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ORIGINAL ARTICLE

New insights from an old mutant: SPADIX4 governs fruiting body development but not hyphal fusion in *Sordaria macrospora*

Ines Teichert[1] · Miriam Lutomski[1,2] · Ramona Märker[1] · Minou Nowrousian[1] · Ulrich Kück[1]

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Abstract During the sexual life cycle of filamentous fungi, multicellular fruiting bodies are generated for the dispersal of spores. The filamentous ascomycete *Sordaria macrospora* has a long history as a model system for studying fruiting body formation, and two collections of sterile mutants have been generated. However, for most of these mutants, the underlying genetic defect remains unknown. Here, we investigated the mutant spadix (spd) that was generated by X-ray mutagenesis in the 1950s and terminates sexual development after the formation of pre-fruiting bodies (protoperithecia). We sequenced the spd genome and found a 22 kb deletion affecting four genes, which we termed *spd1-4*. Generation of deletion strains revealed that only *spd4* is required for fruiting body formation. Although sterility in *S. macrospora* is often coupled with a vegetative hyphal fusion defect, ΔSPD4 was still capable of fusion. This feature distinguishes SPD4 from many other regulators of sexual development. Remarkably, GFP-tagged SPD4 accumulated in the nuclei of vegetative hyphae and fruiting body initials, the ascogonial coils, but not in sterile tissue from the developing protoperithecium. Our results point to SPD4 as a specific determinant of fruiting body formation. Research on SPD4 will, therefore, contribute to understanding cellular reprogramming during initiation of sexual development in fungi.

Keywords Fungal sexual development · Vegetative hyphal fusion · Whole-genome sequencing · *Sordaria macrospora*

Introduction

Multicellularity permits the formation of specialized cells and tissues, and multicellular organisms have evolved independently several times in different eukaryotic clades. Filamentous ascomycetes have been shown to be excellent model organisms for studying multicellular development. These fungi are characterized by the formation of long tubular cells, the hyphae, which are compartmentalized by septa that permit cytoplasmic and organellar movement through septal pores (Gull 1978). In contrast to yeasts, filamentous fungi are truly multicellular, in that they form large colonies of interconnected hyphae, the mycelium. Mycelia contain different cell types, such as leading hyphae at the colony periphery and highly vacuolated trunk hyphae in the colony interior (Bistis et al. 2003).

The fungal mycelium is able to generate higher order structures, such as conidiophores for asexual propagation, fruiting bodies for sexual propagation, and sclerotia for long-term survival. The generation of these structures has been studied in a number of model ascomycetes. For example, conidiophore development has been analyzed in *Aspergillus nidulans, Aspergillus fumigatus,* and *Penicillium chrysogenum* (e.g., Hoff et al. 2010; Harting et al. 2013; Cai et al. 2015; Chi and Craven 2016), fruiting body formation in *A. nidulans*, *Neurospora crassa*, *Podospora*

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✉ Ines Teichert
ines.teichert@rub.de

[1] Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität Bochum, ND6/166, Universitätsstrasse 150, 44780 Bochum, Germany

[2] Present Address: Abteilung Molekulare Genetik I, Universität Duisburg-Essen, 45141 Essen, Germany

anserina, and *Sordaria macrospora* (e.g., Fleissner et al. 2009; Kim et al. 2009, 2012; Lord and Read 2011; Coppin et al. 2012; Voigt and Pöggeler 2013; Lehr et al. 2014; Sarikaya-Bayram et al. 2014; Teichert et al. 2014b), and sclerotia formation in *Botrytis cinerea* and *Sclerotinia sclerotiorum* (e.g., Duan et al. 2013; Siegmund et al. 2015). Among these higher order structures, fruiting bodies are the most complex and harbor many cell types that are not present in the vegetative mycelium (Bistis et al. 2003; Lord and Read 2011). Therefore, vast changes in the hyphal architecture and function are a prerequisite for fruiting body formation (Pöggeler et al. 2006).

In this study, we investigated sexual development in the filamentous ascomycete *S. macrospora,* which has a long history as a model for fruiting body formation and meiotic recombination (Esser and Straub 1956; Esser and Straub 1958; Arnaise et al. 1984). *S. macrospora* is a saprophytic fungus that forms closed pear-shaped fruiting bodies (perithecia) after 7 days of growth under laboratory conditions. Sexual development starts with the formation of curled hyphae, termed ascogonia, which represent the female gametangia. Subsequently, ascogonia are wrapped by enveloping hyphae, leading to the formation of the spherical protoperithecium (Esser 1982; Lord and Read 2011). Though the young protoperithecium is loosely tangled, maturing protoperithecia develop a dense outer layer of adhered hyphae termed peridium.

Many factors required for perithecia formation were described recently for *S. macrospora*. These include transcription factors, mating type proteins, signaling components, subunits of the striatin-interacting phosphatase and kinase (STRIPAK) complex, and proteins involved in autophagy (reviewed in Kück et al. 2009, 2016; Teichert et al. 2014a). Interestingly, most of these factors are also required for vegetative hyphal fusion (VHF), a process assumed to enable a fungal colony to rapidly establish a mycelial network due to fast distribution of nutrients, signaling molecules, and organelles (Aanen et al. 2008; Read et al. 2009; Simonin et al. 2012). A general phenomenon in ascomycetes seems to be that proteins required for VHF are also required for other developmental processes. For example, NADPH oxidase 1, MAP kinase MAK1 of the cell wall integrity pathway, and the scaffold protein PRO40/SOFT are not only involved in VHF, but also in fruiting body formation, symbiotic or pathogenic life style, and/or the development of epigenetically controlled growth phenotypes in diverse ascomycetes (Malagnac et al. 2004; Fleißner et al. 2005; Kicka et al. 2006; Engh et al. 2007; Rech et al. 2007; Prados Rosales and Di Pietro 2008; Lichius 2010; Charlton et al. 2012; Lichius et al. 2012; Kayano et al. 2013; Dirschnabel et al. 2014; Teichert et al. 2014b; Tong et al. 2014; Becker et al. 2015; Turra et al. 2015).

