Phosphoproteomic analysis of STRIPAK mutants identifies a conserved serine phosphorylation site in PAK kinase CLA4 to be important in fungal sexual development and polarized growth

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Abstract

The highly conserved striatin-interacting phosphatases and kinases (STRIPAK) complex regulates phosphorylation/dephosphorylation of developmental proteins in eukaryotic microorganisms, animals and humans. To first identify potential targets of STRIPAK, we performed extensive isobaric tags for relative and absolute quantification-based proteomic and phosphoproteomic analyses in the filamentous fungus *Sordaria macrospora*. In total, we identified 4,193 proteins and 2,489 phosphopeptides, which are represented by 10,635 phosphopeptides. By comparing phosphorylation data from wild type and mutants, we identified 228 phosphoproteins to be regulated in all three STRIPAK mutants, thus representing potential targets of STRIPAK. To provide an exemplarily functional analysis of a STRIPAK-dependent phosphorylated protein, we selected CLA4, a member of the conserved p21-activated kinase family. Functional characterization of the ∆cla4 deletion strain showed that CLA4 controls sexual development and polarized growth. To determine the functional relevance of CLA4 phosphorylation and the impact of specific phosphorylation sites on development, we next generated phosphomimetic and -deficient variants of CLA4. This analysis identified (de)phosphorylation of a highly conserved serine (S685) residue in the catalytic domain of CLA4 as being important for fungal cellular development. Collectively, these analyses significantly contribute to the understanding of the mechanistic function of STRIPAK as a phosphatase and kinase signaling complex.

KEYWORDS

iTRAQ-based proteomic and phosphoproteomic analyses, p21-activated kinase CLA4, sexual development, *Sordaria macrospora*, STRIPAK

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In memory of Prof. Dr. h.c. mult. Karl Esser (1924–2019), who passed away on December 3rd, 2019. In 1958, Karl Esser initiated genetic studies on fruiting body development in *S. macrospora*.
INTRODUCTION

The striatin-interacting phosphatases and kinases (STRIPAK) complex is a highly conserved macromolecular signaling complex that is involved in numerous cellular and developmental eukaryotic processes. In humans, malfunctions of STRIPAK subunits are linked with various diseases and cancer, whereas in eukaryotic microorganisms, several processes, such as cell fusion, fruiting body formation, as well as symbiotic and pathogenic interactions, are controlled by STRIPAK (Kück, Radchenko, & Teichert, 2019). Although architecture, function and regulation of STRIPAK are well characterized in diverse experimental systems (Hwang & Pallas, 2014; Kück, Beier, & Teichert, 2016; Shi, Jiao, & Zhou, 2016), our understanding of how STRIPAK regulates the phosphorylation of developmental proteins is still rudimentary. Further, it also currently remains elusive as to how the inactivation or inhibition of subunits of the STRIPAK complex affects the phosphorylation status of STRIPAK target proteins.

Previously, we have used a large set of mutant and deletion strains to identify and structurally and functionally characterize STRIPAK subunits in the model fungus Sordaria macrospora. In extensive tandem affinity purification followed by mass spectrometry (TAP-MS) and yeast-two-hybrid analyses, we identified the structural (PP2AA) and catalytic (PP2Ac1) subunits of PP2A, the B‴ regulatory subunit of PP2A (striatin), the striatin-interacting protein PRO22, SmMOB3, which is homologous to the mammalian vesicular trafficking protein Mob3, and PRO45, a homolog of the mammalian sarcomembrane-associated protein SLMAP (Kück et al., 2016, 2019). Recently, a STRIPAK complex interactor 1 (SCI1) was identified that exhibited structural similarity to SIKE, a coiled-coil protein that serves as a negative regulator of pathological cardiac hypertrophy in humans (Reschka, Nordzieke, Valerius, Braus, & Pöggeler, 2018). Further, two STRIPAK-associated germlinal center kinases (GCKIII) were also functionally characterized (Frey, Reschka, & Pöggeler, 2015; Radchenko, Teichert, Pöggeler, & Kück, 2018), linking STRIPAK with the fungal septation initiation network (SIN). Of interest is that SIN is homologous to the human HIPPO pathway, which is negatively regulated by STRIPAK (Bae et al., 2017).

Quantitative MS-based phosphoproteomics offers the possibility to monitor thousands of phosphorylation sites even from minute sample amounts. This approach enables the large-scale screening and identification of potential targets of phosphorylation or dephosphorylation by identification of the modified peptides with high confidence (Pagel, Loroch, Sickmann, & Zahedi, 2015). Besides label-free quantification and metabolic labeling (e.g., stable isotope labeling by/with amino acids in cell culture (SILAC)), reporter ion-based quantification by usage of isobaric chemical labels (e.g., isobaric tags for relative and absolute quantification (iTRAQ), tandem mass tag (TMT)) has emerged as an effective technique for large-scale relative protein quantification. Due to the fact that iTRAQ allows simultaneous analysis of up to eight protein samples from different experimental conditions, this technique minimizes random errors during sample preparation, and therefore, proved to be especially suitable for phosphoproteomic analyses (Sachon, Mohammed, Bache, & Jensen, 2006; Wiese, Reidegeld, Meyer, & Warscheid, 2007; Yang et al., 2007).

Here, we performed extensive iTRAQ-based proteomic and phosphoproteomic analyses. This approach measures the relative abundance of phosphorylated peptides by comparing the wild-type proteome and phosphoproteome with those from three mutants lacking distinct subunits of STRIPAK, namely the catalytic subunit of phosphatase PP2A, the B‴ regulatory subunit of PP2A or the striatin-interacting protein. Thus we identified 228 phosphoproteins with up- or downregulated phosphorylation sites in all three STRIPAK mutants. Among these, we identified CLA4, a member of the p21-activated kinase (PAK) family. Our analyses revealed a differentially phosphorylated residue, which is highly conserved in PAKs from human to eukaryotic microorganisms. The functional relevance of this phosphorylation site on fungal development was tested using both phosphomimetic and -deficient variants of CLA4. Our study provides an in-depth quantitative phosphoproteomics data set and extends the number of phosphorylated proteins that are putative targets of STRIPAK. Finally, we present the first evidence that STRIPAK regulates phosphorylation of a kinase from the PAK family.

