diamide causing redox imbalance. Diamide shifts the cellular ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Accordingly, GSSG had a greater limiting impact on growth of the Δ*csnE* strain than GSH. Treatment with either GSH or GSSG resulted in concentration-dependent appearance of red colouring in the growth medium of the mutant, and to a lesser extent in the wild-type. It had been described earlier that aging *csn* mutant colonies also accumulate a red pigment even in the absence of any redox stress (Busch *et al*., 2003). Under conditions inducing asexual development, low GSH concentrations led to increased number of sexual structures in all strains, whereas higher concentrations increased asexual conidiation (data not shown). The growth of the Δ*csnE* strain was also impaired on medium supplemented with 4% ethanol as an inducer of oxidative stress (data not shown). Furthermore, Δ*csnE* was sensitive to increased temperature (Fig. S2).

A functional CSN deneddylase is required for efficient redox and temperature stress response of *A. nidulans*. Both transcriptome and proteome analysis support a strong connection between oxidative stress and fungal development and point towards CSN-mediated regulation of catalase D as well as many still uncharacterized oxidoreductases in this process, ensuring resistance to a wide variety of oxidizing and other stress factors.

Loss of CSN leads to significant changes of the fungal metabolome, inducing accumulation of orsellinic acid-derived metabolites and sterigmatocystin intermediates

We investigated the chemical composition of compounds producing the red colour released to the growth medium in the mutant to explore the link between oxidative stress and the mutant phenotype. The yield of the compounds increased after cultivating the wild-type and Δ*csnE* strains on the surface of liquid medium for 10 days. Whereas several high polymeric compounds could not be identified, extraction of culture filtrates with ethylacetate and thin-layer chromatography revealed many more unique red-coloured substances in the Δ*csnE* mutant than in the wild-type (Fig. 5B). Strains grown on ammonium instead of nitrate as a nitrogen source produced highest yields. Pure compounds were isolated and five orsellinic acid-related metabolites could be identified: orcinol, diorcinol, cordyol C, violaceol I and violaceol II (Figs 5C, S3 and S4). Four colourless substances were obtained, probably undergoing polymerization to produce yellow, orange or red coloured substances when dissolved in methanol.

The archetypal polyketide orsenillic acid is produced by enzymes encoded by a recently discovered *A. nidulans* gene cluster (Schroeckh *et al*., 2009). Indeed, several orsellinic acid synthesis genes are differentially expressed in the *ΔcsnE* mutant (Table S4). *orsB*, encoding an amidohydrolase and *orsC*, encoding a tyrosinase

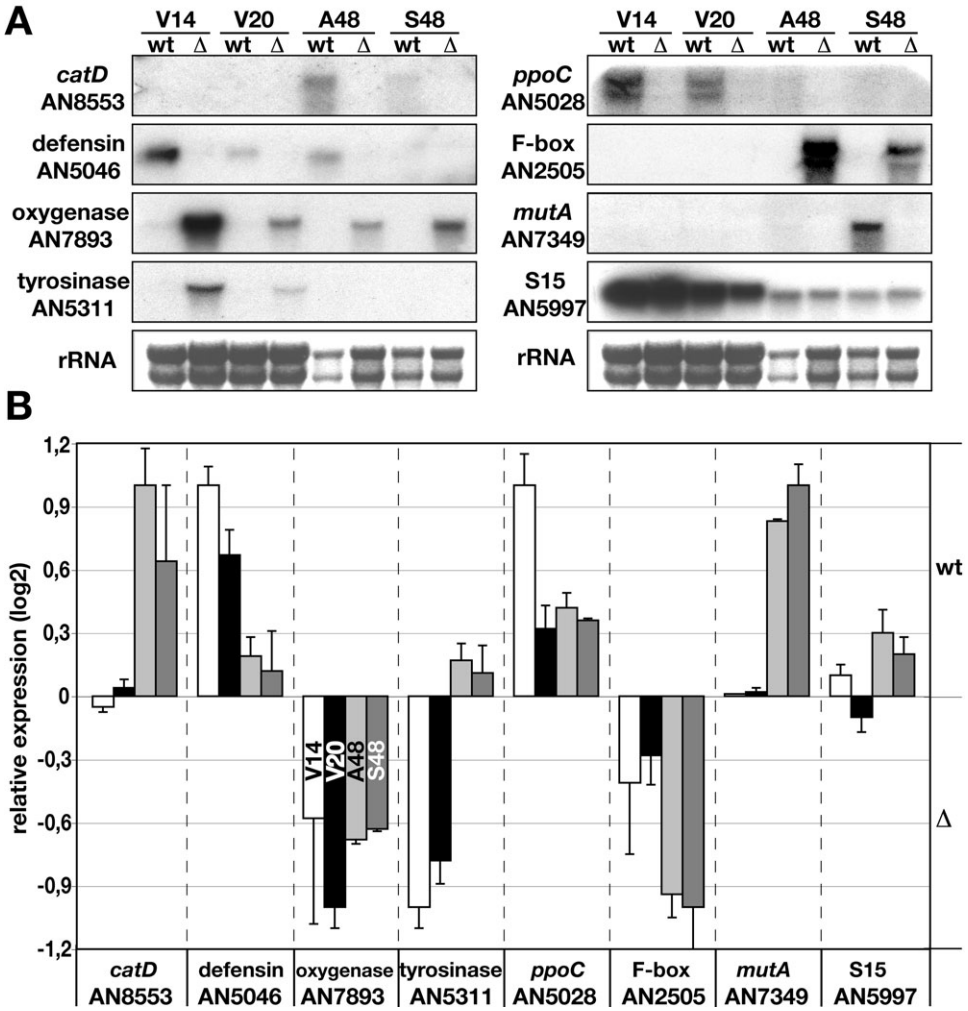
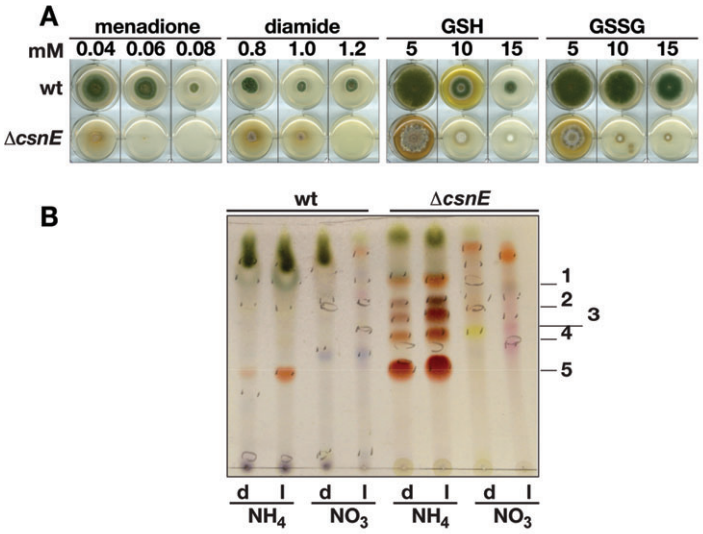


