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Interplay of the fungal sumoylation network for control of multicellular development

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Summary

The role of the complex network of the ubiquitin-like modifier SumO in fungal development was analysed. SumO is not only required for sexual development but also for accurate induction and light stimulation of asexual development. The Aspergillus nidulans COMPASS complex including its subunits CclA and the methyltransferase SetA connects the SumO network to histone modification. SetA is required for correct positioning of aerial hyphae for conidiophore and asexual spore formation. Multicellular fungal development requires sumoylation and desumoylation. This includes the SumO processing enzyme UlpB, the E1 SumO activating enzyme AosA/UbaB, the E2 conjugation enzyme UbcN and UlpA as major SumO isopeptidase. Genetic suppression analysis suggests a connection between the genes for the Nedd8 isopeptidase DenA and the SumO isopeptidase UlpA and therefore a developmental interplay between neddylation and sumoylation in fungi. Biochemical evidence suggests an additional connection of the fungal SumO network with ubiquitination. Members of the cellular SumO network include histone modifiers, components of the transcription, RNA maturation and stress response machinery, or metabolic enzymes. Our data suggest that the SumO network controls specific temporal and spatial steps in fungal differentiation.

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Introduction

Post-translational protein modifications by ubiquitin-like proteins are rapid and efficient processes to modulate the localization, activity, stability and interactions of target proteins. The importance of such modifications for cellular processes and diseases as cancer is a major focus of current research (Zhao, 2007; Lipkowitz and Weissman, 2011; Watson et al., 2011; van der Veen and Ploegh, 2012). The small ubiquitin-related modifier Sumo is a member of the ubiquitin-like protein family. It has a size of around 10 kDa and displays high structural similarity to ubiquitin, although it only shares low similarity at the level of the primary sequence (Bayer et al., 1998). Sumo is attached to target substrates by a similar enzymatic cascade as described for the ubiquitination process. In the first step of sumoylation, Sumo precursor protein is processed by a specific Sumo protease resulting in the exposure of two glycine residues at the protein's C-terminus which is essential for the attachment pathway (Mukhopadhyay and Dasso, 2007). In Saccharomyces cerevisiae, the mature Sumo (Smt3) protein is activated by the heterodimer Aos1/Uba2 (E1 enzyme) in an ATP-dependent reaction (Johnson et al., 1997). Next, it is transferred to the single Sumo E2 conjugating enzyme, Ubc9 (Johnson and Blobel, 1997). Structural analyses of mammalian Ubc9 in complex with the Sumo substrate RanGAP1 showed that the E2 enzyme can recognize a consensus motif in a target protein (Bernier-Villamor et al., 2002). Many sumoylation reactions require E3 ligases which facilitate the final reaction of the sumoylation process. In this step, an isopeptide bond is formed between the di-glycine of Sumo and a lysine residue in the target substrate (Geiss-Friedlander and Melchior, 2007). In S. cerevisiae many proteins become sumoylated by the action of the E3 ligases Siz1 and Siz2 (Johnson and Gupta, 2001; Takahashi et al., 2001; Ferreira et al., 2011). These proteins belong to the family of Siz/PIAS RING finger-like domain proteins. In mammals there are five PIAS domain proteins that function as E3 ligases (Palvimo, 2007). Additionally, there are other known Sumo E3 ligases of other protein families (Geiss-Friedlander and Melchior, 2007; Wang and Dasso, 2009).

Many target proteins of sumoylation share a common short amino acid motif that marks the attachment site. This motif is composed of a hydrophobic amino acid, followed by a lysine, a variable residue and an acidic amino acid (ΨKxE/(D) with Ψ representing a large, hydrophobic amino acid and x any amino acid) (Rodriguez et al., 2001). Sumo can also interact with proteins by a so-called Sumo-interaction motif (SIM). Different potential SIMs were described containing a hydrophobic core. SIMs play a role in Sumo attachment as they can mediate the interaction between Sumo and the enzymes involved in the sumoylation pathway but they are also important for the non-covalent modification of target substrates (Kerscher, 2007).

Sumoylation is involved in cellular processes like DNA damage repair (Dou et al., 2011), transcription (Gill, 2005) and nucleocytoplasmic transport (Matunis et al., 1998). It is a reversible, dynamic process and many proteins go through cycles of sumoylation and desumoylation (Geiss-Friedlander and Melchior, 2007). Sumo-specific isopeptidases can, besides Sumo processing, cleave the Sumo protein from substrates. In S. cerevisiae this function is conducted by the Ubl (Ubiquitin-like protein)-specific proteases Ulp1 and Ulp2 (Li and Hochstrasser, 1999; 2000). Ulp1 is essential for viability (Li and Hochstrasser, 1999) and deletion of the gene encoding Ulp2 has pleiotrophic effects, such as abnormal cell morphology and sporulation as well as temperature sensitivity (Li and Hochstrasser, 2000). The two proteins display a diverse localization and function within the cell. Ulp1 is responsible for the maturation of the yeast Sumo protein, Smt3, and desumoylation of target proteins and localizes to the nuclear envelope (Li and Hochstrasser, 1999). Ulp2 cleaves poly-Sumo chains (Bylebyl et al., 2003) and is present in the nucleoplasm (Li and Hochstrasser, 2000). In mammals six Sumo isopeptidases, the Sentrin-specific proteases Senp1-3 and Senp5-7, have been described. Senp1-3 and 5 resemble Ulp1, whereas Senp6 and 7 share similarity with Ulp2 (Mukhopadhyay and Dasso, 2007).

Only one Sumo protein is expressed in many lower eukaryotes, like S. cerevisiae, Schizosaccharomyces pombe or A. nidulans. In Arabidopsis thaliana eight potential Sumo-encoding genes are present (Kurepa et al., 2003), whereas in mammals four Sumo proteins were described (Geiss-Friedlander and Melchior, 2007). Research until today was mainly focused on Sumo1 to 3. Sumo2 and Sumo3 share high sequence similarity (around 95%), whereas Sumo1 shows 45% identity (Wang and Dasso, 2009). Sumo chain formation is possible via a conserved lysine residue in Sumo2 and Sumo3, as well as in the yeast Sumo (Smt3) (Tatham et al., 2001; Bylebyl et al., 2003). Sumo1 is missing this lysine residue and therefore might act as chain terminator (Matic et al., 2008; Wang and Dasso, 2009). Biological function of Sumo chains is not fully understood but in S. pombe a lack of the lysines 14 and 30 and a resulting loss of Sumo chains leads to cellular defects (Skilton et al., 2009). In human organs, such as kidney, lymph nodes or spleen an additional Sumo protein, Sumo4, is known (Guo et al., 2004; Geiss-Friedlander and Melchior, 2007). It is so far poorly investigated, but mutations in Sumo4 might be connected to diabetes (Wang and She, 2008). Sumo proteins are essential in many organisms, such as S. cerevisiae (Johnson et al., 1997), A. thaliana (Saracco et al., 2007) or mice (Nacerddine et al., 2005). This is different in S. pombe and in A. nidulans, where deletion of the Sumo encoding genes, pmt3 and sumO, leads to strong phenotypical changes but viable strains (Tanaka et al., 1999; Wong et al., 2008).

Aspergillus nidulans is a filamentous fungus that grows vegetative in liquid culture. Under nutrient starvation conditions on solid medium, the organism induces sexual or asexual development to form spores. Asexual development is favoured in light with normal carbon dioxide pressure and leads to the formation of conidiophores, releasing the asexual spores into the air (Adams et al., 1998; Bayram and Braus, 2012). In contrast, darkness and elevated carbon dioxide pressure triggers sexual reproduction and the formation of fruiting bodies, named cleistothecia (Braus et al., 2002). Deletion of the sumO gene revealed that the SumO protein of A. nidulans supports the formation of sufficient asexual spores, the transition of microcleistothecia to mature sexual fruiting bodies and co-ordinated secondary metabolite production (Szewczyk et al., 2008; Wong et al., 2008). The roles of sumoylation or desumoylation, substrates and members of the fungal SumO network responsible for multicellular development are yet unknown.

Here we demonstrate that sumoylation as well as desumoylation are essential processes for cellular differentiation in A. nidulans. This fungus with a single non-essential SumO protein is highly suitable for genetic studies. Members of the SumO interaction network were biochemically identified and include among others the SumO activating enzymes AosA and UbaB, the E2 conjugating enzyme UbcN, the E3 enzyme SizA and a subunit of the COMPASS complex, CclA. We have shown that this SumO network, consisting of proteins for sumoylation and desumoylation, their substrates and interaction partners, is not only required for development as the induction of conidiophore formation but also for the response to light. This also includes cross-talk between the ubiquitin-like protein family. We show, that the COMPASS complex subunit SetA has a specific morphological function in providing the right position within a colony to form aerial hyphae as initial step to produce air-borne asexual spores.

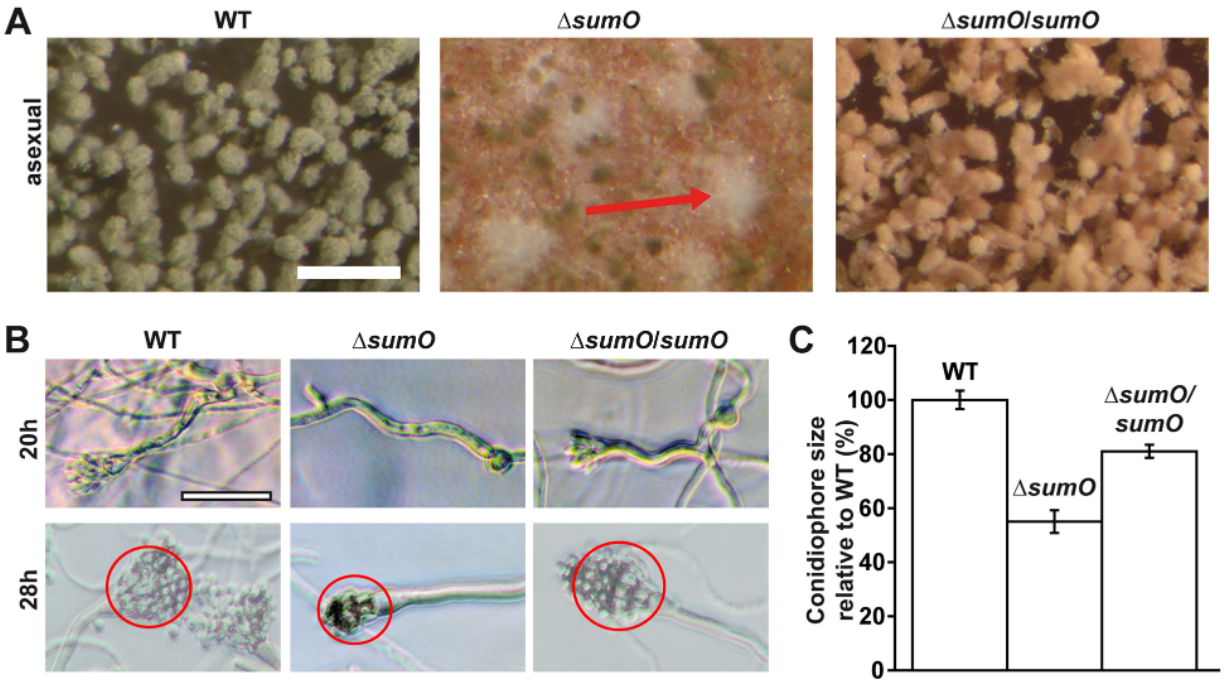

Fig. 1. The sumO deletion strain shows defects in light control and multicellular development.
A. Impact of sumO deletion on asexual development. Only the sumO deletion strain shows sexual nest formation (red arrow) and few asexual spores after 5 days of incubation of 1 × 10^6 spores on minimal medium at 37°C under asexual development inducing conditions. The wild type strain and the complementation strain produce asexual spores (which are yellow in the complementation strain due to a yA mutation).
B. The sumO deletion strain displays a delayed conidiophore formation. Strains were grown on solid MM containing the appropriate supplements on oblique thin agar layers on microscopy slides for 20 h or for 28 h at 37°C. Scale bar 20 µm.
C. Quantification of conidiophore size. Strains were grown as described in B for 28 h at 37°C. Circles were drawn around the upper part of the conidiophores (red circles) and the surface was calculated using the cellSens software (Olympus). Ratios were calculated relative to the WT in %. Data are derived from four experiments with 50 conidiophores each. Shown are the mean values with standard deviations.