RESULTS

This section provides proteomic and phosphoproteomic analyses of STRIPAK mutants, followed by a functional analysis of a potential target of STRIPAK. This includes the investigation of phosphoimetic and -deficient mutants.

2.1 | iTRAQ-based proteomic and phosphoproteomic analyses revealed putative dephosphorylation targets of the STRIPAK complex

To identify phosphorylation/dephosphorylation targets of STRIPAK, we performed iTRAQ-based LC-MS/MS proteomic and phosphoproteomic analyses to directly compare relative changes in protein expression (Figure 1). The iTRAQ strategy is based on the N-terminal labeling of peptides with up to eight amino group-reactive reagents coupled to different reporter and balancer groups (Ross et al., 2004; Wu, Wang, Baek, & Shen, 2006). Thus, we were able to conduct a simultaneous analysis of up to eight protein samples from eight fungal cultures. While these isobaric tags have the same mass, fragmentation during tandem MS (MS/MS) leads to the generation of mass-specific reporter ions whose abundances are used for protein quantification. Using the same sample for the simultaneous analysis of both the global protein expression as well as the phosphoproteome further allows a better differentiation of true changes in phosphorylation levels from mere fluctuations of the associated proteins (Solari,
FIGURE 1  iTRAQ-based mass spectrometry workflow for proteomic and phosphoproteomic analysis of STRIPAK deletion mutants. For protein extraction, wild type, Δpp2Ac1, Δpro11 and Δpro22 were grown for 3 days. Two biological replicates were used for each strain. Following tryptic digestion, peptides of each sample were labeled at the N-terminus with isobaric tags for relative and absolute quantification (iTRAQ). After sample mixing, fractionation for the global proteome analysis and enrichment of phosphopeptides by titanium dioxide (TiO$_2$) and hydrophilic interaction liquid chromatography (HILIC) were conducted. Subsequently, high-performance liquid chromatography (HPLC) and tandem mass spectrometry analysis (MS/MS) generated reporter ions with specific masses, which were used for quantification.
Kollipara, Sickmann, & Zahedi, 2016). For proteomic and phosphoproteomic analyses, we used probes from wild-type and three different STRIPAK deletion mutants lacking the catalytic subunit of PP2A (PP2Ac1), the B‴ regulatory subunit of PP2A (PRO11) or the striatin-interacting protein PRO22 (Beier, Teichert, Krisp, Wolters, & Kück, 2016; Bloemendal et al., 2012; Pöggeler & Kück, 2004). All strains were grown under the same controlled growth conditions for three days in liquid surface cultures, containing bio malt maize media (BMM). As shown in Figure S1a, wild type and mutants had an identical dry weight. As depicted in the workflow of Figure 1, we used two biological replicates per strain. After protein extraction and tryptic digestion, the peptides of each sample were labeled at the N-terminus with the different isobaric tags. After mixing the samples, high-pH reversed-phase fractionation was performed for global proteome analysis, followed by nano-flow high-performance liquid chromatography (nano-HPLC) and MS/MS. In parallel, enrichment of phosphopeptides by titanium dioxide (TiO₂) and fractionation by hydrophilic interaction liquid chromatography was conducted, followed by nano-HPLC and MS/MS. For both data sets, ratios of the reporter ion intensities of the deletion strains were calculated relative to the wild type. To determine differentially regulated candidates, the total variation of ratios for each strain was determined and only phosphopeptides or proteins displaying a fold change of more than two times the standard deviation were considered as regulated.

As shown in Figure 2a, the global proteome analysis of the wild-type and the deletion strains, namely ∆pro11, ∆pro22 and ∆pp2Ac1 revealed a total of 4,193 quantified proteins, identified with a minimum of two unique peptides per protein at 1% false discovery rate (FDR) on the peptide-spectrum match (PSM) level (Dataset S1). These proteins were detected in all four strains and the quantification is independent of their phosphorylation status.

**FIGURE 2** Proteins and phosphoproteins identified in the wild-type and STRIPAK deletion strains (a) The total numbers of proteins and phosphoproteins from a proteomic and phosphoproteomic analysis of ∆pro11, ∆pro22 and ∆pp2Ac1, as well as the wild type, are shown. The number in the intersection displays proteins detected in both analyses. Furthermore, the number of regulated phosphoproteins from all strains are given that were identified with similar abundances in the global proteome. (b) Venn diagram of 781 phosphoproteins with regulated phosphorylation sites in STRIPAK deletion strains. The comparative phosphoproteomic analysis of the wild-type and the deletion strains ∆pp2Ac1, ∆pro11 and ∆pro22 identified 781 phosphoproteins with regulated phosphorylation sites in the deletion strains. However, these proteins show similar abundances in all strains in the global proteome. The Venn diagram shows the number of phosphoproteins with regulated phosphorylation sites in each deletion strain. Numbers in intersections display phosphoproteins with phosphorylation sites that are regulated in two or three deletion strains. Some phosphoproteins are given in more than one intersection due to the fact that they exhibit multiple regulated phosphorylation sites.
Phosphopeptide enrichment and fractionation led to the identification of 13,506 peptides, 11,161 of which harbored a single or multiple phosphorylations, indicating a relative enrichment efficiency of almost 85% on the PSM level. For 10,635 of those peptides, the phosphorylation site could be localized with high confidence (phosphoRS probability ≥ 90%), cumulating in a total of 2,489 phosphoproteins (Dataset S2). Moreover, 39% of the phosphoproteins exhibited one phosphorylation site, while 61% contained multiple phosphorylation sites (Figure S2a). Further, 79%, 20% and 1% of the 10,635 peptides were phosphorylated on serine, threonine and tyrosine residues respectively (Figure S2b). The proteomic and phosphoproteomic analyses showed an overlap of 1,727 proteins. Among them, 781 phosphoproteins were differentially phosphorylated or dephosphorylated at one or more phosphorylation sites. In addition, these proteins were stable in the global proteome, that is, they showed similar abundances in the deletion strains and wild type (Figure 2a).