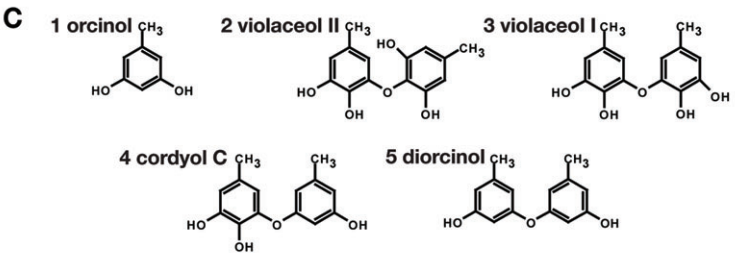
Fig. 4. Comparison of CSN effect on gene expression by Northern and microarray analysis.
A. Northern hybridization. Ribosomal protein S15 (AN5997) was used as internal control. *mutA*, *ppoC* and *catD* transcriptional activation fails in the *ΔcsnE* strain at S48, V14/V20 and A48 respectively. Genes for an F-box protein (AN2505) and tyrosinase (AN5311) are misactivated in the mutant strain at A48/S48 and V14/V20 respectively. The AN7893 oxygenase gene expression is activated and AN5046 defensin gene expression inhibited at all times in *ΔcsnE* strain. V, vegetative growth; A, asexual development (in light); S, sexual development (in the dark).
B. Transcript quantification. Normalized $\log_2$ ratios of expression levels of genes of (A) derived from two biological and two technical replicates of microarray experiments. The time point with the highest expression value of each gene was set to 1 (100%).

are upregulated in the *ΔcsnE* strain under asexual conditions and *orsB* also under sexual conditions. In contrast, the *orsA* alcohol dehydrogenase gene is repressed during late vegetative growth in the mutant (V20). This suggests that the production of these secondary metabolites by the mutant is mainly a result of transcriptional derepression.

A more comprehensive untargeted metabolite fingerprinting analysis was then performed at different time points to investigate intracellular compounds. Overall, the analysis revealed more than 100 metabolite markers differing in their occurrence at least at one developmental stage between wild-type and *ΔcsnE* mutant. Intensity-based clustering by one-dimensional self-organizing maps (1D-SOMs) (Meinicke et al., 2008) was used to get a global overview, shown here for marker candidates of the non-polar extracts (negative ionization mode). The intensity profiles of the marker candidates are represented by 20 prototypes separated in blocks of different intensity patterns (Fig. 6A). A first dominant block represents *ΔcsnE*-specific marker candidates, which accumulate mainly under asexual (prototypes 6–8) or sexual conditions (prototype 13) in the mutant. A second block (prototypes 16–20) contains developmental markers specific for the wild-type and downregulated in *ΔcsnE* strain. Prototypes 10–12 and 14–15 can be combined to a block representing mutation-independent markers specific for asexual and/or sexual development.

Prototype 7 revealed several markers, identified as intermediates of the sterigmatocystin (ST) biosynthetic pathway and strongly accumulated in the *ΔcsnE* strain at





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

Fig. 5. Δ*csnE* strain has impaired resistance to oxidative stress and produces an unusual secondary metabolite pattern.
A. Sensitivities of wild-type (wt) and Δ*csnE* strains to the indicated concentrations of menadione, diamide, GSH or GSSG.
B. Secondary metabolite production of wild-type and Δ*csnE* under grown in NH_4-medium (NH_4) or NO_3-medium (NO_3) in dark (d) or light (l). The numbers '1–5' indicate the identified orcellinic acid derivates.
C. Structures of the identified substances: 1, orcinol; 2, violaceol II; 3, violaceol I; 4, cordyol C; 5, diorcinol.