Results

The sumO deletion strain displays sexual reproduction in light and a delay in conidiophore production

Deletion of the SumO encoding gene in A. nidulans leads to viable cells with multiple developmental phenotypes. These include a block in sexual development combined with changes in secondary metabolism, a decrease in asexual spore production and sensitivity to DNA damaging agents (Szewczyk et al., 2008; Wong et al., 2008). We analysed the cause of the decreased amount of asexual spore formation (Fig. 1A) in more detail.

Sumoylation is important for the developmental control of light-dependent repression of sexual development in A. nidulans. Incubation of the ΔsumO mutant in constant white light for five days led to a strong development of nests as intermediates of sexual development (Fig. 1A). By contrast, the wild type strain as well as the sumO reconstitution strain formed hardly any sexual structures when grown in the light for the same period of time (Fig. 1A). The timing of conidiophore formation in asexual development was investigated on oblique thin agar layers. After approximately 20 h of incubation at 37°C, asexual structures were missing in the sumO deletion strain but were already displayed in the wild type and the sumO complementation strain (Fig. 1B, top). After further incubation at 37°C, the wild type and sumO reconstitution strain developed conidiophores with asexual spores, while the ΔsumO strain showed reduced and delayed conidiophore formation (Fig. 1B, bottom). Conidiophore sizes were quantified by measuring the surface of circles surrounding the conidiophore top (Fig. 1B, red circles) after 28 h of incubation at 37°C. The size of the sumO deletion strain conidiophores was reduced to approximately 60% of the wild type conidiophores, which was complemented by ectopical expression of sumO (Fig. 1C).

Our data demonstrate an additional function of SumO beside its contribution to the morphological transition of microcleistothecia to mature fruiting bodies in sexual development. SumO is part of the control which reduces the number of sexual structures in response to external triggers as light and controlling the correct timing of asexual spore formation induction.

The SumO proteases UlpA and UlpB are required for fungal development

The developmental control defects of the sumO deletion strain suggest the presence of sumoylated substrates which are required for fungal differentiation. We analysed whether only sumoylation has a strong impact on fungal development, or a dynamic SumO network including the desumoylation machinery is also required. Sumo-specific isopeptidases exhibit two functions. They are part of the sumoylation machinery when they process the Sumo precursor. Cleavage of the C-terminal peptide extension of the immature Sumo protein exposes the glycine that forms the isopeptide bond with the substrate. Sumo-specific isopeptidases are part of the desumoylation machinery if they cleave Sumo from substrates. BLAST searches revealed homologues of the S. cerevisiae Sumo (Smt3)-specific isopeptidases Ulp1 and Ulp2. The protein that showed 28% amino acid identity to Ulp1 was named UlpA (AN2689) and the protein with 34% identity to Ulp2 was named UlpB (AN8192). Both proteins carry a Ulp protease domain (Fig. 2A) (PS50600; http://www.ebi.ac.uk/Tools/pfa/iprscan; Quevillon et al., 2005).

Ulp1 is the main processing enzyme of yeast Sumo (Smt3) (Li and Hochstrasser, 1999). Here, the coding region of the corresponding A. nidulans gene was deleted. The ΔulpA strain showed a reduced asexual spore production and a strong induction of sexual fruiting body formation during asexual development but was not able to form mature cleistothecia (Fig. 2B and C). The cleistothecia of the deletion strain did not contain any ascospores. This ΔulpA phenotype is reminiscent to the ΔsumO phenotype and could be complemented by ectopical reintegration of the gene. It was investigated whether the phenotype of the ΔulpA strain results from defects in sumoylation due to a lack of mature SumO within the cell. Western hybridizations were performed, using protein extract of a wild type, the ulpA deletion and the corresponding complementation strain with an antibody specific for A. nidulans SumO (Fig. 2D). In the wild type strain only few potential sumoylated proteins were detected. In contrast, a strong increase (approximately 25-fold) in signals of potential modified SumO substrates was observed in the ulpA deletion strain that decreased back upon ectopical introduction of a wild type copy of ulpA (Fig. 2D and E). This suggests that UlpA is not the processing enzyme for SumO and therefore not part of the sumoylation reaction but an enzyme responsible for the desumoylation process.

Deletion of the ulpB gene resulted as well in similar phenotypes in multicellular development as seen for both, ulpA and sumO deletion strains (Fig. 3A and B). Western analyses of the cellular sumoylation pattern revealed only free SumO protein but no potentially sumoylated proteins could be detected in the ΔulpB strain (Fig. 3C). This suggests, that UlpB is the processing enzyme for SumO.

We compared the role of UlpA and UlpB, for stress response and accurate secondary metabolism to the corresponding described phenotypes of the sumO deletion strain (Szewczyk et al., 2008; Wong et al., 2008). All three strains showed defects in growth on 4-Nitroquinoline 1-oxide (Fig. S1A), an agent that induces DNA damage stress. In addition, the ΔsumO and the ΔulpB deletion strain were defective in the production of the secondary metabolite sterigmatocystin, whereas the ulpA deletion strain was not (Fig. S1B). These results indicate the importance of sumoylation and desumoylation for stress response as well as a role for sumoylation in secondary metabolism.

Our data suggest UlpA as desumoylation and UlpB as SumO processing enzyme of A. nidulans and revealed that there is a contribution of sumoylation as well as desumoylation for accurate fungal multicellular development and stress response as well as a role for sumoylation in secondary metabolism.

Cross-talk between the SumO and the Nedd8 fungal network

UlpA represents one of only three proteases with Ulp domain in A. nidulans (Fig. 2A). DenA is a second Ulp domain protein which was first described as Sumo isopeptidase Senp8 (Mukhopadhyay and Dasso, 2007) and acts as one of two described fungal deneddylases (Christmann et al., 2013). Deletion of denA results in a decrease in asexual spore formation and a shift to sexual development under asexual development (Christmann et al., 2013), which is similar to the ulpA developmental defect. We analysed whether DenA and UlpA share overlapping functions. The denA gene was ectopically expressed in the ulpA deletion strain under the control of the inducible nitrate promoter to test, if there is a connection of DenA to the sumoylation process in A. nidulans. The phenotype of ulpA mutant strain, overexpressing DenA, was investigated and compared with the control strain, carrying the same construct in wild type background. High levels of DenA had no effect on wild type. In the ulpA deletion strain, denA overexpression resulted in a significant increase in cleistothecia size (Fig. 2B). The size of the biggest cleistothecia in the SumO isopeptidase deletion strain is around 55% of wild type cleistothecia. Overexpression of denA rises the size significantly to around 75% compared with the wild type (Fig. 2C), but the strain is still defective in the formation of ascospores. This suggests an effect of DenA on fungal strains defective in SumO mediated development.

Western hybridization showed that the enrichment of sumoylated proteins in the ΔulpA strain was not