The strong correlation of a sexual developmental defect and a VHF defect has led to the hypothesis that VHF is a prerequisite for fruiting body formation. However, recent results challenge this hypothesis; mutants have been identified that are either sterile and fusion-competent or fertile and fusion-deficient (for an overview, see Lichius and Lord 2014). For example, the *S. macrospora* autophagy mutants ΔSmatg4, ΔSmatg8, and ΔSmjlb1, as well as *N. crassa* prm-1, belong to the sterile, fusion-competent category (Fleissner et al. 2009; Voigt et al. 2013; Voigt and Pöggeler 2013), and *N. crassa* mutants ham-4 and ham-11 belong to the fertile, fusion-deficient category (Simonin et al. 2010; Fu et al. 2011; Leeder et al. 2013). The question of how the specific factors control fruiting body formation and regulate others and themselves is still unanswered.

In this study, we analyzed the sexual mutant spadix (spd) from *S. macrospora* that was generated by X-ray mutagenesis and previously described to form aberrant ascogonia and few very small protoperithecia (Esser and Straub 1958; Lord and Read 2011). We found that spd has a pleiotropic phenotype exhibiting sterility, pigment leakage into the medium, and cell lysis, but the mutant is capable of VHF. We identified the mutations underlying the spd phenotype and describe *spd4,* a new gene essential for sexual fruiting body formation.

Materials and methods

Strains and growth conditions

All *S. macrospora* strains used in this study are listed in Table S1. Unless stated otherwise, growth conditions were as described previously (Kamerewerd et al. 2008; Dirschnabel et al. 2014). Transformation was carried out by protoplast formation as described (Dirschnabel et al. 2014), and transformants were selected on medium containing either nourseothricin (50 µg/ml) or hygromycin B (80 U/ml). For measuring vegetative growth, strains were pre-cultured on corn meal-malt fructification medium (BMM) for 2 days, and standard inocula were transferred to synthetic Westergaard's (SWG) medium (Nowrousian et al. 2007). The growth front was marked after one and two days, and the distance between these two marks was measured. Data are from three independent experiments with three technical replicates each.

DNA preparation, Illumina sequencing, and mapping

Sterile mutant lu/spd from our laboratory collection was back-crossed several times to wild-type, developmental mutants, or brown-spored fus to reduce unrelated background mutations (Nowrousian et al. 2012). For

whole-genome sequencing, the spd isolate S102018 was crossed to fus (Fig. S1). DNA was extracted from 40 fertile and 40 sterile progeny of this cross as described previously (Nowrousian et al. 2012). Five micrograms of pooled genomic DNA from each spd and wt was subjected to 50 bp single read Illumina/Solexa sequencing with a HiSeq2000 at GATC Biotech (Konstanz, Germany). Cleaning of raw data, mapping to the *S. macrospora* reference genome (Nowrousian et al. 2010; Teichert et al. 2012), analysis of sequence variants, and detection of uncovered regions were performed as described (Nowrousian et al. 2012) using the Burrows Wheeler Alignment tool (Li and Durbin 2009), SAMtools (Li et al. 2009), and custom-made Perl scripts. Raw sequence data from sequencing mutant spd and wildtype were submitted to the NCBI sequence read archive (accession no. SRX1868445 and SRX1867979).

Generation of deletion strains

Plasmids and oligonucleotides used in this study are listed in Tables S2 and S3, respectively. To generate deletion strains for *SMAC_01961* (*spd1*), *SMAC_01962* (*spd2*), and *SMAC_01963* (*spd3*), plasmids pKO-spd1, pKO-spd2, and pKO-spd3, respectively, were cloned by yeast recombination. For this purpose, 5′ and 3′ flanking regions were amplified from *S. macrospora* genomic DNA for *spd1* (5′, 1961-5fw/1961-5rv, 1065 bp; 3′, 1961-3fw/1961-3rv, 956 bp), *spd2* (5′, 1962-5fw_IT/1962-5rv_IT, 987 bp; 3′, 1962-3fw/1963-3rv, 1000 bp), and *spd3* (5′, 1963-5fw/1963-5rv, 1000 bp; 3′, 1963-3fw/1963-3rv, 1000 bp), and transformed into yeast strain PJ69-4a (James et al. 1996) together with *Eco*RI/*Xho*I-digested pRS426 (Christianson et al. 1992) and a 1.4 kb *hph* cassette, derived from pDrivehph after *Eco*RI hydrolysis (Nowrousian and Cebula 2005).

For generation of a *spd4* deletion strain, plasmid pFlip-spd4 was constructed as follows: 5′ and 3′ flanking regions of *spd4* were amplified from *S. macrospora* genomic DNA, using primer pairs 1964-KO1-EcoRV/1964-KO2-PstI (1099 bp) and 1964-KO3-HindIII/1964-KO4-BglII (1037 bp), respectively, and sub-cloned into pDrive (Qiagen, Germany), generating pD5-spd4 and pD3-spd4. The 3′ flanking region was cut from pD3-spd4 with *Hin*dIII and *Bgl*II and ligated into pFlip (Bloemendal et al. 2014), generating pFlip3-spd4. The 5′ flanking region was cut from pD5-spd4 with *Eco*RV and *Pst*I and ligated into the corresponding sites of pFlip3-spd4, generating pFlip-spd4.