A48 time point (Fig. 6B, Table S6). Accordingly, transcription of genes of ST and ST-like pathways, presumably involved in the biosynthesis of polyketide secondary metabolites (Brown *et al*., 1996), was upregulated in the mutant at this time (Table S4). Figure 6C summarizes the correlation between Δ*csnE* metabolome data and the corresponding upregulated genes of the *A. nidulans* ST gene cluster: *stcE* encodes a putative norsolorinic acid reductase producing averantin (Brown *et al*., 1996), *stcW* encodes a monooxygenase synthesizing 1′-hydroxyversicolorone (Keller *et al*., 2000), the *stcU*-encoded versicolorin reductase catalyses demethyl-ST production, and *stcV* encodes another norsolorinic acid reductase with a predicted role in reduction of averufanin. The function of *stcC* is still unknown.

We next analysed secretion of ST to the growth medium and found that the Δ*csnE* mutant produced significantly less ST than wild-type (Fig. S5). It seems that transcriptional *stc* gene upregulation in the mutant (Table S4) resulted in an accumulation of ST intermediates (Fig. 6B), but also in a defect in production and secretion of the end product, which normally protects developmental structures against threatening organisms in the soil.

CSN controls the ratio of psi factors acting as A. nidulans *sexual hormones*

Light normally stimulates formation of asexual spores and blocks sexual development in wild-type *A. nidulans,* but not in Δ*csnE.* Two genes, *ppoA* and *ppoC,* encode antagonistic dioxygenases involved in biosynthesis of psi factors, lipogenic developmental hormones. A subtle balance between different psi factors is required to shift between developmental programs (Tsitsigiannis *et al*., 2004b). *ppoA* is necessary for formation of sexual and *ppoC* of asexual spores. Both overexpression of *ppoA* and deletion of *ppoC* shift the developmental balance towards sexual sporulation (Tsitsigiannis *et al*., 2004a). *ppoC* expression is necessary already during vegetative growth to enable normal development, but it is present at

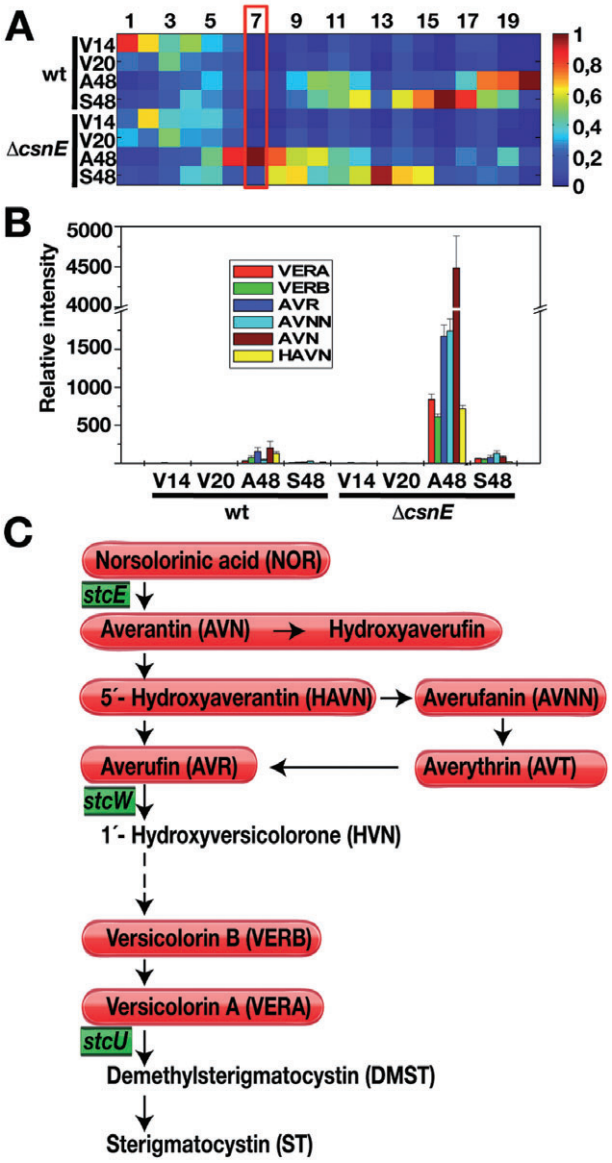


Fig. 6. CSN-dependent metabolite fingerprinting during fungal development.
A. 1D-SOM matrix after metabolite-based clustering of the non-polar extracts analysed with negative ionization mode of 443 marker candidates. Intensity profiles of 20 prototypes (horizontal dimension) of wild-type and Δ*csnE* were compared; time points correspond to these of the transcriptome analysis. Colours of matrix elements represent average intensity values.
B. Intensity profiles of six selected ST biosynthesis intermediates of prototype 7, which accumulate only in the Δ*csnE* mutant at A48 and to a lower extent at S48.
C. Accumulated intermediates of the sterigmatocystin (ST) pathway. The indicated ST intermediates (red boxes) and *stc* transcripts (green boxes) were increased in the Δ*csnE* mutant at A48.

much higher levels present in light (at A48) than in dark (at S48), which is necessary to induce asexual development in the wild-type (Fig. S6). The Δ*csnE* mutant showed a strong upregulation of the *ppoA* gene in light (at A48), accompanied by a reduction in *ppoC* expression (Table S4) not only during development but also in early vegetative growth phase, prior to any visible morphological change (Fig. 4, Table S4).