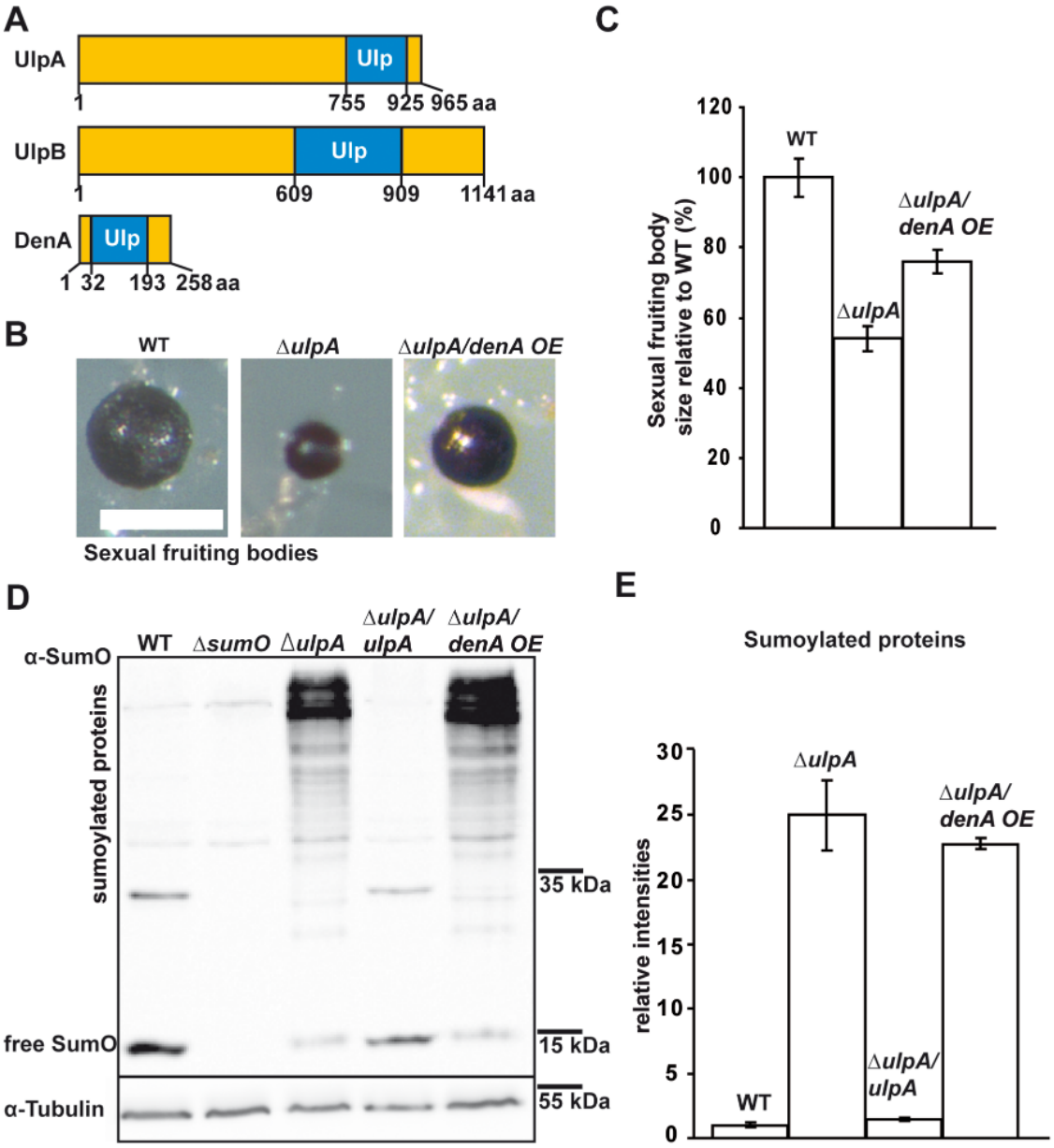

Fig. 2. UlpA is a SumO-specific isopeptidase.
A. Protein domain structures of the proteases UlpA, UlpB and DenA. Ulp marks the Ulp protease domain (PS50600) according to the InterProScan results (http://www.ebi.ac.uk/Tools/pfa/iprscan).
B and C. Comparison (B) and quantification (C) of the cleistothecia size of wild type, ulpA deletion strain and the strain, overexpressing denA in the ulpA deletion background. Strains were plated on solid minimal medium containing appropriate supplements and grown for 7 days at 37°C. Scale bar 100 µm. The diameter of 25 of the biggest cleistothecia formed by the strains were measured using the cellSens software (Olympus). Ratios were calculated relative to the wild type in %. Data are derived from four experiments.
D. Immunoblot of wild type, sumO deletion strain, ulpA deletion, ulpA complementation strain and the strain, overexpressing denA in ulpA deletion background with SumO antibody (weak exposure) and tubulin antibody as control. Crude extracts were prepared from 20 h vegetative grown mycelium.
E. Quantification of signal intensities using Fusion-SL7 system with Bio1D software (Peqlab). The relative intensity of the overall amount of sumoylated proteins (all proteins migrating higher than 25 kDa) was normalized to the wild type, where the relative intensity of the sumoylated proteins corresponds to 1. The signal for tubulin was used as internal standard. Data are derived from two experiments with two replications each. Shown are the mean values with standard deviations.

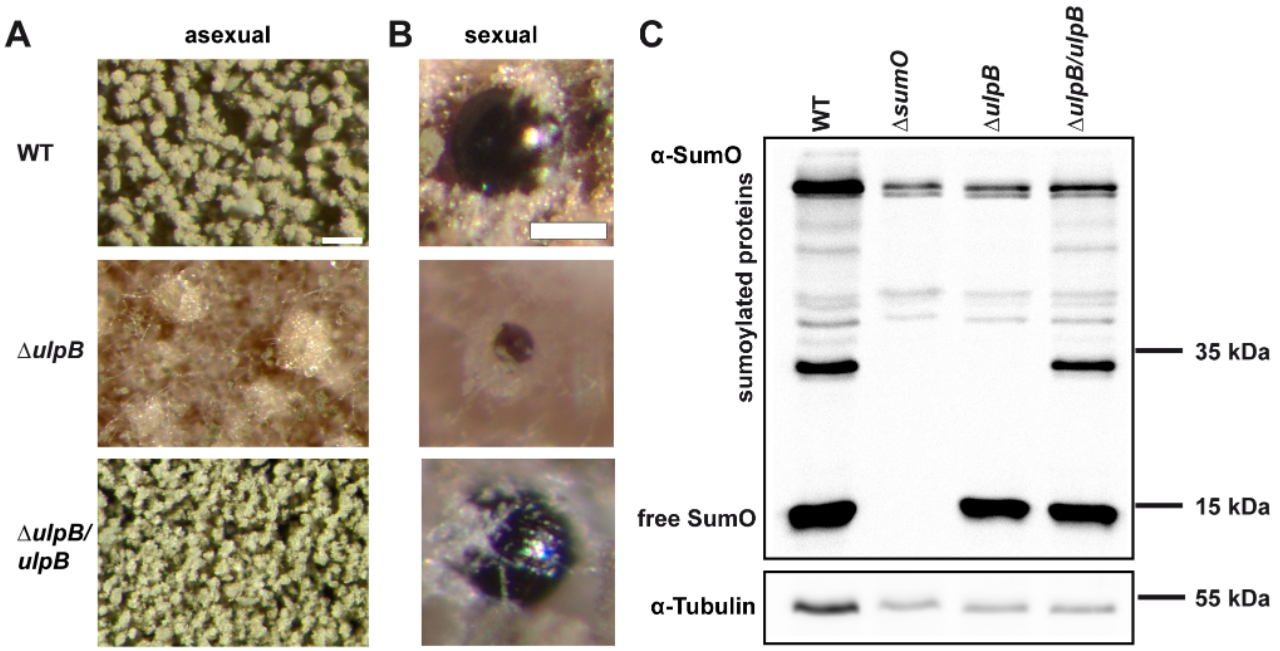

Fig. 3. UlpB is a SumO processing enzyme.
A. Comparison of asexual development of wild type, ulpB deletion and ulpB complementation strain. A total of 1 × 10^6 spores were plated on solid minimal medium containing appropriate supplements and grown for 5 days at 37°C. Scale bar 100 µm.
B. Comparison of the cleistothecia of wild type, ulpB deletion and complementation strain. Strains were plated on solid minimal medium containing appropriate supplements and grown for 7 days at 37°C under sexual development inducing conditions. Scale bar 100 µm.
C. Immunoblot of wild type, sumO and ulpB deletion and ulpB complementation strain with SumO antibody and tubulin antibody as control. Crude extracts were prepared from 20 h vegetative grown mycelium.

significantly reduced upon overexpression of DenA (Fig. 2D and E). This suggests an effect on individual proteins rather than an influence on the global sumoylation pattern in the cell. Alternatively, the observed phenotype on sexual fruiting body size could be the result of a DenA deneddylation reaction. There seems to be a subtle cellular balance between desumoylation/deneddylation, which might be due to a cross-talk between the Nedd8 and SumO pathway.

Identification of members of the SumO network

Our Western analyses suggested the presence of various fungal proteins which are either sumoylated or might interact with sumoylated proteins. A tap::sumO fusion was introduced into the original sumO locus in wild type and ulpA deletion strain to identify both types of proteins as part of the fungal SumO network. The expression of the fusion protein was confirmed in Western hybridizations using a calmodulin binding protein antibody (Fig. 4A). Analyses of the phenotype revealed that TAP::SumO is partially functional. Asexual spore formation was similar in both, wild type and tap::sumO strain (Fig. 4B), but sexual fruiting body formation is impaired. Less than 5% of the developed cleistothecia contained ascospores. However, the strain forms intermediate cleistothecia, significantly larger than the sumO deletion strain but smaller than wild type (Fig. 4C).

The TAP::SumO expressing strains as well as their corresponding parental strains were used for purification of SumO network members. Data were collected from three independent experiments with the strain expressing TAP::SumO in wild type background and from two independent experiments with the ΔulpA/tap::sumO strain. The corresponding parental strains were used as controls. Proteins, identified in the control experiments, were considered as unspecific and are not included in the data set. Candidates for members of the SumO network required at least two identifications according to the parameters mentioned in experimental procedure. A complete list of all candidates including their putative homologues, functions and number of sumoylation motifs is given in Table S1. The proteins were categorized according to their potential functions using the databases AspGD (http://www.aspergillusgenomes.org; Arnaud et al., 2012), CADRE (http://www.cadre-genomes.org.uk; Mabey Gilsenan et al., 2012), SGD (http://www.yeastgenome.org; Cherry et al., 2012), PomBase (http://www.pombase.org; Wood et al., 2012) and NCBI (http://www.ncbi.nlm.nih.gov). Furthermore, phosida