Plasmids pKO-spd1, pKO-spd2, and pKO-spd3 were digested with *Eco*RI, and each construct was transformed into *S. macrospora* Δku70 (Pöggeler and Kück 2006). Preparation of DNA and Southern hybridization were performed as described (Kamerewerd et al. 2008). PCR-verified primary transformants (see Figs. S2–S4 for oligonucleotides) were crossed to spore color mutant fus (Nowrousian et al. 2012), and ascospore isolates showing hygromycin B resistance and nourseothricin sensitivity were analyzed by PCR and Southern hybridization (Figs. S2–S4). To construct a Δspd4 strain, pFLIP-spd4 was digested with *Eco*RV and *Bgl*II, the deletion cassette was transformed into Δku70, and primary transformants were analyzed by PCR. The *spd4* deletion cassette contains an *flp* recombinase gene controlled by the inducible *Smxyl* promoter for marker recycling (Bloemendal et al. 2014), and thus, tetrad analysis was employed to identify hygromycin B- and nourseothricin-sensitive homokaryotic deletion strains. For verification, these strains were analyzed by PCR and Southern hybridization (Fig. S5).

Restoration of the *spd1* ORF in the spd mutant background

To complete the *spd1* ORF in the spd background, we first generated spd/Δku70 by crossing the single mutants to generate an spd strain favoring homologous recombination. Plasmid pKI1961 was generated by yeast recombination, containing the complete *spd1* ORF, an *hph* resistance cassette, and the region located downstream of the spd deletion. PCR fragments were amplified from genomic DNA using primer pairs 1961-KI-01/1961-KI-02 (2546 bp) and 1961-KI-03/1961-KI-04 (1231 bp) and transformed into PJ69-4A together with *Eco*RI/*Xho*I-digested pRS426 (Christianson et al. 1992) and a 1.4 kb *hph* cassette, derived from pDrivehph after *Eco*RI hydrolysis (Nowrousian and Cebula 2005). pKI1961 was digested with *Mun*I and *Xho*I and transformed into spd/Δku70. PCR-verified primary transformants (see Fig. S6 for oligonucleotides) were crossed to fus, and ascospore isolates showing hygromycin B resistance and nourseothricin sensitivity were tested by PCR and Southern analyses (Fig. S6) to confirm the restoration of *spd1*. Strain S131717 was used for further analysis and designated spd::*spd1*.

Generation of plasmids

Cloning and propagation of plasmids were performed using the standard laboratory protocols (Sambrook and Russel 2001) and *Escherichia coli* XL1 Blue MRF' (Jerpseth et al. 1992) as a host strain. Alternatively to restriction-ligation-mediated cloning, yeast recombination was applied as described previously (Colot et al. 2006; Bloemendal et al. 2012) using *Saccharomyces cerevisiae* PJ69-4A (James et al. 1996) as a host.

All plasmids used in this study are listed in Table S2. Vectors with PCR fragments missing in the 22 kb deletion in spd were generated by amplifying fragments from *S. macrospora* wild-type DNA and sub-cloning into

pDrive (Qiagen) or pTOPO (LifeTechnologies). Specifically, pRR343-3 and pRR345-1 are based on pDrive and were generated with primer pairs spd_07/spd_10 and spd_13/spd_04, respectively. pRR350-10, pRR351-6, and pRR352-4 are based on pTOPO and were generated with primer pairs spd_09/spd_12, spd_11/spd_18, and spd_14/spd_17, respectively.

Vectors p1935_OE, p-SPD1_OE, p-SPD2_OE, and pSPD3_OE were generated by homologous recombination in yeast. For *SMAC_01935*, a 1956 bp PCR fragment was generated with primers 1935-01/1935-02. For *spd1*, a 1530 bp PCR fragment was generated with primers 1961-01/1961-02. For *spd2*, a 2218 bp and a 2756 bp PCR fragment were generated with primers 1962-01/1962-02 and 1962-03/1962-04, respectively. For *spd3*, a 1152 bp PCR fragment was generated with primers 1963-01/1963-02. The PCR fragments for each gene were transformed into yeast together with *Hin*dIII-digested pDS23 (Schindler and Nowrousian 2014) as recipient vector.

Vector pSPD4_OE was generated by amplification of the complete *spd4* ORF using primer pair 1964-09/1964-10, sub-cloning into pDrive, and ligation of the *Not*I and *Spe*I cut 2032 bp fragment into *Not*I and *Spe*I sites of pEHN1nat, carrying the *A. nidulans gpd* promoter and *trpC* terminator and the *nat1* resistance gene (Dreyer et al. 2007). Vector pSPD4_NA, carrying *spd4* together with native upstream and downstream regions, was generated by yeast recombination of *Pvu*II-digested pDS23 (Schindler and Nowrousian 2014) and three PCR fragments amplified from *S. macrospora* genomic DNA (1964-11/spd_13, 1970 bp; 1964-12/1964-13, 1380 bp) and pSPD4_OE (1964-09/1964-10, 2029 bp).

For localization of SPD4, GFP fusions were generated as follows: Vector pDS23 was linearized with *Not*I and *Bgl*II and transformed into yeast together with an *Not*I/*Bgl*II digested *egfp* fragment from pDS23, and two PCR fragments amplified from *S. macrospora* genomic DNA (1964-11/1964P-GFP, 1857 bp; GFP-1964T/1964-13, 1360 bp) to generate pSPD4PT-GFP. N- and C-terminally *egfp*-fused *spd4* constructs pGFP-SPD4 and pSPD4-GFP were generated by yeast recombination using *Bgl*II and *Not*I-linearized pSPD4PT-GFP, respectively, and PCR fragments amplified from *S. macrospora* genomic DNA (GFP-1964/spd_33 and spd_14/1964-GFP, respectively).

Microscopy

Microscopic investigations were carried out with an AxioImager M.1 microscope (Zeiss) equipped with a CoolSnap HQ camera (Roper Scientific) and a SpectraX LED lamp (Lumencor). GFP fluorescence was analyzed using filter set 41017 (Chroma Technology; HQ470/40, HQ525/50, Q495lp). To analyze sexual development over time, strains were grown on BMM-covered slides (Engh et al. 2007) for two to seven days. For detection of VHF, strains were grown on cellophane-covered MMS plates (Rech et al. 2007) for two days, and pieces of cellophane were cut and used for microscopy. Images were taken and edited with MetaMorph (version 7.7.0.0; Universal Imaging).