We went on to investigate whether these differences in *ppoA* and *ppoC* expression are affecting the actual psi hormone factor levels, which would confirm an early role of CSN in regulation of psi factor biosynthesis (Figs S6 and S7). Three oxylipins, the PpoA products 8-hydroxy-9-octadecanoic acid (8-HOE) and 8-hydroxy-9,12-octadecadienoic acid (8-HOD) (Brodhun *et al.*, 2009a) as well as the PpoC product 10-hydroxy-9,12-octadecadienoic acid (10-HOD) (Brodhun *et al.*, 2009b) could be detected in cultures. Concentration of PpoC-dependent 10-HOD required for asexual sporulation is reduced at all time points in the absence of CsnE, which is the most likely cause of the increased initiation of sexual structures by the mutant (Fig. 7A). Concentration of PpoA-derived 8-HOD is similar in wild-type and mutant strains during vegetative growth, but reduced in the mutant during development (Fig. 7B), whereas levels of 8-HOE are reduced in the mutant during late vegetative growth and asexual development (Fig. 7C). The ratio of both PpoA-derived psi factors, 8-HOE and 8-HOD, is important for the balance between sexual and asexual spore development (Tsitsigiannis *et al.*, 2004b). Sexual and asexual development are characterized by different 8-HOE to 8-HOD ratios; 1:8.3 under sexual conditions as compared with 1:2.7 under asexual conditions (Table 1). High *ppoA* expression in the Δ*csnE* mutant perturbs this balance resulting in a constantly high 8-HOD proportion both in light (1:18.7) and in darkness (1:15.1), which is likely the driving factor behind uncontrolled initiation of sexual structures.

Our data indicate that CSN is required prior to initiation of development during vegetative growth, as well as at early asexual developmental stages, to ensure the right balance of sexual hormones. This is critical for the expression of the master transcriptional regulator of asexual sporulation *brlA*, which was indeed downregulated in the mutant in light (Table S4). The *brlA*-dependent *wA* encoding a polyketide synthase producing the conidial yellow pigment was also downregulated (Table S4).

CSN controls the activation of cell wall degrading enzymes including β-glucanases

The main metabolic transcriptional networks involved in carbon metabolism were not significantly affected in the Δ*csnE* mutant. However, expression of a large group of genes associated with cell wall degradation was very strongly dependent on a functional CSN. They were mainly downregulated in the mutant after the initiation of development, comprising 21% and 39% of all downregu-

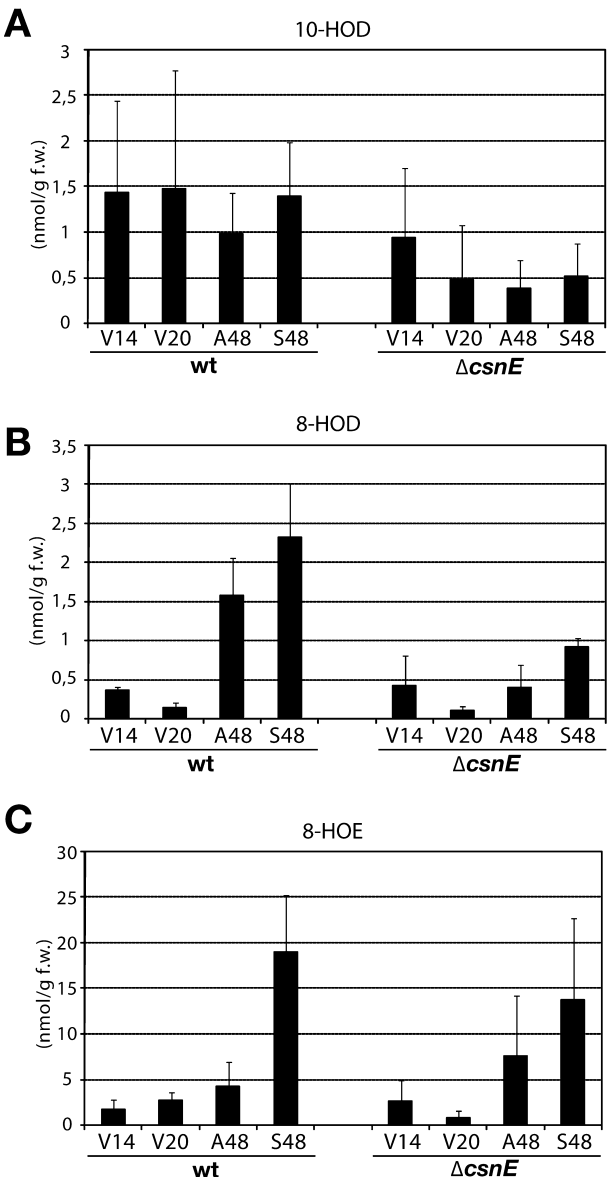


Fig. 7. Amounts of the PpoA and PpoC-derived psi factors 8-HOE, 8-HOD and 10-HOD produced in the Δ*csnE* mutant and the wild-type.
A. 10-HOD levels (B) 8-HOD and (C) 8-HOE levels during different developmental stages of the Δ*csnE* mutant and the wild-type. Psi factors were purified using reversed phase HPLC and detected by GC MS. Measurement was performed for both strains at all developmental time points.

Table 1. Ratios of oleic to linoleic acid-derived psi factors in the *A. nidulans* Δ*csnE* mutant and the wild-type at different developmental time points.