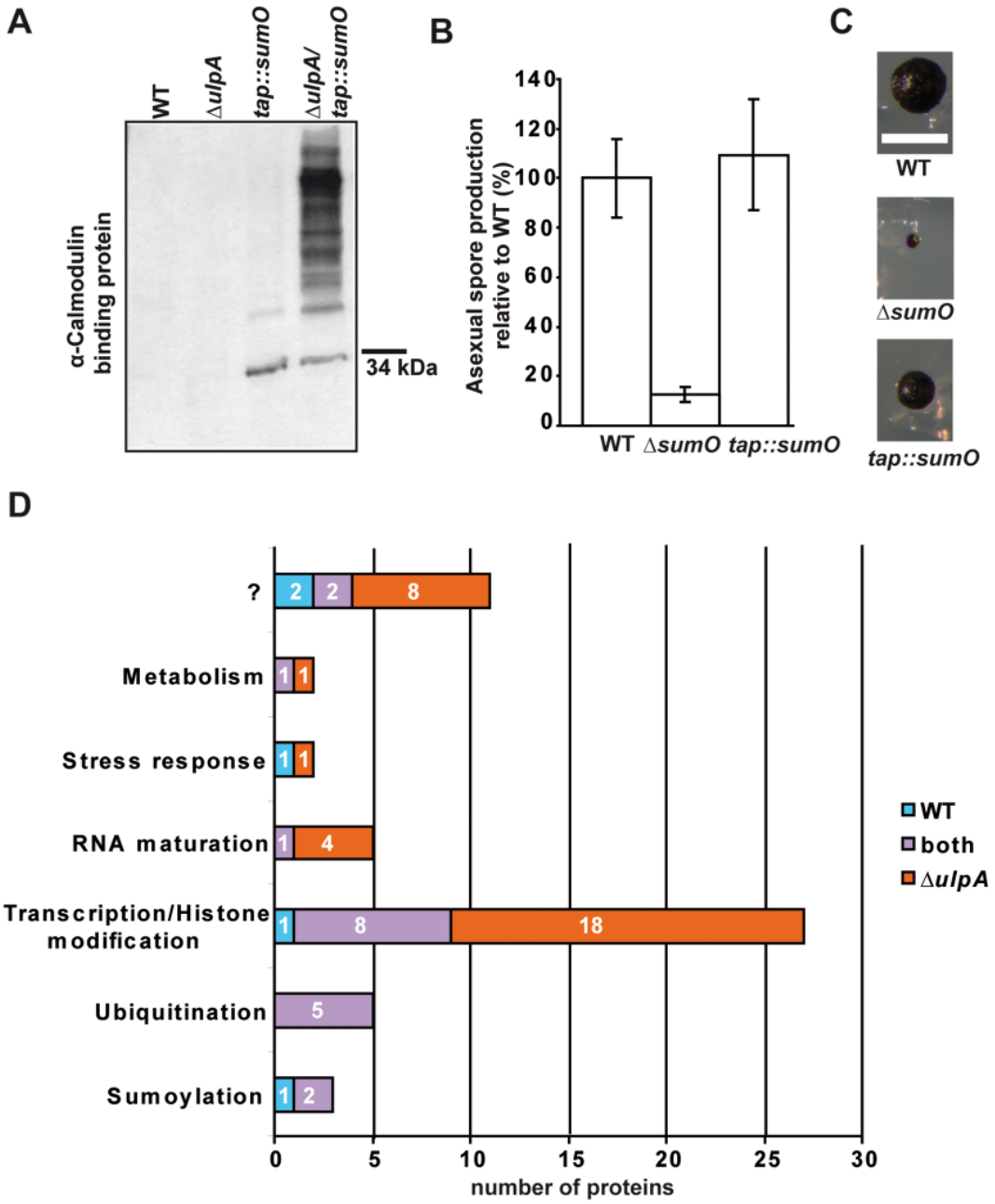

Fig. 4. Expression of TAP::SumO and categorization of co-purified proteins.
A. Immunoblot of the wild type, the ulpA deletion strain and the strain expressing TAP::SumO in wild type and ulpA deletion background with calmodulin binding protein antibody. Crude extracts were prepared from 20 h vegetative grown mycelium.
B. Quantification of asexual spores produced by the wild type, the sumO deletion strain and the strain expressing the TAP::SumO fusion protein. A total of 1×10^6 spores were plated on solid minimal medium containing appropriate supplements and grown for 3 days at 37°C. Ratios were calculated relative to the wild type spore number in %. Data are derived from four experiments. Shown are the mean values with standard deviations.
C. Representative sexual fruiting bodies of the wild type, the sumO deletion strain and the strain expressing the TAP::SumO fusion protein. Scale bar 200 µm.
D. Diagram of the proteins co-purified with TAP::SumO. Chart gives an overview of the different categories and the number of proteins that were identified in wild type, ulpA deletion, or both strain backgrounds respectively. Question mark indicates proteins of unknown functions.

(http://www.phosida.com; Gnad et al., 2007; 2011) was used to predict potential sumoylation motifs. The data sets from the purifications of the candidate proteins are given in Table S6.

A total of 56 candidates for sumoylated or sumo-interacting proteins were identified (Fig. 4D). Homologous proteins for enzymes involved in the sumoylation pathway were found: the E1 enzyme AosA, the E2-conjugating enzyme UbcN, and one potential E3 ligase, SizA. The E1 and E3 enzyme were found in wild type and ulpA deletion background, whereas the E2 enzyme UbcN was only identified in wild type background (Table S1). In addition, a number of proteins of the ubiquitination pathway were identified in both, wild type and ulpA deletion strain background. This includes a homologue of the E1 enzyme for Ubiquitin (AN10266) as well as five putative subunits of the proteasome. These proteins represent candidates for cross-talk of the sumoylation and ubiquitination pathways.

The largest group of proteins identified has a predicted role in transcription. In total 27 proteins were co-purified with TAP::SumO, that are involved in histone modification or transcription. The majority was identified only in the ulpA deletion background (18 proteins), suggesting that transcriptional regulators are only sumoylated in a small subpopulation in the cell and the sumoylation state becomes stabilized in the isopeptidase deficient strain. A number of proteins were identified which have homologues that are incorporated in or interacting with the chromatin modifying SAGA or transcription factor complexes, such as the yeast proteins Sgf29 (AN0668), Spt20 (AN0976), Toa2 (AN2181), Taf12 (AN2769), Taf1 (AN3907), Spt7 (AN4894), Tfa2 (AN5498), Gcn5 (GcnE), Ada2 (AdaB). We also found one subunit of the COMPASS complex, CclA (AN9399) (Bok et al., 2009). The COMPASS complex is responsible for methylation of histone H3 at lysine 4, that is involved in transcriptional regulation (Wood et al., 2005). Several subunits of the RNA polymerase were identified. Interestingly, also an important regulator of development in A. nidulans, the WD repeat protein RcoA was co-purified with TAP::SumO. A rcoA deletion strain displays reduced growth rates, decreased asexual spore production, altered secondary metabolism and a block in sexual development (Hicks et al., 2001; Todd et al., 2006). Additionally, five proteins were identified, which are not characterized in A. nidulans yet, but the corresponding yeast homologues play a role in RNA maturation, such as subunits of cleavage and polyadenylation factors. As observed for the group of transcriptional regulators, most of the proteins were only identified in the ulpA deletion strain.

Two of the proteins which were co-purified are involved in stress response, the superoxide dismutase SodA (Oberegger et al., 2000) and the homologue of yeast Ntg1, a protein that is important for the response to oxidative stress (Alseth et al., 1999). Another group of potential substrates are enzymes involved in metabolic processes, the aldehyde dehydrogenase AldA (AN0554) (Pickett et al., 1987) and the homologue of yeast Ura6 (AN4258), which is an uridylate kinase. Both proteins contain potential sumoylation sites and might reflect an influence of sumoylation on metabolic processes.

These data support a specific role of sumoylation and desumoylation on gene expression during fungal development, which might explain the pleiotropic phenotypes of the sumO deletion strain. The large group of additional enriched proteins in the strain deficient in the SumO isopeptidase UlpA in comparison to wild type corroborates a highly dynamic turnover between sumoylated and desumoylated target proteins which is required for correct multicellular fungal differentiation.

The A. nidulans E1 SumO activating and E2 SumO conjugating enzymes are required for multicellular development

We analysed the enzymes, catalysing the individual steps of the sumoylation process, and their roles in fungal development. The sumO activating protein AosA (AN2298), the E2 enzyme UbcN (AN4399) and SizA (AN10822) were identified as SumO-interacting proteins. The second activating enzyme, UbaB (AN2450) was also identified in the control strains, which might be due to unspecific binding or high cellular expression levels. These proteins resemble their yeast homologues. AosA displays around 33% identity to Aos1, UbaB around 42% identity to Uba2, UbcN around 63% identity to Ubc9 and SizA displays around 33% amino acid identity to Siz1 respectively. The genes encoding the four proteins were deleted and the strains were phenotypically investigated.

The deletion strains of aosA, ubaB (both E1) and ubcN (E2), coding for the SumO E1 and E2 enzymes respectively, resemble the sumO deletion phenotype in asexual and sexual development with a similar decrease in asexual spore production as in the sumO deletion strain (Fig. 5A) and a block in sexual fruit body formation, accompanied with the inability to form ascospores. A combined deletion of both E1 enzyme encoding genes led to the same phenotype as the single deletions. In contrast to ΔsumO, point inoculation of the deletion strains resulted in an irregular colony formation accompanied by the production of a reddish/brown dye (Fig. 5B). The overall cellular sumoylation pattern was analysed by Western hybridization using a SumO antibody (Fig. 5C). In the aosA (E1), ubaB (E1) and ubcN (E2) deletion strains the free SumO protein pool was still present whereas signals of potentially sumoylated proteins were not detectable. The phenotype of the deletion strains was complemented by

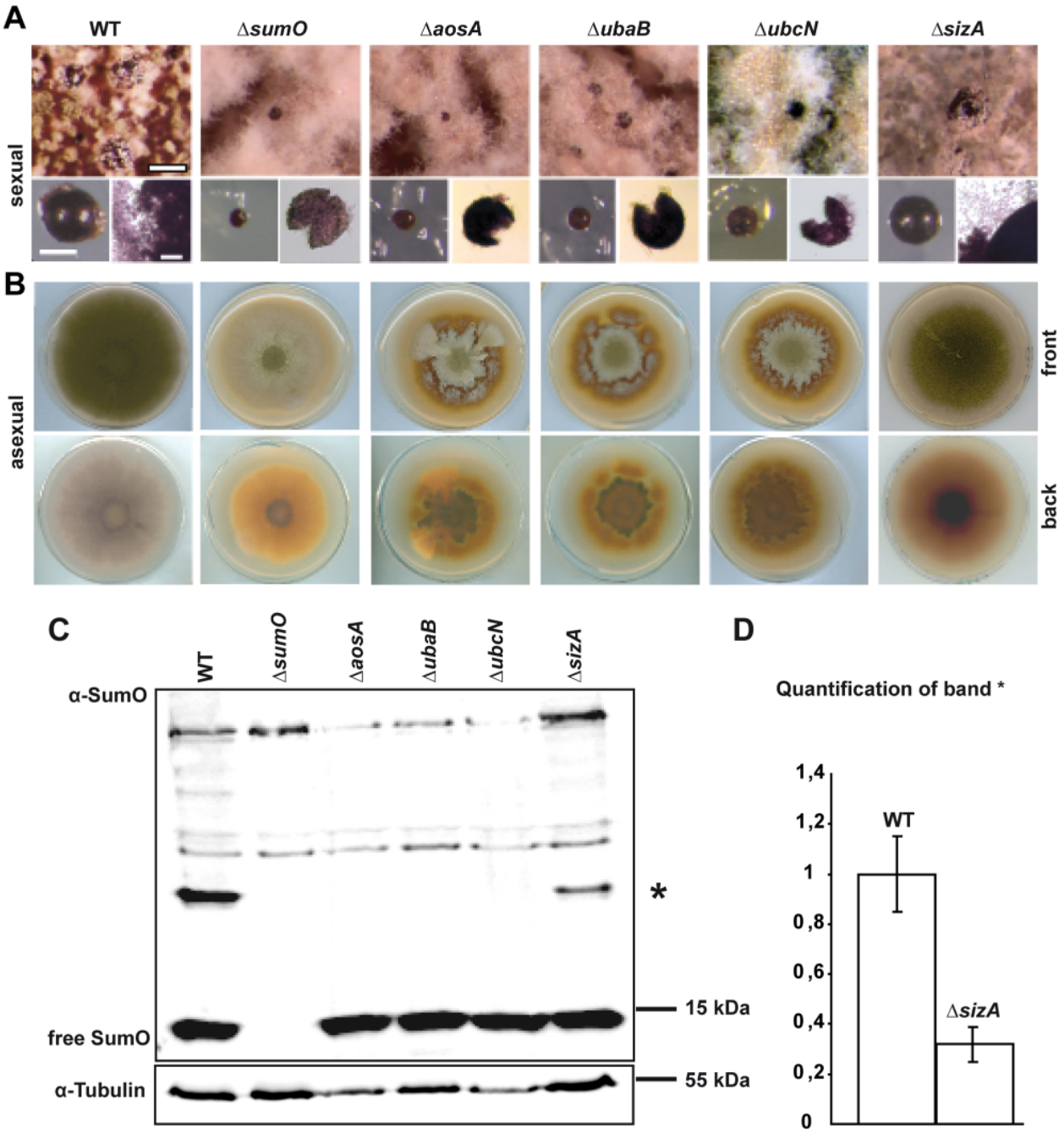

Fig. 5. Characterization of the enzymes of the sumoylation pathway.
A. Sexual development of the wild type, the deletion strains of sumO, aosA, ubaB (both coding for the SumO E1 enzyme), ubcN (coding for SumO E2 enzyme), sizA (coding for a SumO E3 ligase). Upper panel: sexual fruiting bodies on plate (scale bar 200 μm), lower panel: close up view of cleistothecia (left, scale bar 100 μm) and squeezed fruiting bodies under the microscope (right, scale bar 20 μm). Strains were grown on solid minimal medium containing appropriate supplements for 7 days at 37°C under sexual development inducing conditions.
B. Colony view of the wild type, the deletion strains of sumO, aosA, ubaB, ubcN and sizA. 500 spores of each strain were point-inoculated on solid minimal medium containing appropriate supplements and grown for 5 days at 37°C under asexual development inducing conditions.
C. Immunoblot of the wild type and the deletion strains of sumO, aosA, ubaB, ubcN and sizA with SumO antibody and tubulin antibody as control. Crude extracts were prepared from 20 h vegetative grown mycelium. The asterisk '*' indicates a certain type of sumoylated protein/SumO interacting protein migrating between 25 and 35 kDa.
D. Quantification of signal intensities using Fusion-SL7 system with Bio1D software (Peqlab). The relative intensity of band * of the sizA deletion strain was normalized to the WT, which relative intensity corresponds to 1. The signal for tubulin was used as internal standard. Data are derived from three experiments with four replications each. Shown are the mean values with standard deviations.