The GO annotation analysis of these phosphoproteins highlights that numerous proteins play a role in metabolic processes and are located in organelles and the nucleus (Figure S3). Interestingly, 228 proteins carried phosphorylation sites that were regulated in all three deletion strains (Figure 2b). The analysis of these putative STRIPAK targets revealed several proteins of known signal transduction pathways that exhibited up- or downregulated phosphorylation sites (Dataset S3). The large number of identified phosphorylation sites permitted us to perform enrichment analyses for phosphorylation motifs of differentially regulated phosphorylation sites. Comparing the total proteome from S. macrospora, we found in upregulated phosphosites six distinct motifs to be significantly enriched in all three deletion strains (Figure 2a). These motifs [RXpS] and [RXXpSP] showed the strongest enrichment (11.3- and 10.4-fold compared to the background; adjusted p values 2.8E−06 and 1.5E−09 respectively) with the latter showing close resemblance to the reported substrate motif of kinases CDK5, ERK1/2 and GSK3 (Jaffe, Vinade, & Nowrouzian, 2019). The predicted protein has a length of 834 amino acids and shows high amino acid sequence identity to homologous CLA4 proteins in Neurospora crassa (EAA28056.1, 96.65% identity), Fusarium graminearum (SCB65154.1, 69.92% identity) and Magnaporthe grisea (AAL15449.2, 69.36% identity) (Figure S4). The protein exhibits 53.81% identity to Saccharomyces cerevisiae Cla4p (CAA96216.1) and 58.82% identity to human PAK1 (AAC50590.1). The conserved domain search using the Conserved Domain Search service (CD Search) at NCBI revealed a pleckstrin homology domain (PH), a p21-binding domain (PBD) and a kinase domain. These domains were present in the S. cerevisiae homolog Cla4p (Figure 3a). The alignment of the amino acid sequences from S. macrospora and other filamentous fungi indicated that among the three identified phosphorylation sites in S. macrospora, only S685 is located in a highly conserved region within the kinase domain (Figure 3b). Importantly, this phosphorylation site has not been functionally characterized in any organism to date, while the related amino acid is located within a substrate-binding site, and thus, seems to be of relevance (Figure S4).

For functional characterization of CLA4 in S. macrospora, a deletion strain was constructed as described in experimental procedures. The homokaryotic Δcla4 strain served for further investigations. As shown in Figure 4a,b, Δcla4 forms ascogonia, protoperithecia and perithecia within 7 days. However, the perithecia of Δcla4 have a shortened neck compared to wild-type perithecia (Figure 4b). Moreover, less than 10% mature asci compared to wild type are generated. This reduced fertility is also indicated by the lack of discharged ascospores after 14 day growth on solid BMM media (Figure 5b). Δcla4 shows swelling and hyperbranching in vegetative hyphae as well as a very dense growth (Figures 5a and 5e). Vegetative growth tests showed that Δcla4 displays a strongly reduced growth rate of 3 mm/d in comparison to the wild type with 28.11 ± 0.79 mm/d on Sordaria Westergaards medium (SWG).
TABLE 1  Phosphoproteins that appear to be regulated in all three investigated STRIPAK mutants Δpp2Ac1, Δpro11 and Δpro22. Given are phosphorylated peptides from a total of 29 selected proteins. For each phosphorylation site, log<sub>2</sub> ratio of reporter ion intensity in deletion strain and wild type relative to the respective standard deviation is given.