Perithecia formation was assayed on BMM plates after 7 days of growth using a Stemi 2000-C stereomicroscope (Zeiss) equipped with a AxioCamERc5 s digital camera (Zeiss) and AxioVision software (Zeiss). Images were processed with Adobe Creative Suite 4 (Adobe Corp.).

Results

Sequencing of the spd genome reveals a 22 kb deletion

The *S. macrospora* spd mutant was previously shown to lack fruiting bodies and thus being sterile (Esser and Straub 1958; Lord and Read 2011), but the underlying genetic defect had not been identified yet. Here, we sequenced the genome of spd using a pipeline previously established for mutant genome sequencing in *S. macrospora* (Nowrousian et al. 2012) (Table S4). The mutant was crossed with spore color mutant fus. DNA from 40 sterile and 40 fertile progeny was pooled and subjected to whole-genome re-sequencing. Comparing the spd genome to the wild-type reference genome (Nowrousian et al. 2010; Teichert et al. 2012), we identified a missense mutation (G502A) in an open reading frame (ORF), *SMAC_01935*, resulting in amino-acid substitution D133N at the protein level. *SMAC_01935* encodes a putative mitochondrial external NAD(P)H dehydrogenase. Further searches for regions not covered in the spd genome identified a 22 kb deletion (contig 2.5:1,274,642–1,296,071) comprising part of the *SMAC_01961* ORF and the complete *SMAC_01962*, *SMAC_01963*, and *SMAC_01964* ORFs (Fig. 1). All mutations are located on scaffold 2.5 of the *S. macrospora* reference genome. PCR and Southern analysis confirmed the point mutation and deletion in spd (Fig. 1), and the sterile phenotype co-segregated with the point mutation and the deletion in 40 progeny from a cross of spd and wildtype (Figs. S7 and S8). Primer walking analysis verified the genomic sequence of the deleted region.

We renamed *SMAC_01961*, *SMAC_01962*, *SMAC_01963*, and *SMAC_01964* as *spd1*, *spd2*, *spd3*, and *spd4*, respectively. SPD1 is a homolog of *A. nidulans* E1 SumO activating enzyme AosA (Harting et al. 2013), SPD2 contains a deleted in azoospermia-associated protein 2 (DAZAP2) (Tsui et al. 2000) domain, SPD3 is a putative S-adenosylmethionine-dependent methyltransferase, and SPD4 contains a domain of unknown function.

Using data from a recent RNA-seq approach (Teichert et al. 2012), we investigated the transcriptional expression

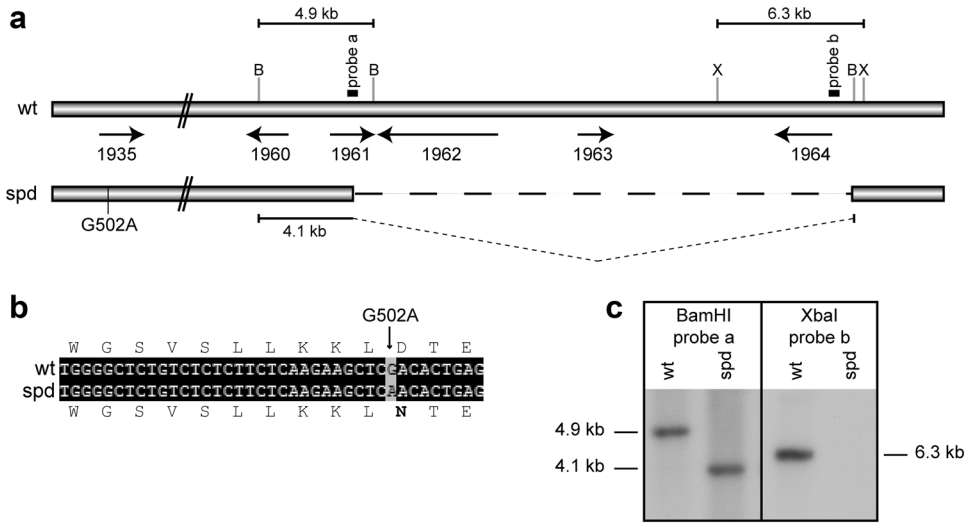

Fig. 1 Mutant spd shows a 22 kb deletion and a point mutation in *SMAC_01935*. **a** Schematic representation of the region affected by the deletion and point mutation in wildtype (wt) and spd. ORFs are indicated by *arrows*; Locus tag numbers are given below the *arrows*. *B* and *X* denote restriction sites for *Bam*HI and *Xba*I, respectively. Probes and predicted signal sizes for Southern analysis are indicated. G502A gives the location of the point mutation in the *SMAC_01935* ORF. **b** G502A substitution in *SMAC_01935* in the spd mutant genome leads to a D133 N amino-acid substitution. **c** Southern analysis of spd and wildtype (wt) verifies the 22 kb deletion in the mutant. Probes were as indicated in (**a**)

of *spd1-4*. Samples were obtained from vegetative and sexual mycelia, as well as from protoperithecia. For technical reasons, reads from protoperithecia samples tended to map preferentially to the 3′ end of the mRNA, and often could not be mapped correctly, if the 3′ untranslated regions (UTRs) were not annotated. Therefore, we manually reannotated the UTRs of the *spd* genes. Compared with sexual mycelium, *spd1* and *spd4* were up-regulated in protoperithecia, whereas *spd4* was down-regulated in vegetative mycelium compared to sexual mycelium (Fig. S9).