reintroduction of the genes. This indicates that all three proteins are involved in the sumoylation process.

In contrast to the impact of E1 and E2 on fungal development the situation is different for sizA encoding a putative SumO E3 ligase. The corresponding deletion strain did display wild type-like conidiospore formation and sexual development, including cleistothecia formation. Investigation of the number and viability of the produced ascospores suggested a similar situation as for the wild type. However, the strain showed an increase in sexual nest formation in comparison to wild type during cultivation in light, which favours asexual development. The cellular pattern of sumoylated proteins of the sizA (E3) mutant strain and wild type primarily differ in a band migrating between 25 and 35 kDa. Signal intensity caused by the SumO antibody is three times stronger in wild type than in the mutant strain (Fig. 5D). This suggests that SizA contributes to fungal development less than the E1 and E2 enzymes and suggests the presence of additional SumO E3 ligases encoded in the genome of A. nidulans. Further E3 ligases might play redundant or different roles in development, whereas SizA might play a specific role in nest formation.

The COMPASS complex controls accurate development of A. nidulans

In this study, CclA was identified as a member of the fungal SumO network representing either a potential SumO-interacting or SumO modified protein (Table S1). CclA is a subunit of the COMPASS (Complex Proteins Associated with Set1) complex. The phenotype of the ΔsumO strain was compared with the described cclA deletion strain (Bok et al., 2013) to further investigate a connection between the COMPASS complex and sumoylation. The cclA deletion and the sumO deletion strain showed changes in colony and medium colour, which support an impaired secondary metabolism (Fig. S2A; Szewczyk et al., 2008; Bok et al., 2009). A quantification of the amount of asexual spores formed by the cclA deletion strain revealed a decrease to approximately 30% in comparison to the wild type as 100% which is similar to the defects of the sumO deletion strain (Fig. S2B; Wong et al., 2008; Giles et al., 2011) The cclA deletion is unable to produce mature fruiting bodies and is blocked in sexual development even earlier than the sumO deletion strain which can still produce microcleistothecia (Fig. S2C; Wong et al., 2008).

CclA is the homologue of the yeast COMPASS complex subunit Bre2 that is important for gene expression through methylation of histone 3 at lysine 4 (Eissenberg and Shilatifard, 2010). All eight subunits of the COMPASS complex including the core subunit SetA are conserved in A. nidulans. We verified the fungal CclA interacting COMPASS complex by analysing the proteins recruited by the SetA core subunit. We replaced the setA locus with a setA::tap fusion and could co-purify six subunits of the COMPASS complex by tandem affinity purification. They included homologues of the yeast proteins Bre2 (CclA, AN9399), Swd2 (AN0065), Shg1 (AN0179), Swd1 (AN0808), Spp1 (AN2850) and Swd3 (AN3926) (Table S2). The only missing protein of the complex was the homologue of Sdc1 (Roguev et al., 2001).

We analysed whether defects in genes for other subunits of the COMPASS complex displayed similar phenotypes in A. nidulans as defects in sumO. The developmental function of the gene for SetA was analysed, because yeast Set1 revealed a biochemical interaction with yeast Sumo (Smt3) (Wohlschlegel et al., 2004) and SetA carries seven putative sumoylation motifs. In addition, PR-Set7 containing a Set domain was suggested to be sumoylated in human (Spektor et al., 2011). The A. nidulans setA deletion strain displayed strong developmental defects and a disturbed secondary metabolism similar to defects in cclA. In sexual development, the strain showed a block in cleistothecia development at an early state. Incubation in darkness and oxygen-limiting conditions resulted in the formation of uneven colonies and a strong red/brown colour (Fig. 6A). Investigation of the nests, still produced by the strain, revealed only the presence of sexual Hülle cells but no fruiting body precursor (Fig. 6A). Upon further incubation, small fruiting body precursors were developed in some individual nests but never mature cleistothecia with ascospores. The complementation strain showed wild type like cleistothecia formation. Secondary metabolism in the setA deletion strain appeared to be disturbed, but the production of sterigmatocystin as a well-characterized metabolite of A. nidulans was unaffected in a setA deletion strain (Fig. S3A). This suggests that other secondary metabolite pathways are affected.

Also during illumination when asexual development is favoured, the colony morphology was drastically disturbed and a dark-red pigment was produced (Fig. 6B). Growth of the setA deletion strain was significantly impaired (Fig. 6B and C). The number of conidia of the setA deletion strain accounts for around 40% of the spores produced by wild type (Fig. 6D). Further investigation of the conidiophore morphology on oblique, thin agar layers showed a defect in positioning and localization of asexual structures. The strains were incubated for approximately 22 h at 37°C. In both, wild type and the complementation strain, asexual spores germinated (Fig. 6B, red arrows) and formed hyphae from which 70–80% develop so-called stalks (Fig. 6B, blue arrows). From these stalks a vesicle buds with metulae, phialides and conidiospores develop on the top. Only a small portion of approximately 20% of wild type spores directly developed conidiophores but with only short stalks. This

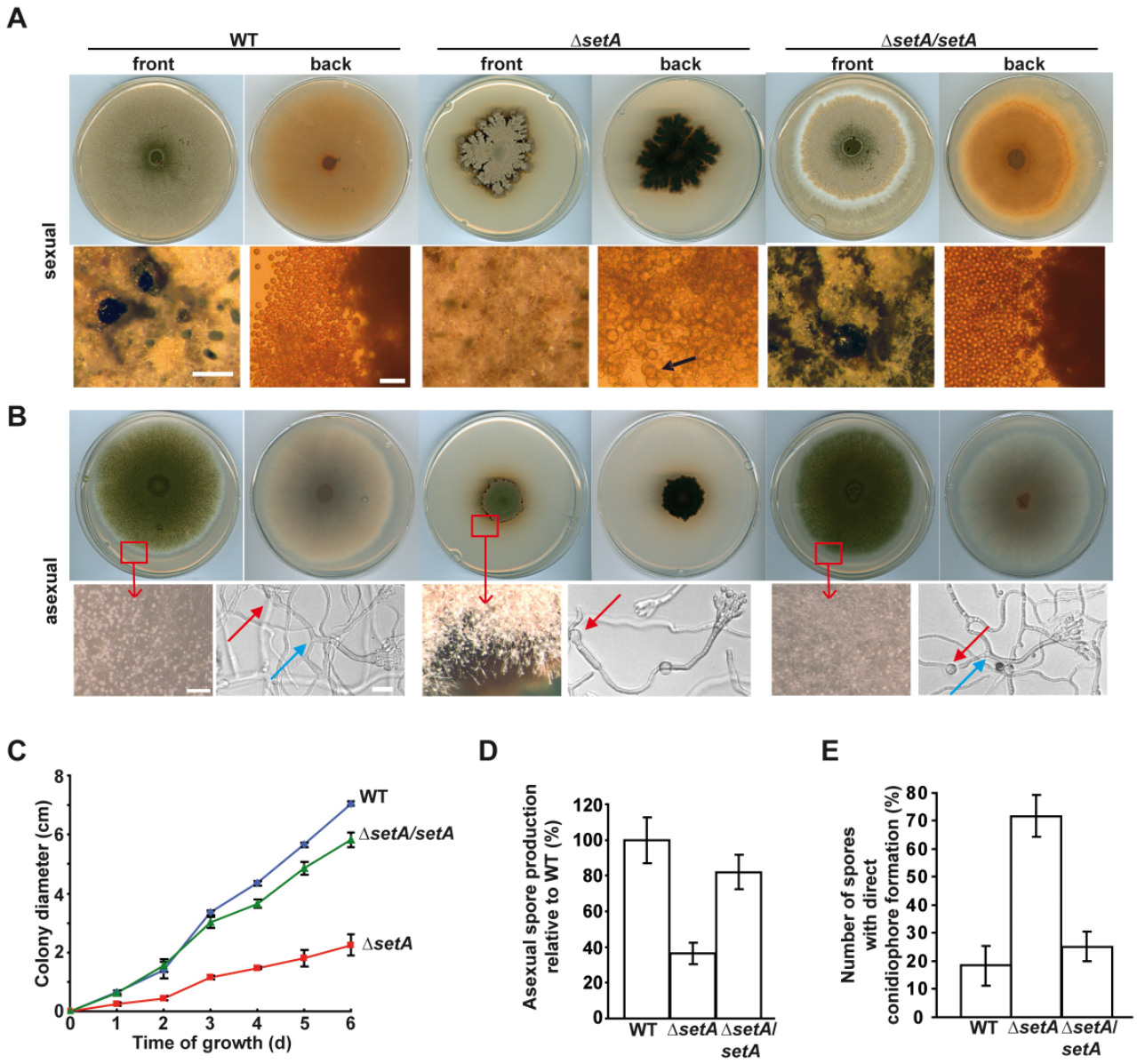

Fig. 6. SetA has pleiotropic effects on all stages of A. nidulans development.
A. Upper panel: colony view of the wild type, the deletion strain of setA and the complementation strain from front and back. A total of 500 spores of each strain were point-inoculated on solid minimal medium containing appropriate supplements and grown for 7 days at 37°C under sexual development inducing conditions. Lower panel, left: close up views of sexual fruiting bodies. Scale bar 200 μm. Lower panel, right: microscopy view on squeezed fruiting bodies/nests. Black arrow marks Hülle cells. Scale bar 20 μm.
B. Upper panel: colony view of the wild type, the setA deletion and the complementation strain from front and back. 500 spores of each strain were point-inoculated on solid minimal medium containing appropriate supplements and grown for 6 days at 37°C under asexual development inducing conditions. Lower panel, left: close-up views of the colony edge. Scale bar 200 μm. Lower panel, right: microscopy of conidiophores. Red arrows mark the germinating spores, whereas blue arrows indicate the foot cells from which the conidiophore stalk is formed. The setA deletion strain directly formed conidiophores at the end of hyphae. Scale bar 20 μm.
C. Colony growth of the wild type, the deletion strain of setA and the complementation strain. A total of 500 spores of each strain were point-inoculated on solid minimal medium containing appropriate supplements and grown for 5 days at 37°C under asexual development inducing conditions. Colony diameter was measured daily. Data are derived from two experiments.
D. Quantification of asexual spores formed by the wild type strain, the setA deletion and complementation strain. A total of 1×10^6 spores were plated on solid minimal medium containing appropriate supplements and grown for 3 days at 37°C. Ratios were calculated relative to the wild type spore number in %. Data are derived from four experiments. Shown are the mean values with standard deviations.
E. Quantification of spores that directly developed conidiophores. Spores were directly inoculated on oblique thin agar layer on microscope slides. Conidiophores were pictured under microscope and the number of spores that directly formed conidiophores was counted and ratios were calculated in %. Data are derived from six experiments with 100 conidiophores each. Shown are the mean values with standard deviations.