Time point	Strain	8-HOE : 8-HOD
V14	Δ*csnE*	1:6.2
	Wild-type	1:4.8
V20	Δ*csnE*	1:8.2
	Wild-type	1:19.6
A48	Δ*csnE*	1:18.7
	Wild-type	1:2.7
S48	Δ*csnE*	1:15.1
	Wild-type	1:8.3

8-HOE, 8-hydroxy oleic acid; 8-HOD, 8-hydroxy linoleic acid.

lated genes at A48 and S48 respectively (Tables S2 and S5). This group of genes encode chitinases, glucanases and enzymes involved in degradation of plant cell wall material, such as cellobiose hydrolases (Table S5). Strikingly, 32 of the 37 glycosyl hydrolases present in the *A. nidulans* genome were differentially expressed at least at one developmental stage and 14 were downregulated during development. Northern hybridization confirmed that Δ*csnE* was unable to express the cell wall degrading α-glucanase gene *mutA* during sexual development (Fig. 4B). Surprisingly, only one more α-glucanase gene was affected, while CSN had most impact on genes encoding putative β-glucanases with a potential to hydrolyse both β-1,3- and β-1,4-glucans (Table S5). Laminarinase (β-1,3-glucanase) activity was reported to increase in developing cultures (Zonneveld, 1975; Bagga *et al.*, 1989), but there was no indication up to date that it is required for sexual development.

We measured cytoplasmic laminarinase activity and confirmed that it was first induced after 48 h of development. The presence of CsnE was necessary for this induction during both asexual and sexual development (Fig. S8). During metabolite fingerprinting we detected accumulation of various disaccharides, including maltose, galactose, galactosamine and *N*-acetylgalactosamine during sexual and asexual development in the wild-type strain, most likely as a result of high glucanase and chitinase gene expression (Table S6). Developmental downregulation of α- and β-glucanases in the Δ*csnE* mutant, concomitant with upregulation of a chitin synthase gene (Table S5), resulted in a reduced accumulation of disaccharides. These findings reveal the importance of CsnE in regulating accumulation of cell wall-derived sugars acting both as a reserve for energy-consuming developmental processes and materials for cell wall remodelling.

The CSN-dependent cellular remodelling also involves membrane transport, which is apparent by major changes in expression of membrane transport-related genes induced by *csnE* deletion (Fig. 2C). The second largest group of genes differentially expressed at all developmental stages (up to 8%) fell into this 'transport' category. They encoded proteins facilitating membrane transport of different metabolites, predominantly carbohydrates, amino acids and toxins. This might be an additional reason why only the wild-type accumulated β-glucan and chitin decomposition products, which provide building blocks for restructuring cell wall during development.

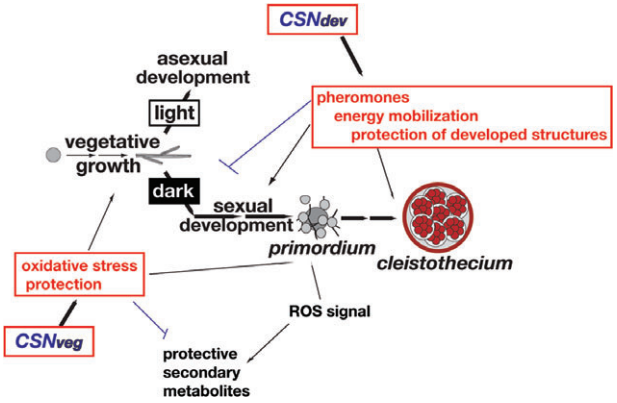


Fig. 8. CSN and fungal development. Vegetative CSN activities (CSN_{VEG}) are required to protect the fungus against oxidative stress. They are prerequisite for proceeding through development beyond the primordial state, where an internal oxidative stress signal is required. Development-specific CSN activities (CSN_{DEV}) inhibit the sexual pathway by light. They shift the ratio of psi factors which are fungal hormones. They also activate cell wall degrading enzymes to restructure the cell wall for development and control the production of protective secondary metabolites.

Discussion

The underlying cause of embryonic lethality in higher eukaryotes deficient in CSN is a crucial question in the field of COP9 signalosome research. We used a filamentous fungus which can survive without CSN, but is impaired in sexual development as a model to investigate the role of COP9 signalosome in development. This work represents the first comprehensive genome-wide transcriptome, proteome and metabolome analysis of both the COP9 signalosome function and of fungal development. As a result we were able to identify multiple processes depending on an intact CSN during the differentiation of the complex multicellular structure of the fruit body (Fig. 8). It is feasible that the role in some of these processes, which are conserved on a molecular level in multicellular eukaryotes, is making CSN indispensable for plant and animal development. The presence of CSN is required to ensure resistance to oxidative stress, the right concentration of sexual hormones, protection of the fruit body (including the production of toxic secondary metabolites) and mobilization of cell wall glucans during development.

A connection between CSN and oxidative stress response was observed in higher eukaryotes, where CSN5 binding partners accumulate under hypoxic conditions. CSN5 associates with several redox-sensitive transcription factors such as AP-1 (Abate *et al.*, 1990), NF-κB (Galter *et al.*, 1994), p53 (Bech-Otschir *et al.*, 2001) and with the antioxidative enzyme thioredoxin (Hwang *et al.*, 2004). Plant seedlings of CSN mutants accumulate the antioxidant anthocyanin, which is upregulated by high levels of light-induced ROS (Dohmann *et al.*, 2005). Protection against oxidative stress is required for fungal development, because a transient increase in ROS levels acts as an internal signal for developmental progression (Aguirre *et al.*, 2005). This resistance, involving the expression of *catD* catalase gene (Fig. 4), is dependent on the COP9 signalosome, and while not essential for normal growth it seems to set the stage for development by allowing the cell to quench the ROS signal and avoid oxidative damage. ROS-mediated signalling balanced by protection conferred by endogenous antioxidants is emerging as a shared feature in the regulation of multicellular development (Covarrubias *et al.*, 2008; Matsuzawa and Ichijo, 2008).