| Sordaria macrospora identifier | Phosphopeptide | Phospho-sites | Protein description | Predicted function | log<sub>2</sub> ratio of reporter ion intensities/standard deviation | Δpp2Ac1 versus wt/0.63 | Δpro11 versus wt/0.61 | Δpro22 versus wt/0.49 |
|-------------------------------|----------------|--------------|---------------------|-------------------|---------------------------------------------------------------|----------------|----------------|----------------|
| Sexual signaling              |                |              |                     |                   |                                                               |                |                |                |
| SMAC_00557                    | YSIGDAsDDEDGQKEK | S634         | Pheromone processing carboxypeptidase KEX1 | Homolog of the Sc α-pheromone processing carboxypeptidase KEX1 (Dmochowska, Dignard, Henning, Thomas, & Bussey, 1987) | 2.06 2.98 2.22 |                |                |                |
| SMAC_02938                    | GGELYDAFAGGqDDEDVDDGGVYR | S805         | Pheromone processing endopeptidase KEX2 | Homolog of the Sc α-pheromone processing endopeptidase KEX2 (Julius, Brake, Blair, Kunisawa, & Thorner, 1984) | 2.32 2.03 2.12 |                |                |                |
| SMAC_00341                    | NIQKPEPQPELPPIEDEPDRK | T271         | HAM-9              | Nc: might connect the STRIPAK complex to MAP kinase pathways (Fu et al., 2011) | 2.57 2.97 2.85 |                |                |                |
| Kinases and phosphatases      |                |              |                     |                   |                                                               |                |                |                |
| SMAC_04619                    | ALSAASSTGGASDAENATAPAEKRLSK | T374         | Calcium calmodulin-dependent protein kinase CAMK-1 | Nc: regulates fertility, growth, thermotolerance, oxidative stress survival (Kumar & Tamuli, 2014) | 2.23 2.01 2.56 |                |                |                |
| SMAC_04538                    | SDSasAPAsPPAPVPADSRR | S27, S31     | Cyclin-dependent protein kinase complex component | Homolog of the Sc cyclin Pcl7p (Lee et al., 2000) | 2.11 2.00 2.30 |                |                |                |
| SMAC_08843                    | sDNVLDDAR      | S685         | CLA4; member of the p21-activated kinase (PAK) family | Nc: involved in vegetative growth, asexual/sexual reproduction (Lichius et al., 2014; Park et al., 2011) | 2.15 3.08 2.81 |                |                |                |
| SMAC_05293                    | VIADTsPNR      | S331         | Protein kinase LKH1 | An: controls vegetative growth, asexual/sexual development (Kang, Kim, Oh, & Park, 2013) | 3.61 2.98 2.77 |                |                |                |
| SMAC_01589                    | sADLLSK        | S272         | Serine protein kinase DSK1 | Sc: modulates salt tolerance (Forment, Mulet, Vicente, & Serrano, 2002) | 2.53 2.57 2.36 |                |                |                |
| SMAC_08582                    | DLDPRPsR       | S125         | Serine threonine-protein kinase STK-57 | Sp: involved in mRNA cis splicing (Schweinhus et al., 2001) | 2.28 2.49 2.54 |                |                |                |
| SMAC_02638                    | sGELQRPR       | S226         | inositol polyphosphate phosphataseinositol-11 |                   | 2.26 2.45 2.60 |                |                |                |
| Transcription factors         |                |              |                     |                   |                                                               |                |                |                |
| SMAC_04034                    | GVSEVSIGsDDSELARPNTR | S353         | bZIP transcription factor ASL-1 | Nc: activated by the OS MAPK pathway, might mediate circadian gene expression (Lamb, Finch, & Bell-Pedersen, 2012) | 2.18 2.42 2.24 |                |                |                |
| SMAC_01749                    | SALDsPPNLRDDK  | S630         | C2H2 finger domain protein ZNF-25 | | 2.36 2.19 2.03 |                |                |                |
(Continues)
| Sordaria macrospora identifier | Phosphopeptide | Phospho-sites | Protein description | Predicted function | log$_2$ ratio of reporter ion intensities/standard deviation |
|-------------------------------|----------------|---------------|---------------------|-------------------|----------------------------------------------------------|
| SMAC_02926                    | RPDSHAAARDDsVDDAsG GDGAEFTPLTEEQFGFTPR | S237, S242 | C2H2 finger domain protein ZNF-22 | Nc: plays a critical role in circadian conidiation rhythm (Gai et al., 2017) | 2.18 2.33 2.22 |
| SMAC_03305                    | T472 | C2H2 zinc finger protein SIP-5 | Sc: involved in the glucose starvation signaling pathway (Sanz, Ludin, & Carlson, 2000) | 3.59 5.51 3.53 |
| SMAC_05375                    | AP1PLR | T434 | C6 transcription factor | 3.99 3.73 2.82 |
| SMAC_06177                    | NASYDAAYSTGPVSGQSEPMGHEMR AEGYEPSPQSNHER | S185, S147 | C6 zinc finger domain-containing protein female fertility-7 | Nc: involved in sexual development (Colot et al., 2006; Carrillo et al., 2017) | 4.16 2.19 3.74 |
| SMAC_09436                    | S237, S242 | | Fungal specific transcription factor domain containing protein ASM2 | Sm: involved in ascospore maturation and discharge (Schumacher et al. 2018) | 2.12 2.81 2.49 |
| SMAC_04294                    | S253 | | HLH transcription factor RES-2 | Nc: required for ER stress response and cellulase synthesis (Fan et al., 2015) | -3.42 2.09 2.22 |
| SMAC_02857                    | KIA1PAEEAEPAKPVAPAsPENK | T267, T269, S285 | HMG box protein | Pa: involved in the distribution of fruiting bodies (Ait Benkhali et al., 2013) | 4.14 3.22 2.68 |
| SMAC_04153                    | AsFDNR ELSPEPKPQQSLDPKPK FPQFDQPTsAATPSsR | S657, S824, S1138, S1144 | Myb-like dna-binding protein SNT1 | Homolog of the Sc Set3C deacetylase complex subunit SNT1 (Pijnappel et al., 2001) | 2.33 2.12 2.14 |
| SMAC_12586                    | TTDGRsPTAIQRPDQQRSPVNEEGR sPVNEEGR | S223, S235 | NOT2 family protein | Sc: involved in mRNA decapping (Alhusaini & Coller, 2016) | 4.32 3.74 6.35 |
| SMAC_06968                    | IILDQTqsPPYPsPK | S470, T468 | PHD finger domain | 2.49 3.47 2.86 |
| SMAC_04251                    | IQAAEELAQLsED5ksVGSGPGR | S393, S398 | PHD finger domain-containing protein | 2.41 3.14 2.43 |
| SMAC_04152                    | NDstTDTNPLLVLSETAEFIPR | S403 | Related to C2H2 zinc finger transcription factor D-SP1 | 2.18 2.12 2.29 |
| SMAC_01859                    | VGSPQDGAIPPVIRQ | T59 | SNF5 | Nc: required for cell fusion, growth, sexual development (Fu et al., 2011) | 2.10 2.70 2.38 |
| SMAC_08303                    | EILSNVsPDAQDRDLER EILSNVsPDQDQDRE | S255, S258 | Transcription factor RRG-2 | Nc: is involved in the oxidative stress response (Banno et al., 2007) | 3.02 2.88 2.55 |
This reduced growth rate is also responsible for the reduced dry weight of the mycelium compared to the wild type (Figure S1a). Previously, we have reported that all STRIPAK mutants have defects in hyphal fusion (Kück et al., 2016). However, in ∆cla4, we observed no fusion defect (Figure S6b). Complementation analysis was performed by transforming plasmid pNA-8843 into the ∆cla4 deletion strain. This plasmid carries the clo4 gene under the control of the native promoter as well as the nourseothricin (nat) resistance cassette. After selection of primary transformants on media containing hygromycin B and nourseothricin, ascospore isolates of fertile transformants were generated and phenotypically characterized. As displayed in Figures 4 and 5, the ectopic integration of the clo4 gene under expression control of its native promoter completely restored the wild-type phenotype in ∆cla4.