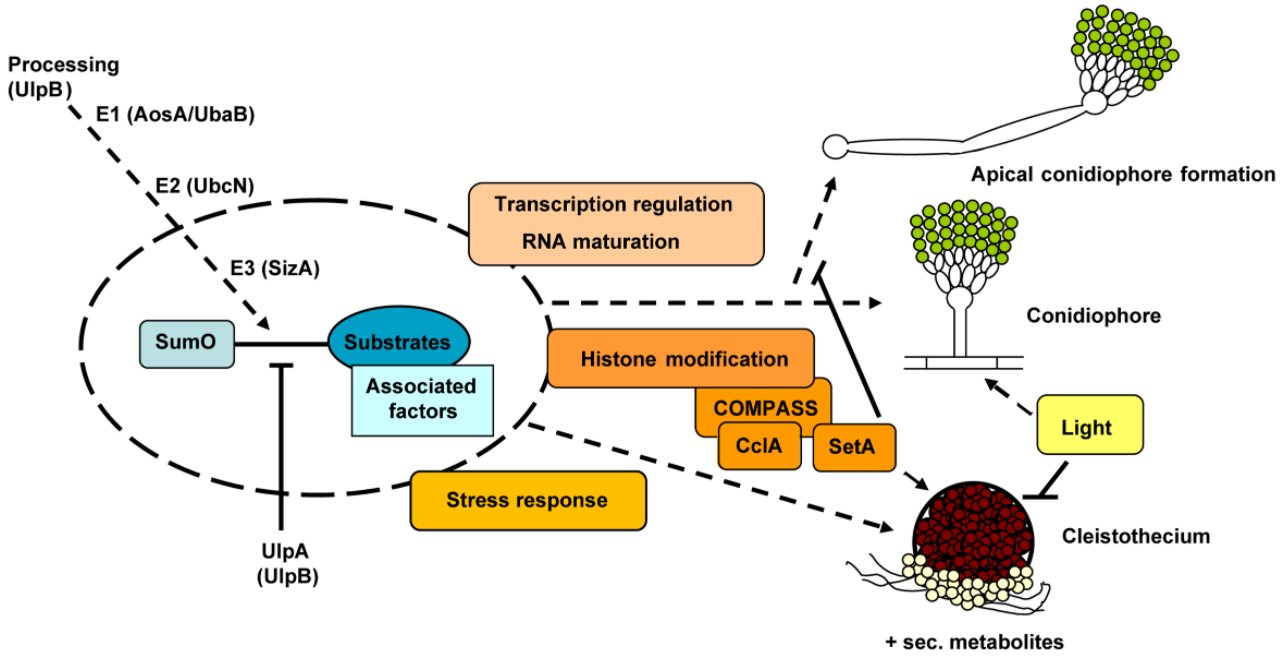

Fig. 7. Aspergillus nidulans development and sumoyation. Sumoylation and desumoylation of putative substrates identified in this study are summarized. Downstream effects depend on environmental stimuli and result in A. nidulans in the formation of sexual fruiting bodies (cleistothecia) or asexual structures (conidiophores). Sumoylation is required (i) for sexual reproduction, (ii) for repression of sexual development in light, (iii) for enhanced conidiophore formation in light. The SumO precursor is processed (UlpB), activated (E1: heterodimer AosA/UbaB), and transferred to the conjugation enzyme (E2: UbcN). SizA and other E3 ligases attach SumO to target substrates. Potential target proteins for sumoylation in A. nidulans are histone modifiers (as the COMPASS complex), transcriptional regulators, RNA maturation proteins as well as metabolic and stress response enzymes. SetA is a subunit of the COMPASS complex and is required for sexual development, secondary metabolism and the repression of apical conidiophore formation.

ratio was reversed in the mutant strain. Only about 30% of the setA deletion mutant spores developed conidiophores originating from stalks. These formed after branching of the hyphae. Hyphal branching was partially disturbed. Approximately 70% of the setA deletion strain spores directly formed conidiophores, partially at the end of very long hyphae (Fig. 6B and E, Fig. S3B). Some of the ΔsetA hyphae which ended in conidiophores could form new stalks with conidiophores (Fig. S3C).

These data show that the COMPASS complex subunits CclA and SetA are linked to multicellular development and secondary metabolism similar to sumoylation. The SetA subunit has an interesting function in asexual development where it is important for the balance between the formation of conidiophores on foot cells or hyphal tips as starting point for conidiophore development. COMPASS might act in development on molecular level by connecting histone methylation and sumoylation pathways.

Discussion

The SumO network and multicellular development

Post-translational modifications of target proteins by sumoylation and the recognition of sumoylated substrates are conserved processes in eukaryotes and play a prominent role in multicellular differentiation of the fungus A. nidulans. Various members of this highly dynamic developmental SumO network could be identified and characterized in this study (Fig. 7). SumO is involved in developmental control because it is required for repression of sexual development during illumination and simultaneously accelerates asexual spore formation. SumO is also required for sexual fruiting body formation and coordinated secondary metabolism (Szewczyk et al., 2008; Wong et al., 2008).

A prominent regulator complex required to link development and secondary metabolism in A. nidulans is the conserved trimeric velvet domain complex VelB/VeA/LaeA (Bayram et al., 2008). Formation of the heterodimer VelB/VeA, which is required for cleistothecia formation is promoted by MAP kinase phosphorylation (Bayram et al., 2012). VeA bridges the complex to LaeA, which is required for Hülle cell formation for nursing the fruiting body and to provide the accurate secondary metabolism (Sarikaya Bayram et al., 2010). The velvet complex connects light sensing of the fungus to development, as VeA shows light-dependent expression and nuclear transport (Bayram and Braus, 2012). RcoA was found to co-purify with

TAP::SumO and is a candidate for a link between the SumO network and the velvet domain complex. RcoA could act downstream of velvet signalling, as deletions in the corresponding gene lead to a block in sexual development, which was dependent on VeA (Todd et al., 2006).

The fungal SumO network might also be connected to development through the control of stress response pathways. This is supported by two members of the SumO network identified here: the superoxide dismutase SodA (Oberegger et al., 2000) and the homologue of yeast Ntg1, which is involved in repair of oxidatively damaged DNA (Bruner et al., 1998). Deletion in a gene coding for the NADPH oxidase NoxA results in a block of fungal sexual reproduction (Lara-Ortiz et al., 2003). Therefore, the balance of reactive oxygen species within fungal cells represents an important internal trigger of development as well as hyphal growth (Lara-Ortiz et al., 2003; Semighini and Harris, 2008) and might represent an interface between the SumO network and fungal development.

Multicellular fungal development requires equally the entire sumoylation pathway including SumO, the SumO processing enzyme UlpB, the E1 SumO activating enzyme AosA/UbaB as well as the E2 conjugating enzyme UbcN. Deletion phenotypes of the corresponding genes share similar pleiotropic effects on fungal cell differentiation. Several E3 SumO ligases might have distinct functions in fungal development as a deletion in the gene coding for the E3 ligase SizA only affected the fungal light response, but no other developmental program. This might be due to redundant and/or additional functions of other E3 ligases in the fungus. A second protein with a Siz/PIAS RING finger-like domain is present in A. nidulans, which is the uncharacterized protein AN4497 (http://www.ebi.ac.uk/Tools/pfa/iprscan/; Prosite accession number PS51044). In addition, a putative homologue of the yeast Sumo (Smt3) E3 ligase Mms21 is expressed in the fungus, which is AN10240. Deletion of the yeast Mms21 encoding gene led to an increased sensitivity against DNA damage stress and defects in nucleolar integrity and telomere functions (Zhao and Blobel, 2005). Both proteins might function as additional E3 ligases in the SumO pathway.

Fungal development requires both sides of the SumO network, sumoylation of substrates and in addition a controlled detachment of SumO. The SumO processing enzyme UlpB might be a dual function enzyme being involved in maturation of the modifier and also in the cleavage of SumO from substrates. In yeast this dual function is suggested for Ulp1, whereas Ulp2 is a desumoylating enzyme (Li and Hochstrasser, 1999; 2000). In addition, Wss1 (weak suppressor of smt3) cleaves Sumo-Ubiquitin as well as Sumo chains in vitro (Mullen et al., 2010). AN0304 represents a putative A. nidulans homologue, which was not investigated so far. UlpA represents the major desumoylating enzyme of A. nidulans. The impact of the UlpA desumoylating enzyme is considerable but less severe in comparison to sumoylation enzymes as the E1 activating or the E2 conjugating enzyme. This might be due to the described additional desumoylation functions, underlining the importance of a dynamic change between the modified and unmodified states of SumO target substrates during differentiation.

The SumO network and other UBL pathways

Cellular target proteins might be modified by sumoylation or ubiquitination at the same lysine residue. Different modifications of the same substrate can result in different molecular readouts which can affect stability, localization or activity of a target protein. If the same lysine residue can be differently post-translational modified, this can lead to competitive situations for two protein modification machineries. Additional interactions of two modification pathways include the attachment of one modifier to the enzymes of the pathway of the other modifier or of enzymes that function in both pathways (Praefcke et al., 2012). Cross-talk can be mediated through SUMO-targeted ubiquitin ligases (STUbLs), which are conserved in eukaryotes. STUbLs can recognize sumoylated proteins through SIM motifs and attach ubiquitin. This ubiquitin can serve as proteasomal degradation signal leading to proteolysis of the Sumo modified substrate (Uzunova et al., 2007). Several subunits of the proteasome were identified through the tagged SumO recruitment approach, hinting to a connection between sumoylated proteins and the 26S proteasome. One scenario is that ubiquitin serves as specific degradation signal for distinct sumoylated substrates. This is also supported by the identification of a putative ubiquitin E1 activating enzyme in the SumO screen. The protein could be SumO modified as it carries four possible sumoylation motifs.