The *A. nidulans csnE* mutant strain develops Hülle cells, but cannot develop sexual structures beyond the stage of primordia. Hülle cells have a nursing function and supply the developing fruit bodies with nutrients. Developmental progression is also accompanied by specific expression of catalase peroxidase and NADPH oxidase (NoxA) in Hülle cells (Scherer *et al.*, 2002). These enzymes lead to the production of superoxide and H_2O_2. Genes for Nox enzymes are restricted to fungi with the potential for fruit body formation, but are common in higher eukaryotes. Fungal *ΔnoxA* strains are blocked at the same primordial state (Lara-Ortiz *et al.*, 2003) as *csn* deletion strains. Thus, the most apparent effect of CSN is involved in the only ROS-dependent developmental transition known so far in fungi. In mammalian development, the first ROS-induced programmed cell death occurs in the blastocyst, causing apoptosis of inner cells destined to develop into trophectoderm (Parchment, 1993). Strikingly, mouse embryos lacking Csn5 survive to the blastocyst state but die soon after implantation without undergoing gastrulation (Tomoda *et al.*, 2004), suggesting a corresponding involvement of COP9 in ROS-mediated signalling. During the acquisition of developmental competence in *A. nidulans* a protective peroxiredoxin PrxB (AN10223) is expressed in emerging Hülle cells of wild-type, but not of the *ΔcsnE* strain (data not shown). The PrxB murine homologue Prdx6 plays an important role in antioxidant defence in the lungs and protects the lens epithelium from UV-induced cell death (Manevich and Fisher, 2005; Kubo *et al.*, 2010). It would be interesting to see if mammalian Prdx6 is also targeted by CSN.

Impaired oxidative stress response resulting in the accumulation of unusual secondary metabolites becomes apparent in the *ΔcsnE* mutant already during vegetative growth. Oxidative stress responses require catalase, superoxide dismutase and peroxiredoxin enzyme activities as well as thioredoxin and glutathione as redox buffering systems (Aguirre *et al.*, 2005). Increased levels of *S*-adenosylmethionine synthetase (required for glutathione biosynthesis) and of HMG-CoA synthase levels in the mutant suggest increased isoprenoid biosynthesis

presumably to produce protective carotenoids (Iigusa *et al*., 2005), providing an additional indication of elevated oxidative stress (Fig. 3).

The red-coloured compounds conspicously released into the medium by the *csn* mutant are more complex than we had anticipated (Figs 5, S3 and S4). They include several orsellinol- and orsellinic acid-derived compounds that have only recently been assigned to *A. nidulans* when co-cultivated with a competitor organism, *Streptomyces hygroscopicus* (Schroeckh *et al*., 2009). Production of these metabolites depends on a physical interaction of *A. nidulans* and *S. hygroscopicus* and comprises a putative defence mechanism induced by the exchange of ROS. Secretion of these phenolic compounds by the hypersensitive mutant could also be interpreted as a visible constititutive reaction to oxidative stress. Moreover, gene expression of several oxidoreductases and a polyketide synthase in several yet unexplored gene clusters involved in the production of unknown secondary metabolites is upregulated in the Δ*csnE* strain (data not shown), suggesting an even broader fungal metabolic repertoire in response to stress.

The presence of CSN during vegetative growth is prerequisite for developmental processes accompanied by rearrangement of the cell wall. This requires the activity of numerous cell wall degrading enzymes and a major adjustment of the membrane transport systems to shuttle metabolites. Δ*csnE* strain showed a marked decrease in expression of genes involved in cell wall and membrane-associated functions (Table S5), indicating that a functional CSN is required for cell wall remodelling. In particular, it is necessary for induction of β-glucanase activity targeting β-1,3-glucans and possibly also β-1,4-glucans (Fig. S8), which appear to be mobilized along with α-glucans during development. The accumulation and subsequent degradation of cell wall α-glucans is necessary for fruit body formation in *A. nidulans* (Zonneveld, 1972; 1974). β-Glucans belong to an alkali-insoluble fraction of the *Aspergillus* cell wall and were thought to be responsible mostly for its structural framework. Production of endo-β-1,4-glucanases is induced during cleistothecial development (Bagga *et al*., 1989), but a requirement for β-glucanase activity for fruit body formation had not been described. The extent of cell wall remodelling necessary for fruit body formation might therefore be much greater than previously anticipated.

Other genes involved in cell wall remodelling are also affected in the Δ*csnE* mutant, which is consequently unable to accumulate decomposition products of α- and β-glucans as well as of chitins during development. Concomitant upregulation of putative genes responsible for synthesis of α-glucan and chitin suggests that CSN is either directly or indirectly required for cell wall recycling during development at many levels. Accumulation of mono- and disaccharides, which is impaired in the mutant (Table S6), might be an early trigger for the initiation of later steps of sexual development that are blocked in the Δ*csnE* mutant.