### 2.3 | Phosphomimetic and -deficient mutants of the PAK kinase CLA4 show defects in sexual development and hyphal growth

In \textit{S. macrospora}, proteomic and phosphoproteomic analyses of the STRIPAK deletion strains ∆pro11, ∆pro22 and ∆pp2Ac1, in comparison to the wild type identified three phosphorylation sites of the PAK1 homolog CLA4. Two phosphorylation sites, S78 and S414, were found in weakly conserved regions, while S685 is located in the catalytic domain (Figure 3). As depicted in Table 1, only the phosphorylation site S685 was identified as differentially regulated. The MS data showed more than two-fold upregulation in ∆pp2Ac1 and ∆pro22 and threefold upregulation in ∆pro11. At the same time, the overall level of CLA4 remained stable in all strains, further strengthening the hypothesis of the STRIPAK complex directly acting on this phosphorylation site. To unravel the role of phosphorylation site S685 in fungal development, related phosphomimetic and -deficient variants of CLA4 were generated (Figure S7). For this purpose, single base pairs of the triplet encoding S685 were mutated, resulting in substitution to either alanine (blocks phosphorylation) or aspartic acid (mimics phosphorylation because of a negative charge) in the derived protein. Mutation of the phosphorylation site S78 to A78 or D78 served as control since it is located in a weakly conserved region.

The four plasmids pNA-8843-S78A, pNA-8843-S78D, pNA-8843-S685A and pNA-8843-S685D, encoding the phosphomimetic and -deficient variants of CLA4 were transformed into the ∆cla4 deletion strain. After selection of primary transformants by hygromycin B and nourseothricin resistance, ascospore isolates of fertile transformants (cla4-S78A, cla4-S78D, cla4-S685A and cla4-S685D) were generated, molecularly characterized and phenotypically analyzed. Representative isolates of all mutants are shown in Figures 4 and 5.

Sexual development and hyphal growth were investigated in all selected strains and compared to wild type, ∆cla4, as well as ∆cla4::clo4. As depicted in Figure 4c,d, the analysis of different developmental stages revealed that ∆cla4::clo4, cla4-S78A and cla4-S78D form wild-type-like ascogonia, protoperithecia, as well as pear-shaped...
FIGURE 3  Protein domain structure and identified phosphorylation sites of CLA4. (a) Primary structure of CLA4 proteins from S. macrospora (SmCLA4), S. cerevisiae (ScCla4p) and human (HsPAK1). Numbers above the SmCLA4 protein indicate residues, representing phosphorylation sites. (b) Comparison of phosphorylated regions from S. macrospora (Sm) CLA4 with other CLA4-like proteins from N. crassa (Nc), F. graminearum (Fg), Magnaporthe grisea (Mg), B. cinerea (Bc), A. nidulans (An), S. cerevisiae (Sc) and H. sapiens (Hs). Phosphorylation sites of CLA4 from S. macrospora are highlighted in red, which were detected in this investigation. Black color indicates highly conserved amino acid residues. Abbreviations: AID, autoinhibitory domain; PBD, p21-binding domain; pleckstrin homology (PH) domain

FIGURE 4  Microscopic analysis of sexual development. As indicated, images were obtained from wild type, Δcla4 and transformants, carrying wild type, phosphomimetic or phospho-deficient versions of cla4. All gene constructs were transferred into Δcla4 strain. (a,c) Images of developmental stages of strains grown on BMM-coated slides at 27°C for 2–4 days. Scale bars indicate 20 µm. (b,d) For microscopic analysis of perithecia and ascospores, strains were grown on BMM medium at 27°C for 14 days. Red arrows indicate ascospores. Scale bar represents 100 µm [Colour figure can be viewed at wileyonlinelibrary.com]
perithecia. Further, vegetative growth of all these strains is identical to the wild type (Figures 5 and S6a). In contrast, cla4-S685A shows swelling of hyphae, hyperbranching and a reduced growth rate, albeit sexual development seems to be wild type like (Figures 4, 5, and S6). Strikingly, a completely different phenotype was observed for cla4-S685D. In detail, this strain generates deformed perithecia and only very few ascospores are detected after mechanical disruption of fruiting bodies. The reduced sexual fertility is also reflected by the fact that no ascospores are discharged (Figure S1b). Moreover, cla4-S685D shows swelling and mycelial hyperbranching as well as dense vegetative growth (Figures 5a and S6). Thus, the phenotype of this mutant is similar to that of Δcla4.

3 | DISCUSSION

The conserved STRIPAK complexes from fungi, animal or humans have the capacity to phosphorylate or dephosphorylate target proteins. The complex interacts with other conserved signaling complexes, which might be potential phosphorylation/dephosphorylation targets (Hwang & Pallas, 2014; Kück et al., 2016). Although some substrates of STRIPAK have been identified, the picture is still far from complete. Thus, to identify targets of STRIPAK likely involved in fungal development, we compared the proteome and phosphoproteome of wild type and three STRIPAK mutants. Here, we used quantitative phosphoproteomics to identify potential targets of STRIPAK.