Among the Ubiquitin family, Nedd8 is most similar to ubiquitin. An interplay of the SumO network with the Nedd8 network of neddylation and deneddylation is also possible. Overexpression of the deneddylase DenA can partially suppress developmental defects caused by a ulpA deletion strain lacking the SumO isopeptidase. The exact molecular interplay is yet elusive and might include also a second more complex deneddylase, which is present in the fungus, the COP9 signalosome (Braus et al., 2010; Nahlik et al., 2010). Both deneddylases physically interact with each other (Christmann et al., 2013). The COP9 signalosome also links deneddylation to development, secondary metabolism, hormone levels, oxidative stress response and light control (Braus et al., 2010). A link between the COP9 signalosome and desumoylation has not been described yet and might be indirectly mediated through the DenA-COP9 interaction, which seems to be conserved in eukaryotes (Christmann et al., 2013).

The SumO network and gene expression

The efficiency of transcription depends heavily on the modification of histones, which can result in activating or completely inhibiting gene expression. Histone modifications include phosphorylation, methylation, acetylation, but also ubiquitination or sumoylation. Histone modifications have been proposed to link development and secondary metabolism in A. nidulans. The LaeA subunit of the heterotrimeric velvet domain complex is a putative histone methyltransferase (Bok and Keller, 2004; Bayram et al., 2008; Sarikaya Bayram et al., 2010) and the single fungal S-adenosylmethionine synthetase, which supplies the cell with donor methyl groups is also involved in fungal development and secondary metabolism (Gerke et al., 2012).

Sumoylation is normally connected to a repressive state of transcriptional control (Gill, 2005). Low-level sumoylation of transcriptional regulators can support binding of additional factors. This could result in an inhibitory complex or in remodelled chromatin, which efficiently represses transcription (Geiss-Friedlander and Melchior, 2007). The A. nidulans SumO network identified in this study includes several possible transcriptional regulators as SrrA. This protein is presumably involved in control of colony shape, conidiophore production, secondary metabolism and oxidative stress response (Hagiwara et al., 2007; Vargas-Perez et al., 2007).

Important chromatin remodelers involved in Sumo-dependent gene repression are histone deacetylases (Gill, 2005) as the homologue of a subunit of the yeast histone deacetylase complex, Sin3, which was identified as part of the A. nidulans SumO network. In addition, we found subunits of the SAGA complex. Gcn5, one subunit of the histone acetyltransferase module of the SAGA complex was shown to be modified with yeast Sumo (Smt3) (Sterner et al., 2006). The COMPASS histone methyltransferase complex and especially its core subunit SetA/Set1 has been suggested to be sumoylated (Wohlschlegel et al., 2004). Another Set domain protein, human PR-Set7, functions in transcriptional repression and is modified by Sumo1. Ubc9 depleted cells revealed a derepression of PR-Set7-regulated genes (Spektor et al., 2011). There are several examples where sumoylation is not part of an inhibitory function of transcription but provides activating Sumo modifications on transcription (Lyst and Stancheva, 2007). COMPASS can be as well involved in gene silencing as in transcriptional activation (Briggs et al., 2001; Bernstein et al., 2002; Sims et al., 2007). The A. nidulans COMPASS complex and especially the catalytic subunit SetA turned out to be highly important for fungal sexual development and co-ordinated secondary metabolism. This is consistent with the recent finding that the COMPASS complex plays an important role in the initiation of meiotic recombination, which is part of the sexual program (Acquaviva et al., 2013). COMPASS is also required for accurate colony growth and efficient production of asexual spores. In an unknown mechanism, COMPASS provides spatial information for the fungal hyphae to properly position the initials of future conidiophores. Without COMPASS many hyphal tips are under the investigated conditions terminally differentiated into conidiophores, whereas an intact COMPASS directs conidiophore formation from the hyphal tip to foot cells. These cells are located within the filament and allow the final tip to continue to grow and enlarge the covered space of the colony. This seems to be an important control mechanism to efficiently populate a surface covered with nutrients. Currently, the factors triggering this developmental program and the connection to the COMPASS complex and subsequent chromatin modifications are unknown. There might be a connection between COMPASS and nutrient sensing where the setA deletion strain is deficient in syntheses or uptake of certain nutrients or sensitive to other environmental stresses, as this phenotype occurs under nutrient-poor conditions on thin agar layers. There might be also a connection between COMPASS and polarity maintenance or redirection as under the investigated conditions, the mutant branching pattern of the hyphae appeared to be partially disturbed.

In summary, we describe elements of the complex and dynamic SumO network, including sumoylation and desumoylation of substrates, which is essential to permit multicellular development in A. nidulans. The organism possesses a single SumO protein, which is non-essential for vegetative growth but required for all multicellular developmental options of the mould. Sumoylation is involved in many cellular processes and diseases in human. It is connected to cancer (Zhao, 2007), neurodegenerative and cardiac diseases (Dorval and Fraser, 2007; Wang, 2011), diabetes (Wang and She, 2008) and viral infections (Zhao, 2007). This makes the understanding of complex sumoylation networks an important task for further research, where a multicellular fungus might be suitable to give some molecular insights.

Experimental procedure

Strains, media and growth conditions

For the preparation of plasmid DNA the Escherichia coli strain DH5α (Woodcock et al., 1989) was used. Bacteria were grown in lysogeny broth (LB) medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl) (Bertani, 1951) in the presence of 100 μg ml^{-1} ampicillin at 37°C. Aspergillus nidulans strains used in this study are listed in Table S3. The respective parental strains of the mutants were used as control for the experiments and are indicated as WT. Minimal medium [MM, 1% glucose, 2 mM $MgSO_4$, 70 mM

$NaNO_3$, 7 mM KCl, 11.2 mM KH_2PO_4, 0.1% trace element solution (Käfer, 1977) pH 5.5, 2% agar] was used for growth of Aspergillus strains, supplemented with the appropriate amounts of pyridoxine-HCl (0.1%), uridine (5 mM), uracil (5 mM), tryptophane (4 mM), pyrithiamine (100 ng ml^{-1}), nourseothricin (120 µg ml^{-1}) and phleomycin (10 µg ml^{-1}).

Transformation procedure

Escherichia coli cells were transformed as described (Inoue et al., 1990). A. nidulans was transformed as described (Punt and van den Hondel, 1992; Eckert et al., 2000). Plasmids used for transformation or carrying cassettes for transformation are listed in Table S4. Primers used in this study are listed in Table S5.

Plasmid and strain construction of the sumO deletion and reconstitution strain

All primers used for plasmid constructions are listed in Table S5. For the purpose of deleting sumO, a deletion cassette containing 2.3 kb of the 5′ and 3.3 kb of the 3′ flanking region of sumO was generated. The flanking regions were amplified from genomic DNA using the primers OLKM34, OLKM35, OLKM36 and OLKM37 containing flanking SfiI sites. The PCR product was ligated with the pyrithiamine (ptrA) resistance cassette (Krappmann et al., 2006), which was cut out of pME3024 using SfiI restriction sites. The ligation product was cloned into pCR-BluntII-TOPO (Invitrogen) yielding plasmid pME3320 and the entire deletion cassette was obtained by digestion of pME3320 with ScaI and transformed into the ΔnkuA strain TNO2A3 resulting in AGB380. Southern hybridization analysis was conducted to confirm homologous recombination of the ptrA knockout cassette at the sumO locus using a PCR amplified flanking region sumO probe (OLKM35 and OLKM42). AGB380 was backcrossed with the strain UI224 resulting in strain AGB339 to remove the nkuA deletion.

For reconstitution of sumO, the plasmid pME3319 was transformed into AGB339 yielding strain AGB350. pME3319 was created by cloning a genomic PCR fragment with the size of 3.3 kb containing 2 kb of the 5′ and 300 bp of the 3′ flanking region of sumO as well as the respective ORF (OLKM44 and OLKM45) into the ApaI restriction site of pNV1. Ectopical integration was confirmed by PCR using primers OLKM42 and OLKM47. We transformed the deletion cassette into AGB551 resulting in AGB801 to generate a sumO deletion strain in veA+ background. The strain was verified by Southern hybridization.

Plasmid and strain construction for TAP::SumO strains

To express a TAP::SumO fusion protein, we amplified the 5′ flanking region of A. nidulans sumO using primers containing flanking ApaI restriction sites (RH1 and RH2). The PCR product was ligated into the ApaI site of plasmid pME2968 that carries a TAP cassette. Afterwards, the 3′ flanking region of sumO, amplified with RH8 and RH37 (with flanking NdeI restriction sites) and ligated into the NdeI site of the plasmid resulting in pME3967. A fusion cassette was generated (primers RH3 and RH38) consisting of the sumO cDNA (amplified with RH3 and RH5 from cDNA), the sumO terminator (amplified with RH4 and RH7 from genomic DNA) and the A. fumigatus pyrG marker (amplified with RH6 and RH38) from pME3160. This cassette was cloned into the ClaI restriction site of pME3967 resulting in pME3968. For A. nidulans transformation the tap::sumO cassette was excised from pME3968 using the restriction enzymes NruI and PscI. The cassette was transformed into the strains TNO2A3 and AGB689 resulting in AGB688 and AGB737. Correct replacement of the sumO locus by the tap::sumO cassette was confirmed by Southern hybridization using the sumO probe described before. Expression of the fusion protein was confirmed by Western hybridization using a calmodulin binding epitope tag antibody (Upstate/Millipore) and horseradish peroxidase-coupled goat anti-rabbit IgG (Invitrogen) as secondary antibody.

With the cassette used to express TAP::SumO, the locus of the neighbouring gene AN1192 is disturbed. We wanted to exclude an influence on the strain and therefore constructed a control strain carrying only the pyrG marker surrounded by the sumO terminator and the 3′ flanking region but not the tap::sumO cassette. The fragment was amplified with RH4 and RH37 from pME3968 and transformed into TNO2A3. Insertion of the cassette was verified in Southern hybridization using a sumO terminator probe amplified with OLKM36 and OLKM43. Phenotypical analyses of the AN1192 deletion strain revealed neither an impact on asexual nor on sexual development suggesting no global impact on development and protein expression.