Several genes are responsible for the pleiotropic spd phenotype

We pursued several strategies to complement the sterile phenotype of spd. Transformation of spd with *N. crassa* and *S. macrospora* cosmids harboring the region deleted in spd whole or in part, *S. macrospora* PCR fragments covering different parts of the region deleted in spd, and five plasmids carrying the candidate genes expressed from the constitutive *A. nidulans gpd* promoter (Table S2) did not yield any fertile strains (Fig. S10). We thus individually deleted each of the genes affected by the 22 kb deletion in spd (Fig. 2a). We identified five to 12 strains for each deleted gene (Table S1), and strains having the same deletion exhibited the same phenotype. In contrast to the other deletion strains, Δspd4 is marker-free, because we employed a recently developed one-step FLP/*FRT* recombination system for its generation (Bloemendal et al. 2014).

The deletion strains were analyzed for sexual development after 7 days of growth. The wildtype formed black, pear-shaped fruiting bodies, and similar fruiting bodies were observed for Δspd1, Δspd2, and Δspd3 (Fig. 2b). However, like spd, Δspd4 exhibited no fruiting bodies (Fig. 2b). Thus, the *spd4* deletion likely causes the protoperithecial arrest. For restoration experiments, we used plasmids pSPD4_OE and pSPD4_NA in which *spd4* was under the control of *A. nidulans gpd* and the native promoter, respectively. Transformation of these plasmids into Δspd4 resulted in the restoration of fruiting body formation (Fig. 2b). However, the plasmids were unable to restore fertility in the spd mutant.

Our results indicate that additional genes are responsible for the pleiotropic spd phenotype. We hypothesized that the N-terminal part of the *spd1* gene still present in the spd mutant (Fig. 2a) interfered with the complementation approach. We thus restored the complete *spd1* ORF in the spd background (see Materials and Methods and Fig. S6 for details). These spd::*spd1* strains still exhibited an spd-like sterile phenotype, but full fertility was regained after transformation of spd::*spd1* with cosmid A12, carrying the entire region deleted in the spd mutant (Fig. S10). However, transformation of spd::*spd1* with plasmids pSPD4_OE und pSPD4_NA did not result in perithecia formation, indicating that additional genomic regions deleted in spd are required for fruiting body formation. Interestingly, RNA-seq analysis revealed a strongly transcribed region in the intergenic region of *spd2* and *spd3* (Teichert et al.

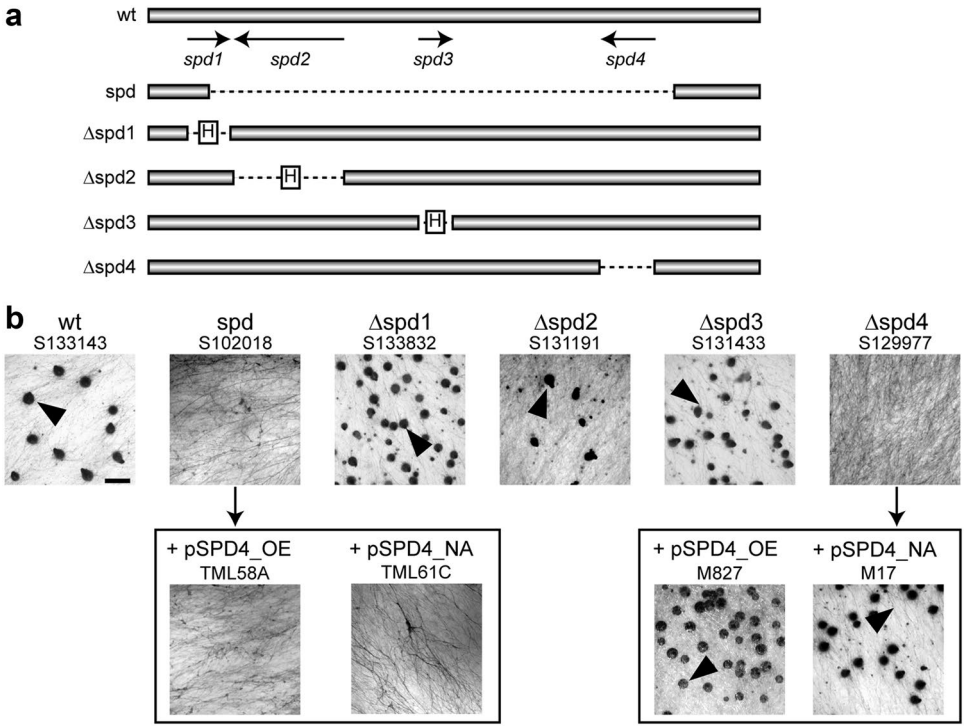

Fig. 2 Characterization of *spd* genes. **a** Schematic representation of the region affected by the spd deletion in wildtype (wt) and spd as well as four deletion strains each lacking one of the four *spd* genes. A *boxed H* indicates a hygromycin resistance cassette used to replace the particular *spd* gene. Δspd4 does not contain a resistance cassette due to marker recycling. **b** Phenotypic analysis of deletion strains and transformants with plasmids carrying *spd4*. Strains were grown on BMM fructification medium for 7 days. Mature perithecia are marked by *arrowheads*. Of the four deletion strains, only Δspd4 has a defect in perithecia formation. Introduction of the *spd4* gene expressed from its native promoter (NA) or the *A. nidulans gpd* promoter (OE) restores perithecia formation in Δspd4, but not spd. The *scale bar* is 1 mm

2012). This region does not overlap any annotated features, and tblastx searches did not reveal homology to annotated fungal genes. Therefore, it might encode a non-protein coding RNA or a small peptide essential for sexual development. The *SMAC_01935* mutation seems to be unrelated to the spd phenotype and was most probably carried along because of its close proximity to the 22 kb deletion.