3.1 | Quantitative phosphoproteomic analysis in fungi

iTRAQ-based phosphoproteomic analysis has already been performed in diverse filamentous fungi such as Aspergillus fumigatus (Adav, Ravindran, & Sze, 2015; Cagas, Jain, Li, & Perlin, 2011), Beauveria bassiana (Wang et al., 2016), Fusarium graminearum (Taylor et al., 2008) and Neurospora crassa (Jonkers et al., 2014; Xiong et al., 2014). In this work, we present an in-depth quantitative phosphoproteomic data set of S. macrospora in conjunction with corresponding global proteome analysis, allowing for differentiation between changes in total protein expression and true alterations of specific phosphorylation levels. With the identification and quantification of 8,908 phosphorylation sites...
across all conditions, our data set exhibits substantially higher coverage than comparable work with other filamentous fungi to date (Franck et al., 2015; Jonkers et al., 2014; Xiong et al., 2014; Zhou, Ye, Zheng, Jiang, & Lu, 2019). Here, we provide the first comprehensive STRIPAK-dependent quantitative proteome and phosphoproteome analyses in a eukaryotic organism. Further, from the sum of all quantitative data, we substantially extend the number of potential STRIPAK targets. Finally, this study also contributes significantly to our current understanding of the mechanistic function of STRIPAK as a phosphatase and kinase signaling complex.

### 3.2 Phosphorylation of the potential STRIPAK target CLA4 controls fungal development

One of the putative potential STRIPAK targets is the PAK kinase CLA4, which was identified with three different phosphorylation sites. The phenotypical analysis of a ∆cla4 deletion strain revealed that CLA4 is involved in sexual development. This finding is similar to previous reports that have investigated the function of CLA4 homologs in other filamentous fungi. For example, in Bipolaris maydis, the CLA4 homolog regulates the formation of fruiting bodies as well as ascospore development (Kitade, Sumita, Izumitsu, & Tanaka, 2019). Similarly, CLA4 homologs in F. graminearum, N. crassa and M. grisea affect ascospore development and release, but not fruiting body morphology (Li, Xue, Bruno, Nishimura, & Xu, 2004; Park et al., 2011; Wang et al., 2011).

Further, ∆cla4 exhibits dense growth and altered branching in vegetative hyphae, suggesting that CLA4 is involved in regulating cell polarity in S. macrospora. This observation is consistent to reports by others, thus indicating that CLA4 function is conserved in filamentous ascomycetes (Kitade et al., 2019; Lichius et al., 2014; Rolke & Tuzynska, 2008; Tian, Zhou, Guo, & Wang, 2015). The branching pattern of hyphae might be due to the nonrandom distribution of the Spitzenkörper, as was suggested for a B. maydis ∆cla4 deletion strain (Kitade et al., 2019). In S. cerevisiae, the PAK1 homolog Cla4p has also been extensively investigated. Several studies indicate that Cla4p regulates important biological processes, including cell polarity, cell cycle progression, cytokinesis and gene transcription (Benton, Tinkelenberg, Gonzalez, & Cross, 1997; Cvrcková, Virgilio, Manser, Pringle, & Nasmyth, 1995; Lin et al., 2009). In addition, overexpression of Cla4p was shown to affect the pheromone-induced cell cycle arrest. Thus, Cla4p has the potential to control the pheromone response (PR) pathway (Heinrich, Köhler, & Mösch, 2007). Taken together, these studies support the view that CLA4 homologs play a significant role in fungal development.

The human CLA4 homolog PAK1 is the best studied member of the PAK family and is involved in a variety of cellular processes such as cytoskeleton remodeling, cell cycle control, the DNA damage response, cell motility, cell apoptosis and neurodevelopment. Several studies in human showed that PAK1 is crucial for cardiac excitation, and muscle contraction dynamics by regulation of ion channel activity (Kumar, Sanawar, Li, & Li, 2017; Wang et al., 2018). Further, PAK1 is upregulated and activated in several human tumor types, including breast, colon and brain tumors (Kumar, Gururaj, & Barnes, 2006). PAK1, a member of group A PAK kinases, is regulated by an auto-inhibition mechanism. In an inactivated state, the autoinhibitory domain (AID) overlaps with the PBD domain and binds the catalytic domain of another PAK1 protein, resulting in the formation of a homodimer. The binding of the small GTPases CDC42 or RAC to the PBD domain leads to a conformational change in the AID, followed by dissociation from the catalytic domain of the other PAK1 molecule. Both PAK proteins become auto-phosphorylated, and therefore, activated at several sites, including the activation loop within the kinase domain (Kumar et al., 2017; Zhao & Manser, 2012). In human as well as yeast, various phosphorylation sites of PAK1 and Cla4p have been detected and experimentally verified. Further, the generation of phosphomimetic and -deficient variants of PAK1 regarding numerous phosphorylation sites, including S144, S223 and T423, revealed their importance for kinase activity (Chong, Tan, Lim, & Manser, 2001; Ng et al., 2010; Shin, Kim, & Kim, 2013). Since PAK1 is associated with several disease phenotypes (Kumar et al., 2017; Wang et al., 2018), PAK1 emerged as a therapeutic target and several PAK1 inhibitors were developed (Kumar et al., 2017).