Synthesis of secondary metabolites by fungi is intimately related to development; toxins such as ST have been proposed to protect against fungivores (Rohlfs *et al*., 2007). CSN appears to be required for production of secondary metabolites coinciding with development. The Δ*csnE* mutant produces almost no ST, which is accompanied by a misregulation of genes in the ST biosynthesis pathway and an accumulation of various ST intermediates during development. CSN was also necessary for the expression of genes encoding cell wall proteases (Table S5) and protective structural components of the spore wall, mainly hydrophobins (Table S7). Taken together, these data suggest that CsnE is crucial in co-ordination of multiple pathways, which ensure the protection of newly produced sexual structures against various external threats.

Aspergillus nidulans Δ*csnE* strain is unable to complete and regulate sexual development, and constitutively develops sexual Hülle cells and primordia. CSN-dependent secondary metabolites also include psi factors, hormone-like oxylipins, which have direct sporogenic effects and regulate the balance of asexual and sexual spore formation in *A. nidulans.* Control of psi synthesis depends on at least two *ppo* genes encoding psi factor-producing fatty acid oxygenases (Tsitsigiannis *et al*., 2005). PpoA and PpoC have antagonistic functions, whereby PpoC ultimately promotes asexual and PpoA sexual sporulation (Brodhun *et al*., 2009a). CSN is required for the expression of *ppoC* in early vegetative stage, which probably contributes to the mutant's inability to inhibit sexual development under asexually inducing conditions such as light. Indeed, misregulation of *ppoA* and *ppoC* results in an imbalance of psi factor biosynthesis. Constitutive *ppoC* downregulation is reflected by permanently low levels of the PpoC-derived 10-HOD during vegetative growth (Fig. S7). Decreased 10-HOD concentation at an early vegetative stage, when developmental competence is not yet achieved, seems to be the underlying cause of the light-insensitive phenotype of the mutant. Moreover, the ratio of the PpoA-derived psi factors 8-HOE to 8-HOD is essential for the timing and balance of asexual and sexual differentiation (Tsitsigiannis *et al*., 2004a). The lower the ratio, the more the developmental balance shifts towards asexual differentiation. Constitutively increased *ppoA* expression in the mutant results in the distortion of the 8-HOE : 8-HOD ratio (Table 1), further enhancing the shift of developmental balance towards sexual differentiation.

The effect of CSN on hormone signals could account for the lack of transcriptional activation of the asexual

master regulatory gene *brlA* by light in the *ΔcsnE* mutant. The BrlA transcription factor initiates a cascade of additional regulatory genes including the AbaA transcription factor, which further activates *brlA* and numerous downstream genes (Andrianopoulos and Timberlake, 1994). CSN is known as a negative regulator of stability of transcription factors in other systems, such as JY5 in plants and p53 in mammals. The stability of these proteins is modulated by CSN-regulated degradation by the ubiquitin system (Bech-Otschir *et al*., 2001; Schwechheimer *et al*., 2002). It is currently unknown whether CSN regulates the stability or activity of developmental transcription factors in *A. nidulans*. However, genes encoding components of the ubiquitin system were upregulated in the mutant during development and include several putative F-box proteins (Table S7, Fig. 4A), which convey target specificity in SCF ubiquitin ligases, a prototypical group of CRLs. Analysis of promoter regions of genes downregulated during development in the *ΔcsnE* mutant revealed statistically overrepresented binding sites for AbaA transcription factor (Andrianopoulos and Timberlake, 1994); McmA, a homologue of yeast transcription factor Mcm1 involved in sexual development (Passmore *et al*., 1989); and stress response elements (STREs) interacting proteins (Marchler *et al*., 1993) (Fig. S6). These factors do not appear to be regulated by CSN on a transcript level, suggesting that it might be acting instead by regulating their protein stability, which remains to be investigated experimentally.

We assume that most of the effects observed in the fungal *ΔcsnE* mutant result from the lack of deneddylase activity, removing Nedd8 ubiquitin-like peptide from CRLs. Deneddylation regulates the activity of CRLs, which are disassembled and can be reassembled only after another round of neddylation (Cope *et al*., 2002). We hypothesize that the wide scope of vegetative and developmental CSN functions in *A. nidulans* reflects the regulation of various CRLs fulfilling regulatory roles in different regulatory networks and time points. As additional function, it has been shown in *Neurospora crassa* that CSN is also involved in the circadian clock (He *et al*., 2005). The identity of neddylated proteins involved in the control of *A. nidulans* development and secondary metabolism remains to be explored. Another open question is whether CSN-associated proteins mediating phosphorylation and deubiquitination of substrates are also involved in these processes (Naumann *et al*., 1999; Sun *et al*., 2002).

In summary, we demonstrate that CSN exerts a broad role, both during vegetative growth when it is crucial for determining later developmental fate and during development itself. Our data suggest a much greater extent of cell wall remodelling prior to fruit body formation than previously recognized in fungi. The broad metabolic repertoire of CSN-deficient strain confirms the fungal potential for producing novel secondary metabolites under stress revealed by recent research. Our data also provide evidence for a crucial biological role of CSN in regulating redox homeostasis of eukaryotic cells, especially during processes dependent on ROS signalling. Combined deficiencies in these functions, although not essential for vegetative growth of a single cell or modular organism under laboratory conditions, might be the reason why the development of multicellular plant or mammalian embryo, or a fungal fruit body cannot be completed. Our comprehensive analysis opens further avenues into research on the molecular functions of the COP9 signalosome in filamentous fungi, both as a way to explore novel natural products and as a model of mammalian development.