Mutant Δspd4 is sterile and exhibits lysis of ascogonia and protoperithecia

Our deletion approach revealed that *spd4* is required for fruiting body formation. Therefore, we performed a time-course analysis of Δspd4 sexual development compared to wildtype and spd. The wild-type formed ascogonia, protoperithecia, and perithecia within the predicted period of 7 days (Fig. 3a). As described previously (Lord and Read 2011), spd generated only aberrant ascogonia and small aberrant protoperithecia (Fig. 3a). We further noticed profound lysis of protoperithecia. The Δspd4 strain exhibited the same phenotype as the spd mutant. Wildtype-like fruiting body development was restored by transformation with pSPD4_OE and pSPD4_NA.

We further analyzed vegetative growth of Δspd4 and wildtype on SWG plates. Growth of both strains was comparable, with 36.7 ± 1.1 and 37.1 ± 0.5 mm/day for wildtype and Δspd4, respectively. As mentioned above, most *S. macrospora* mutants with developmental arrest at the protoperithecia stage have a defect in VHF. However, Δspd4 is still capable of VHF, as is mutant spd (Figs. 3b and S11).

SPD4 localizes to the nucleus

Spd4 is conserved in ascomycetes, but its function has not yet been elucidated. To gain insight into SPD4 function, we performed in silico analysis of the predicted polypeptide using the ELM resource (Dinkel et al. 2016), revealing a nuclear localization signal (NLS), a nuclear export signal (NES), and a domain of unknown function (DUF2841). This domain structure is similar to the uncharacterized yeast homolog YDR124W (Fig. 4a). We performed localization studies with SPD4 containing an N-terminal (pGFP-SPD4) or C-terminal (pSPD4-GFP) tag. Both constructs were able to restore fertility in Δspd4 (Fig. 4b). GFP-tagged SPD4 localized to

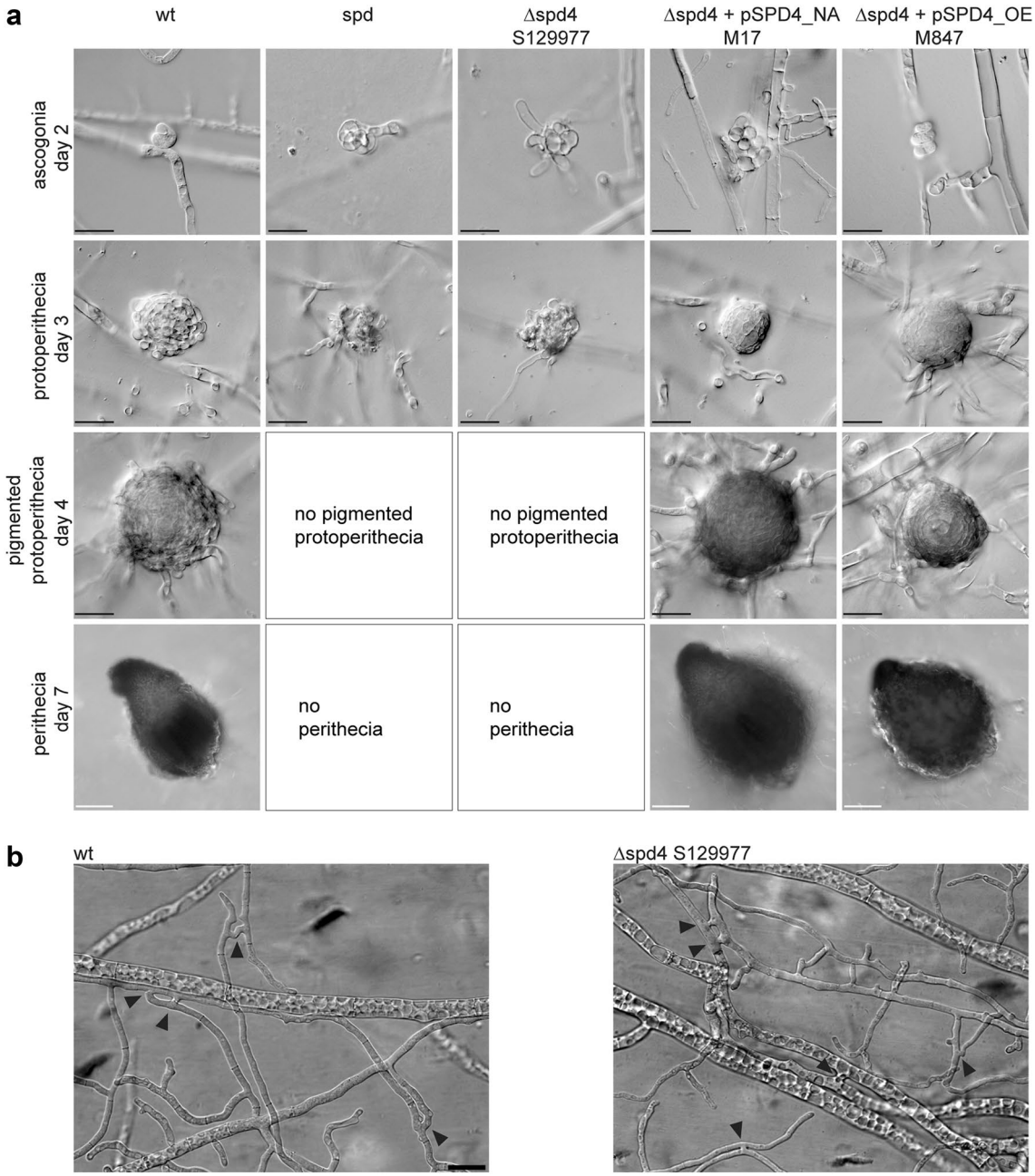

Fig. 3 Phenotypic characterization of Δspd4. **a** Sexual development was assessed microscopically after 2 to 7 days of growth on BMM-covered microscope slides. The wildtype (wt) forms ascogonia (day 2), non-melanized (day 3), and melanized protoperithecia (day 4), and mature perithecia (day 7). Mutants spd and Δspd4 form only ascogonia and non-melanized protoperithecia-like structures that show profound lysis. Perithecia are never observed. Sterility can be complemented in Δspd4 by transformation with plasmids pSPD4_NA (strain M17) and pSPD4_OE (strain M847), expressing *spd4* from native and the *A. nidulans gpd* upstream regions, respectively. The *black scale bar* is 20 µm, the *white scale bar* is 100 µm. **b** VHF in wildtype (wt) and Δspd4. Strains were analyzed after 2 days of growth on cellophane-covered MMS. *Arrowheads* indicate fusion bridges. The *scale bar* is 20 µm