Our phosphoproteomic analysis of the STRIPAK deletion strains and the wild type revealed three different phosphorylation sites. S78 and S414 are located in weakly conserved regions of the protein, while S685 locates in the highly conserved catalytic domain. Only phosphorylation site S685, which has neither been characterized in human nor yeast, was regulated in the STRIPAK deletion strains. The phospho-deficient mutation of this site leads to reduced growth as well as slightly enhanced swelling and hyperbranching in vegetative hyphae, but does not affect the formation of fruiting bodies and ascospore development. These results indicate that phosphorylation is important for the functional mechanism of CLA4 in vegetative hyphal growth. In contrast, the phosphomimetic mutant S685D resulted in severe defects of sexual development and vegetative hyphal growth comparable to the ∆cla4 deletion strain, thereby suggesting that dephosphorylation of S685 might be even more critical for proper CLA4 function. Thus, phosphorylation/dephosphorylation of S685 from CLA4 resulted in severe defects of sexual development and vegetative hyphal growth comparable to the ∆cla4 deletion strain, thereby suggesting that dephosphorylation of S685 might be even more critical for proper CLA4 function. Thus, phosphorylation/dephosphorylation of S685 from CLA4, a member of the conserved PAK family, seems to be STRIPAK dependent. Previous studies in human already described this amino acid as a potential substrate-binding site of PAK1 (Ng et al., 2010), thus emphasizing the significance of the phosphorylation site S685.

### 3.3 CLA4 plays an important role in connecting the STRIPAK complex

From our analysis, we conclude that CLA4 is a putative phosphorylation/dephosphorylation target of the STRIPAK complex, and AP-MS analysis indicates further an association with the striatin-interacting protein PRO22 (Märker, 2019). Previous studies in human showed that the association of the CLA4 homolog PAK1 with PP2A plays a
role in regulating Ca\(^{2+}\) homeostasis in the heart. In particular, these studies indicated that PAK1 induces auto-dephosphorylation of the catalytic subunit PP2Ac at the phosphorylation site Y307 through a scaffolding mechanism. Subsequently, PP2A antagonizes the effects of cAMP-dependent kinase (PKA) on Ca\(^{2+}\) channels activity (Ke, Lei, & Solaro, 2008; Ke, Lei, Wang, & Solaro, 2013). Further, there is evidence that PAK1 and PP2A form a signaling module in mast cells, where PP2A dephosphorylates PAK1 at T423 in the activation loop, resulting in the disassembly of PP2A (Staser et al., 2013).

In conclusion, our study indicates that the STRIPAK complex regulates numerous signaling pathways controlling different biological processes in Sordaria macrospora. These results increase our current basic knowledge about the function of the eukaryotic STRIPAK complex and further support the notion that STRIPAK is a central regulator of diverse cellular processes. Moreover, our study provides clear evidence that phosphorylation and dephosphorylation of residue S685 of Cla4 is crucial for fungal cellular development. Finally, since this amino acid has not yet been reported to be phosphorylated in other eukaryotes, our findings will encourage future investigations of this phosphorylated site in Cla4 homologs, which in humans were considered to be therapeutic targets in cancer and allergen-induced disorders (Kichina, Goc, Al-Husein, Somanath, & Kandel, 2010; Pandolfi et al., 2015).

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Strains and growth conditions

All S. macrospora strains (Table S1) were grown under standard conditions, unless otherwise described (Kamerewerd, Jansson, Nowrousian, Pöggeler, & Kück, 2008). DNA-mediated transformation was performed as described previously (Nordzieke et al., 2015), but lacking caspase treatment during protoplast formation. Transformants were selected on medium containing hygromycin B (80 U/ml) and/or nourseothricin (100 µg/ml). Growth tests were performed three times with three technical replicates per strain, and hyphal growth was measured as described before (Teichert, Lutomski, Märker, Nowrousian, & Kück, 2017). For each experiment, Petri dishes with solid SWG medium were inoculated with an 8 mm diameter agar plug and incubated for 2 days. Quantification of fertility was done by counting discharged ascospores and perithecia from strains grown on solid BMM media for 14 days with two and three independent cultures per strain respectively. Dry weight of mycelia was calculated from three independent cultures per strain grown for 3 days in liquid BMM media at 27°C and 40 rpm. Further details are given in the legend of Figure S1. Isolation of DNA and Southern hybridization were performed as described previously (Kamerewerd et al., 2008).

For cloning and propagation of recombinant plasmids, E. coli XL1-Blue MRF’ (Jerpseth, Greener, Short, Viola, & Kretz, 1992) and NEB5α (New England Biolabs, Frankfurt, Germany) were used under standard laboratory conditions (Sambrook & Russell, 2001). For plasmid construction, homologous recombination in S. cerevisiae PJ69-4a was performed (James, Halladay, & Craig, 1996) as described previously (Colot et al., 2006). Recombinant yeast strains were selected on minimal medium, lacking uracil; all other experiments with yeast were carried out according to standard protocols (Clontech Yeast Protocol Handbook, PT3024-1).