Experimental procedures

Aspergillus nidulans *culture*

Aspergillus nidulans strains were cultivated at 37°C or 30°C in minimal medium and fungal development was induced as described (Clutterbuck, 1974). Briefly, asexual sporulation was induced by incubation in continuous white light and cleistothecia formation by oxygen-limiting conditions on plates sealed with cellophane film grown in the dark. Oxidative stress assays were performed with 4% ethanol, menadione (0.04, 0.06, 0.08, 0.10 mM), diamide (0.8, 1.2, 1.4, 1.6 mM), GSH or GSSG (5, 10, 15, 20 mM), added to the medium. Growth, conidiation and cleistothecia development of *A. nidulans* was observed by differential interference contrast microscopy (DIC).

Microarray RNA extraction, labelling and hybridization

RNA was extracted from 2 ml of ground fungal mycelium with Trizol (Invitrogen), followed by a double phenol/chlorophorm extraction (Chomczynski, 1993). The RNA was then treated with DNase I for 20 min at 37°C in the presence of RNAseOUT (Invitrogen). RNA was extracted with phenol/chlorophorm/isoamyl alcohol and additionally purified using RNeasy columns (Qiagen, Hilden, Germany). For each array hybridization, 1 μg RNA was amplified using the Message Amp II kit (Ambion, Darmstadt, Germany) and 2 μg resulting aRNA was labelled with NHS-activated Cy-3 or Cy-5 dyes (Amersham Bioscience). RNAs from two independent biological replicates were analysed and four technical replicates were performed.

The TIGR *A. nidulans* microarrays were provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA. The polymer-coated glass arrays contained 11 481 70-mer oligonucleotide probes spotted in duplicate, providing 98% genome coverage of 10 701 predicted genes. The slides were prehybridized in 5× SSC, 0.1% SDS, 1% BSA for 1 h at 65°C. After washing in distilled water and isopropanol the slides were dried in nitrogen stream. Approximately 1.5 μg (*c*. 130 pmol) of each labelled RNA was hybridized to the slides using the Agilent '60-mer Oligo Microarray Processing' protocol. The slides were washed and

dried in a nitrogen stream. Following hybridization, spot intensities were determined using an Agilent G2505B microarray scanner.

Data analysis

Microarray intensity data were extracted using the 'Automatic Image Processing for Microarrays' software (Katzer *et al*., 2003). Normalization of the raw intensity data was performed with a non-linear loess regression method. Differentially expressed genes were identified by an ANOVA fixed effects model (Landgrebe *et al*., 2004). Normalization and statistical computation was performed for two independent data sets derived from a high gain and a low gain scan. 'Contrasts' refer to $\log_2$ normalized intensity ratios between wild-type and *ΔcsnE* samples. Genes with $\log_2$ ratios of ≥ 3.0 and adjusted *P*-values of ≤ 0.01 were regarded as significantly regulated and genes with $\log_2$ ratios of ≥ 2 and adjusted *P*-values of ≤ 0.01 were regarded as moderately regulated. The data are deposited in NCBI's Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pluhlwwaocgqmpa&acc=GSE22442 and were generated conforming to the MIAME guidelines. Gene probes were annotated and assigned to functional categories manually by homology searches via translated nucleotide BLAST algorithm (blastx). Each gene probe was assigned to one functional category only.

Immunoblotting

Approximately 15–20 μg of crude extracts was separated by SDS-PAGE, electroblotted and hybridized with rabbit anti-calmodulin binding protein epitope tag (Upstate/Milliore, Schwalbach, Germany), mouse anti-GFP (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) or rabbit anti-actin C11 peptide (Sigma-Aldrich, Steinheim, Germany) antibodies.

Two-dimensional electrophoresis, protein visualization and image analysis

Approximately 70 μg of protein were treated with chloroform and methanol, pelleted and resolved in rehydration buffer to a final volume of 350 μl. The mixture was used for the hydration of IPG strips, followed by the multistep focusing protocol using the IPGphor (GE Healthcare Life Sciences, Munich, Germany).

After the first dimension, the individual strips were equilibrated for 60 min. The second dimension was performed on homogeneous polyacrylamide gels, which were silver stained and analysed with PDQuest (Bio-Rad Industries, München, Germany). Extracts of three independent cultures were analysed with at least two replicate gels for each.

In gel digestion and nano-ESI-MS

Tryptic peptides were separated by water-acetonitrile gradients on Dionex-NAN75-15-03-C18-PM columns on an ultimate-nano-HPLC system (Dionex, Sunnyvale, CA, USA). Online ESI-MS/MS2 spectra were generated on an LCQ-DecaXP^{plus} mass spectrometer (Thermo Finnegan, San Jose, CA, USA).

Summary of metabolite analysis

Untargeted metabolic fingerprinting was performed by Ultra Performance Liquid Chromatography (UPLC) coupled with time-of-flight mass spectrometry (TOF-MS). ST extracts were analysed by thin layer chromatography. Psi factor composition was analysed by gas chromatography mass spectrometry (GC MS). Isolated orsellinic acid derivatives were analysed by HPLC. Details are described in *Supporting information*.

Supplementary information

Aspergillus nidulans strains (Table S8), plasmids (Table S9), primers (Table S10), complete molecular biology, protein extraction, electron microscopy, chemical extraction and analysis procedures as well as full characteristics of the metabolites produced by the *A. nidulans* Δ*csnE* strain are listed in *Supporting information*.

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