spherical structures that stained with the nuclear dye NucBlue (Fig. 4c). This nuclear localization was consistent in hyphal tips and hyphae from the colony periphery (Fig. 4d). When analyzing older regions of mycelial colonies, we found that nuclear fluorescence accumulated in ascogonial coils (Fig. 4e) and was restricted to ascogonial tissues in the developing protoperithecium (Fig. 4f). Fluorescence was not observed in melanized protoperithecia, which may be due to either pigment accumulation or signal dissipation (Fig. 4g).

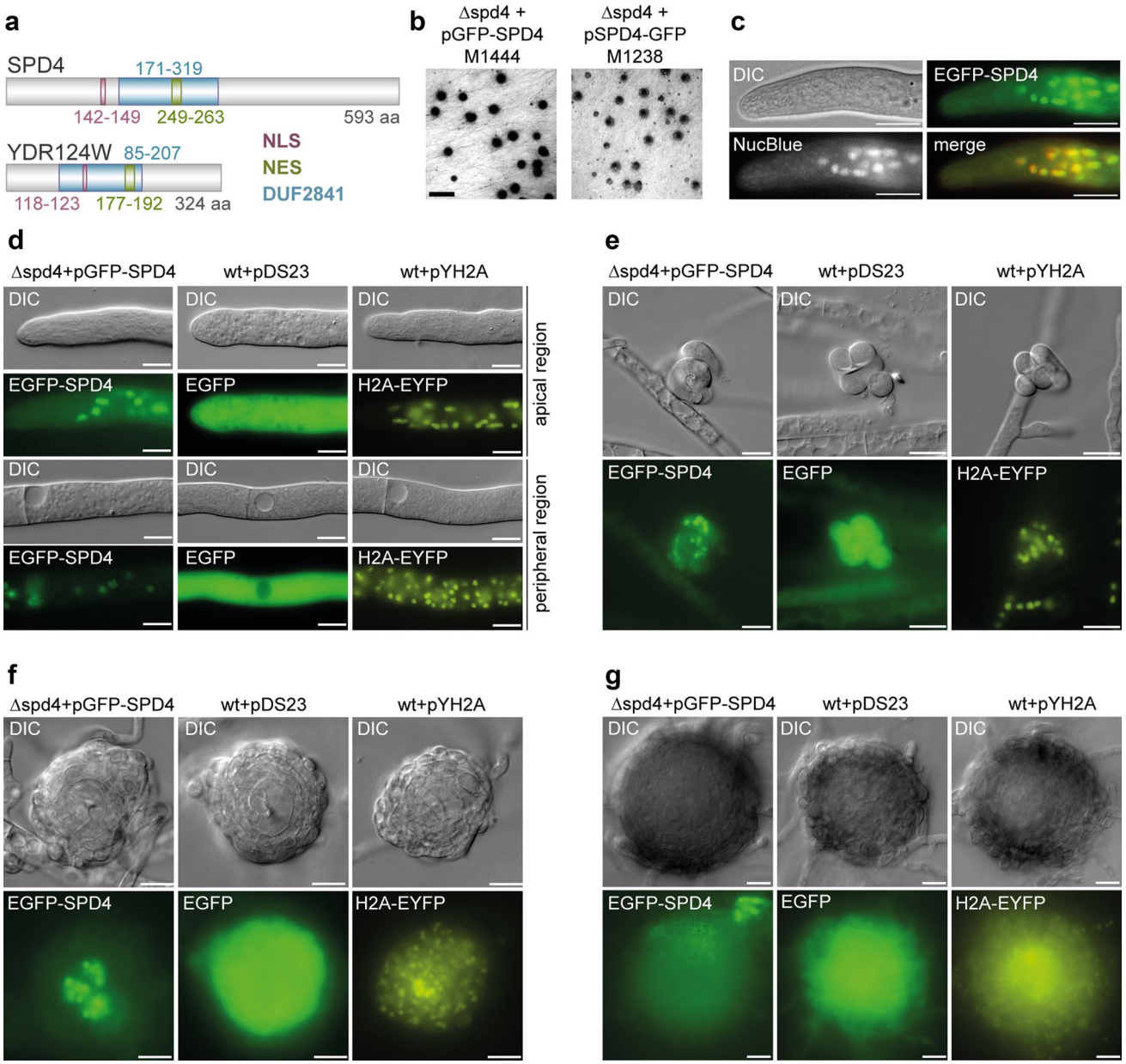

Fig. 4 Localization of SPD4. **a** Domain structure of SPD4 and its yeast homolog. Protein domains predicted in silico are shown in different colors. NLS, nuclear localization signal; NES, nuclear export sequence; DUF, domain of unknown function. **b** N-terminally (M1444) and C-terminally (M1238) GFP-tagged versions of SPD4 are able to restore fertility in Δspd4. Scale bar is 1 mm. **c** GFP-tagged SPD4 (Δspd4 + pGFP-SPD4) localizes to spherical structures that are identified as nuclei by staining with the nuclear dye NucBlue. GFP-SPD4 localizes to nuclei in hyphal tips (apical region) and vegetative hyphae from the peripheral region (**d**) as well as ascogonial coils (**e**), but not in peridial tissue of non-melanized (**f**) and melanized protoperithecia (**g**). Strains carrying EGFP alone (wt + pDS23) or EYFP-tagged histone H2A (wt + pYH2A) are shown for comparison in (**d-g**). In these strains, cytoplasmic and nuclear fluorescence, respectively, appears evenly distributed through all cells. The scale bar is 10 µm in (**c-g**)