PCR-mediated construction of deletion cassettes and deletion strain construction for ulpA, ulpB, aosA, ubaB, ubcN, sizA

We replaced the coding region of the genes ulpA, ulpB and ubcN as well as the complete genes (including UTRs) aosA, ubaB and sizA with cassettes constructed by fusion PCR (Szewczyk et al., 2006). The 5′ and 3′ flanking regions were amplified from genomic DNA of the A. nidulans wild type strain TNO2A3 (Nayak et al., 2006) using the following primers: ulpA-RH78 + RH79, RH82 + RH83; ulpB-RH84 + RH96, RH88 + RH89; aosA-RH254 + RH255, RH258 + RH259; ubaB-RH248 + RH249, RH252 + RH253; ubcN-RH19 + RH21, RH23 + RH24; sizA-RH260 + RH261, RH264 + RH265. For the knockout

cassettes the ptrA marker was used for ulpB (amplified with RH95 + RH87), aosA (amplified with RH256 + RH257), ubcN (amplified with RH20 + RH22) and sizA (amplified with RH262 + RH263). For the deletion cassette of ulpA the pyroA marker amplified with RH80 and RH81 and for ubaB the pyrG marker amplified with RH250 and RH251 was used. The corresponding fusions were generated with the following primers: ulpA-RH78 + RH83; ulpB-RH84 + RH89, aosA-RH254 + RH259; ubaB-RH248 + RH253; ubcN-RH19 + RH24; sizA-RH260 + RH265. The cassettes were subcloned into the pJET1.2/blunt vector (Thermo Scientific) resulting in plasmids pME3979, pME4064, pME3977; pME3976; pME3984 and pME3978. For A. nidulans transformations the deletion cassettes were excised with appropriate restriction enzymes. All cassettes were transformed in TNO2A3 resulting in AGB692, AGB717, AGB693, AGB690; AGB691 respectively. The double deletion strain AGB694 was constructed by transformation of AGB692 with the ubaB deletion cassette from pME3976. Deletion strains were confirmed by Southern hybridizations using appropriate restriction enzymes and probes.

Plasmid and strain construction for complementations of ulpA, aosA, ubaB and ubcN deletion strains

In order to complement the constructed deletion strains, a cassette for ectopical reintroduction of ulpA was constructed. ulpA with 5′ and 3′ flanking regions was amplified from genomic DNA with the primers RH234 and RH235. The fragment was cloned into the EcoRV restriction site of pME3281 resulting in pME3985 and the plasmid was transformed into AGB689 resulting in AGB707. Ectopical integration was confirmed by Southern hybridization.

The genes encoding aosA, ubaB and ubcN were reintroduced at their original loci. For complementation of aosA the 5′ flanking region with the gene and the terminator region was amplified from genomic DNA using the primers RH360b and RH319. The pyroA marker of A. fumigatus was amplified using the primers RH316 and RH317. The 3′ flanking region was amplified using the primers RH320 and RH361b. The three fragments were fused using the primers RH360b and RH361b. The cassette was subcloned into the pJET1.2/blunt vector resulting in pME3994. For transformation the cassette was amplified from pME3994 with the primers RH360b and RH361b. The cassette was transformed into AGB692 resulting in AGB704.

For complementation of ubaB the 5′ flanking region together with the gene and a terminator region was amplified from genomic DNA using the primers RH395 and RH397. The fragment was subcloned into the pJET1.2/blunt vector resulting in pME3986. An A. fumigatus pyroA marker was amplified with RH316 and RH317 and a 3′ flanking fragment with RH318 and RH396. The two fragments were fused with RH316 and RH396 and inserted in the StuI restriction site of pME3986 resulting in pME3987. The cassette was restricted from the vector and transformed into AGB693 resulting in AGB705. Integration at the ubaB locus was confirmed by Southern hybridization.

For complementation of the ubcN deletion strain we amplified the 5′ flanking region together with the gene and the 3′ flanking region with the primers RH19 and RH24. The fragment was subcloned into the pJET1.2/blunt vector. The plasmid was then linearized with EcoRV, which restriction site was inside the 3′ flanking region behind the terminator. The A. fumigatus pyroA marker was amplified with RH80 and RH81 and ligated into the plasmid resulting in pME3995. The cassette was restricted from the plasmid and used for transformation into AGB690 resulting in AGB706. Correct integration was confirmed by Southern hybridization.

Plasmid and strain construction of setA deletion

The cassettes for deletion of setA were constructed by fusion PCR (Szewczyk et al., 2006). The 5′ and 3′ flanking regions were amplified from genomic DNA of the A. nidulans wild type strain TNO2A (Nayak et al., 2006), using the primers RH201, RH202, RH205 and RH206. For the knockout cassette the ptrA marker was amplified from pME3024 using the primers RH203 and RH204 and fused to the flanking regions of setA. The cassette was subcloned into the pJET1.2/blunt vector resulting in the plasmid pME3996. The deletion cassette was excised from pME3996 and transformed into the A. nidulans strain AGB551 resulting in AGB712. Deletion strains were confirmed by Southern hybridizations using appropriate restriction enzymes and probe.

Complementation of setA and ulpB deletion

We constructed a plasmid for ectopical reintroduction of the setA and the ulpB locus respectively. Therefore, the setA gene including around 500 bp of the 5′ and 3′ flanking regions was amplified from genomic DNA of a wild type strain using the primers RH212 and RH215. We ligated the PCR fragment into the EcoRV restriction site of a vector carrying a bleo resistance cassette (pME3281), mediating resistance against phleomycin. The resulting plasmid was named pME3997. The ulpB gene including promoter and terminator region was amplified with UW45 and RH89 and ligated into the EcoRV restriction site of pME3281 resulting in pME4067.

Due to the very sensitive nature of the setA and the ulpB deletion strain we applied the following strategy. For complementation of the setA deletion strain we first ectopically integrated the plasmid pME3997 into AGB551,

resulting in AGB713, and confirmed the integration as well as the integrity of the setA wild type locus by Southern hybridizations using appropriate enzymes and the 5′ flanking region as probe. Next, we used this strain for transformation with the setA deletion cassette and confirmed the replacement of the wild type locus as well as the ectopical presence of the complementation construct using Southern hybridizations as described before. The resulting strain was named AGB714. For complementation of the ulpB deletion strain we ectopically integrated pME4067 in TNO2A3 and confirmed both, the ectopical integration as well as the integrity of the original locus, with Southern hybridization using the 5′ flanking region as probe. The strain was named AGB733. Next, the ulpB deletion cassette was restricted from pME4064 and introduced in AGB733 resulting in AGB734. The replacement of the endogenous locus and the maintenance of the ectopical complementation construct was confirmed by Southern hybridization as described before.

Plasmid and strain construction for SetA::TAP

To replace the wild type locus by a setA::tap gene fusion we amplified the 5′ flanking region with the setA gene from wild type DNA using the primers RH206 and RH227. In addition, we amplified a fusion construct of the tap and a gpdA promoter driven nourseothricin resistance cassette from pME3928 using the primers OZG209 and OZG192. We also amplified the 3′ flanking region of setA from genomic wild type DNA using the primers RH223 and RH201. In order to fuse the tap-marker construct to the gene and the 3′ flanking region we applied fusion PCR with the primers RH221 and RH224. We subcloned the fusion construct into the pJET 1.2/blunt vector resulting in pME4066. For transformation we used the strain AGB551 and the cassette from pME4066. Transformants were verified by Southern hybridizations using appropriate enzymes and the 3′ flanking region as probe. The strain was named AGB736.

Plasmid and strain construction for denA overexpression

For overexpression of denA we used the plasmid pME3160 and cloned denA genomic DNA amplified with MC30 and MC31 in the SwaI restriction site resulting in pME4068. The plasmid was transformed into the wild type AGB152 and the ulpA deletion strain AGB689 resulting in AGB695 and AGB696 respectively. Ectopical integration was confirmed by Southern hybridization using appropriate restriction and a nitrate promoter probe (amplified from genomic DNA with OLKM67 and OLKM68). High expression levels of denA were confirmed using qRT-PCR with the primers RT-MC1 and RT-MC2.

Tandem affinity purification (TAP)-tag purification

For purifications of TAP tagged SumO, substrates and interacting proteins, we used the strains AGB688 and AGB737. For purifications with TAP tagged SetA we used strain AGB736. Purifications were performed as reported before (von Zeska Kress et al., 2012). Protein bands from SDS gels were in-gel digested with trypsin (Shevchenko et al., 1996).

NanoLC-nanoESI mass spectrometry analysis and database searching

Peptides of 2 µl sample solution from trypsin-digested proteins were trapped and washed with 0.05% trifluoroacetic acid on an Acclaim® PepMap 100 column (75 µm × 2 cm, C18, 3 µm, 100 Å, P/N164535 Thermo Scientific) at a flow rate of 4 µl min^{-1} for 12 min. Analytical peptide separation by reverse phase chromatography was performed on an Acclaim® PepMap RSLC column (75 µm × 15 cm, C18, 3 µm, 100 Å, P/N164534 Thermo Scientific) running a gradient from 96% solvent A (0.1% formic acid) and 4% solvent B (acetonitrile, 0.1% formic acid) to 50% solvent B within 25 min at a flow rate of 250 nl min^{-1} (solvents and chemicals: Fisher Chemicals).

Peptides eluting from the chromatographic column were online ionized by nano-electrospray (nESI) using the Nanospray Flex Ion Source (Thermo Scientific) and transferred into the mass spectrometer. Full scans within m/z of 300–1850 were recorded by the Orbitrap-FT analyser at a resolution of 60 000 at m/z 400. Peptides were fragmented by collision-induced decay (CID) in the LTQ Velos Pro linear ion trap. LC/MS method programming and data acquisition was performed with the software XCalibur 2.2 (Thermo Fisher).

Orbitrap raw files were analysed with the Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA) using the Mascot and Sequest search engines against the A. nidulans protein database with the following criteria: Peptide mass tolerance 10 ppm; MS/MS ion mass tolerance 0.8 Da, and up to two missed cleavages allowed. The variable modification considered was methionine oxidation, and carbamidomethylation was considered as fixed modification. For protein identification at least two different high ranking peptides were required that after validation against a decoy database had a false discovery rate of 0.01 or less.

Protein assays and Western hybridization analysis

In general, experiments were carried out as described before (von Zeska Kress et al., 2012). Protein was extracted from ground mycelium with extraction buffer (300 mM NaCl, 100 mM Tris-HCl pH 7.2, 10% glycerol,

0.1% NP-40, 1 mM DTT, and protease inhibitor cocktail – Complete, EDTA-free; Roche). Protein concentration was determined by a modified Bradford assay (Bradford, 1976). Proteins were separated in SDS-PAGE 8–20% gradient or non-gradient 12% gels. Gels were transferred onto nitrocellulose membrane (Whatman). The membrane was blocked in 5% dried milk dissolved in TBS/T buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). As primary antibodies we used Calmodulin binding protein antibody (Upstate/Millipore), SumO antibody (Genescript) or tubulin antibody (Sigma-Aldrich) respectively. As secondary antibodies horseradish peroxidase-coupled rabbit antibody (Invitrogen) or mouse antibody (Jackson ImmunoResearch) were used. For developing, the enhanced chemiluminescence method (Tesfaigzi et al., 1994) was applied. The signal intensity was quantified with the Fusion-SL7 system and the Bio1D software (Peqlab).

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