As a control for cellular localization, we analyzed two strains with cytoplasmic EGFP and nuclear EYFP fluorescence. Strain S106352 (Teichert et al. 2012) expresses *egfp* from the *A. nidulans gpd* promoter, which led to cytoplasmic fluorescence in vegetative hyphae and evenly distributed fluorescence in different stages of fruiting body development (Fig. 4d–g). The nuclear fluorescence by strain S107299 carrying the H2A-EYFP fusion protein (Rech et al. 2007) was evenly distributed throughout hyphae, ascogonia, and protoperithecia (Fig. 4d–g). Therefore, during sexual development, SPD4 specifically localizes to nuclei in ascogonial cells.

Discussion

In this study, we identified the developmental gene *spd4* required for fruiting body development by genome sequencing of the sterile spd mutant. While restoration of sexual development in spd requires additional genomic regions besides *spd4*, the *spd4* deletion mimics the fruiting body defect of spd and thus likely causes the developmental arrest in the mutant.

Expression studies have shown that *spd4* transcript levels are up-regulated in protoperithecia compared with sexual mycelium (Teichert et al. 2012). In *S. cerevisiae*, expression of the uncharacterized gene *YDR124W,* which is homologous to *spd4,* has been shown to be induced by α factor mating pheromone (Harris et al. 2001). We found no regulation of *spd4* in Δppg1 lacking the α-factor-like pheromone gene *ppg1* (M. Lutomski and I. Teichert, unpublished), but the Δppg1 mutant from *S. macrospora* is fertile. Only strains lacking components of both pheromone/pheromone receptor pairs are sterile in *S. macrospora* (Mayrhofer et al. 2006).

We observed nuclear localization of SPD4 in vegetative hyphae and ascogonial coils, which is consistent with the presence of an NLS in SPD4. Interestingly, we did not detect SPD4 in peridial tissues. To the best of our knowledge, this is the first description of a protein that exhibits tissue-specific nuclear localization during fruiting body formation. It was suggested previously that distinct nuclear populations and developmental proteins are present in the fertile tissue inside the fruiting body (hymenium) and others in the peridium (Johnson 1976; Debuchy et al. 2010). For example, analysis of genetic mosaics of *P. anserina* indicated that *PaNox-1* and *IDC1,* which is homologous to *N. crassa ham-5* recently described to be a scaffold for the MAK-2 MAPK pathway during VHF (Dettmann et al. 2014; Jonkers et al. 2014), are required in the peridium, whereas thioredoxin genes are required in the hymenium (Jamet-Vierny et al. 2007; Malagnac et al. 2007). Localization of SPD4 to ascogonia may indicate that this protein is required in the hymenium. However, the mutant is blocked much earlier than the beginning of hymenium development, forming abnormal ascogonial coils and rudimentary protoperithecia. SPD4 may be required for proper ascogonium formation (Lord and Read 2011) or the ascogonium to protoperithecium transition, which requires the formation of enveloping hyphae that adhere to form the peridium (Kück et al. 2009; Debuchy et al. 2010; Lord and Read 2011). Ascogonium-derived signals have been suggested to be required for the formation of these hyphae (Bloemendal et al. 2010; Debuchy et al. 2010). Further studies have to clarify the nature of this signal and whether it is SPD4-dependent.

The question remains as to whether VHF is a prerequisite for fruiting body development or whether the finite number of signaling proteins in the fungal cell is just reused for different signaling processes with additional factors conferring specificity to signaling outputs. A prominent example of a reusable signaling protein is the yeast MAPKKK STE11, which participates in three MAPK pathways: pheromone, pseudohyphal growth, and osmostress (reviewed by Saito 2010). In this case, signaling specificity is provided by diverse mechanisms, including scaffold proteins and cross-pathway inhibition, leading to interactions with different downstream effectors. A growing number of fungal proteins have been identified that regulate just one of the two processes, VHF or fruiting body formation. Similar to SPD4, *S. macrospora* autophagy proteins SmATG4, SmATG8, and SmJLB1, as well as *N. crassa* PRM-1, are essential for completion of the sexual cycle, but not VHF (Fleissner et al. 2009; Voigt et al. 2013; Voigt and Pöggeler 2013), though the underlying mechanisms may be different. Autophagy probably provides the fruiting body with energy and nutrients from the underlying mycelium (Khan et al. 2012). Similarly, peroxisomes mobilize reserve compounds and are necessary during fruiting body formation (Peraza-Reyes and Berteaux-Lecellier 2013). Interestingly, *S. macrospora* mutants lacking the transcription factor gene *Smjlb1* form either protoperithecia or perithecia devoid of ascospores, depending on the complexity of the medium (Voigt et al. 2013). In contrast, the *N. crassa* prm-1 mutant is sterile in sexual crosses, because it lacks croziers, hook-shaped dikaryotic hyphal tips that give rise to ascus initials. PRM-1 has been shown to be involved in membrane merger after fusion, and the mutant exhibits a 50 % reduction in germling fusion and fusion of the conidium and trichogyne (a protoperithecial protrusion) (Fleissner et al. 2009). Therefore, PRM-1 and autophagy proteins may be required at a much later stage during sexual development than SPD4.

In summary, we identified SPD4 as a nuclear protein essential for sexual development. Though numerous developmental factors have already been identified, SPD4 is involved specifically in the early sexual fruiting body formation, but not VHF. Furthermore, SPD4 is restricted to ascogonial cells in the developing protoperithecium. Future studies should analyze its role as a determinant of cellular signaling specificity during the early sexual development, thereby enhancing our understanding of this reprogramming step during the fungal life cycle.

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Compliance with ethical standards

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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