### 4.2 | Protein extraction, enrichment and fractionation

For protein extraction, we followed in principle a protocol described previously (Teichert et al., 2014). S. macrospora strains were precultured in Petri dishes with 20 ml liquid BMM with two biological replicates per strain at 27°C for 2 days. Three standardized inoculates of each BMM preculture were transferred in Petri dishes with 20 ml liquid BMM and grown at 27°C and 40 rpm for 3 days. For cell wall lysis and protein extraction, mycelium was harvested and freeze-dried to 60 s. The frozen mycelium was ground in liquid nitrogen and suspended in FLAG extraction buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 10% glycerol, 0.05% NP-40, 1 mM PMSF, 0.2% protease inhibitor cocktail IV (Calbiochem, 539136), 1.3 mM benzamidine, as well as 1% phosphatase inhibitor cocktails II and III (Sigma Aldrich, P5726, P0044). Afterward, the samples were centrifuged at 4°C and 15,000 rpm for 30 min. The same lysates were used for proteomic and phosphoproteomic analyses. Protein concentration in all lysates was determined by a calorimetric bicinchoninic acid assay (Pierce BCA protein concentration assay kit) following the manufacturer’s protocol. Carbamidomethylation was performed by reduction of free cysteine residues by the addition of dithiothreitol (DTT) to a final concentration of 10 mM and incubation for 30 min at 56°C followed by alkylation with 30 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. Excess of IAA was quenched by further addition of 10 mM fresh DTT. Samples were purified prior to digestion by ethanol precipitation and resuspended with 40 µl of 6 M guanidinium hydrochloride, followed by dilution with ammonium bicarbonate buffer (pH 7.8) to a final concentration of 0.2 M and addition of CaCl\(_2\) to a final concentration of 2 mM. Trypsin was added at a 1:20 (protease:substrate (w/w)) ratio and samples were incubated for 14 hr at 37°C. Digestion was stopped by addition of 10% trifluoroacetic acid (TFA) to a final concentration of 1%. Acidified peptides were desalted and quality controlled as described previously (Burkhart, Premslser, & Sickmann, 2011). Peptides were then dried down completely using a SpeedVac and resuspended in 0.5M triethylammonium bicarbonate (pH 8.5). Per sample, 150 µg of tryptic peptide were labeled with iTRAQ 8-plex reagents (AB Sciex, Darmstadt, Germany) following the manufacturers protocol. After pooling and quenching, an aliquot corresponding to 70 µg of total peptide amount was taken for global proteome analysis. From this, 35 µg were fractionated by high-pH reversed-phase chromatography using an Ultimate 3000 HPLC (Thermo Scientific) equipped with a C18 column (BioBasic-18, 5 µm particle size, 300 Å pore size, 150 x 0.5 mm). A total of 20 fractions was collected in 1 min windows using a concatenated collection mode.
For protein enrichment, the remaining multiplexed sample (1,130 µg) was dried under vacuum and subjected to a phosphopeptide-enrichment protocol using titanium dioxide (TiO₂. Titansphere TiO, 5 µm particle size, GL Sciences Inc., Japan) adapted from a previous report (Engholm-Keller et al., 2012). For protein fractionation, phosphopeptides were fractionated by hydrophilic interaction liquid chromatography on an Ultimate 3000 HPLC (Thermo Scientific, Dreieich, Germany) and a total of 13 fractions was collected.

### 4.3 LC-MS/MS analysis

Samples for global proteome analysis were subjected to LC-MS/MS analysis using an Ultimate 3,000 nanoRSLC HPLC coupled to a Q Exactive HF mass spectrometer (both Thermo Scientific). Preconcentration of peptides was performed on a precolumn (Pepmap RSLC, Thermo Scientific, C18, 100 µm x 2 cm) for 10 min at 20 µl/min flow (0.1% TFA) followed by separation on a 75 µm x 2 cm C18 main column (Pepmap RSLC, Thermo Scientific). A binary gradient was used with 0.1% formic acid (FA) as solvent A and 84% acetonitrile (ACN), 0.1% FA as solvent B. A linear gradient was used with solvent B increasing from 3% to 35% in 120 min. The MS was operated in the data-dependent acquisition (DDA) mode, first performing a survey scan from 300 to 1,500 m/z at a resolution of 60,000, with the AGC target set to 3 x 10⁶ and the maximum injection time to 120 ms. The polysiloxane ion at m/z 371.101236 was used as lock mass and the top 15 most intense ions were subjected to higher energy collisional dissociation (HCD) and subsequent MS/MS analysis. HCD normalized collision energy was set to 31% and quadrupole isolation was performed with a 0.7 m/z window and all other settings were kept as described above. Maximum injection times were 50 and 200 ms and HCD collision energy was 40%. Quadrupole isolation was performed with a 0.8 m/z window and all other settings were kept as described above.

### 4.4 Proteomics data analysis

Acquired raw data were analyzed with Proteome Discoverer 1.4 (Thermo Scientific), incorporating the search algorithms Mascot (Version 2.4.1, Matrix Science), Sequest HT and MS Amanda. The searches were conducted in a target/decoy manner against a S. macrospora protein sequence database (10,091 target sequences) with the same settings for all three algorithms: Precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 0.02 Da. Cleavage specificity was set to trypsin with a maximum of 2 allowed missed cleavages. iTRAQ-8 plex on peptide N-terminals and lysines as well as carbamidomethylation of cysteines was set as fixed modification, while oxidation of methionine and phosphorylation (only phosphoproteome data) of serine, threonine or tyrosine was allowed as variable modifications. For phosphoproteome analysis, the phosphoRS (version 3.1) node (Taus et al., 2011) was used to determine modification site confidence. Percolator was used to filter the results to a false discovery rate (FDR) of 1% on the PSM level and only rank 1 hits were allowed. For global proteome data, a minimum of two uniquely identified peptides per protein were required, while for the phosphoproteome data, only quantified PSMs with identified phosphorylations and a phosphoRS site probability ≥ 90% were exported.

To correct for systematic errors during sample labeling, global proteome data was normalized by correction factors calculated from the summed total intensities of all iTRAQ channels. Mean ratios of biological replicates were calculated and proteins with a change in abundance greater than two times the standard deviation of the respective condition were considered as regulated. For analysis of phosphoproteome data, an Excel macro was used provided by Taus et al. (2011). Data were normalized using the correction factors determined from the global data. Only ratios of confidently localized phosphorylations were used in the analysis and mean of biological replicates was calculated. Two times the standard deviation of the total data set of the respective condition was used as the criterion to determine regulation of each phosphopeptide.

### 4.5 Phosphorylation motif analysis

To identify overrepresented consensus motifs of the identified phosphorylation sites, seven flanking amino acids up- and downstream of the modified residues were extracted. The motifs of up- or downregulated sites in the individual deletion strains were uploaded to the MoMo web server (Cheng, Grant, Noble, & Bailey, 2018). Significantly enriched motifs were identified using the motif-x algorithm and the S. macrospora protein database (10,091 sequences) as context sequence and requiring a minimum number of 20 occurrences and a p value of threshold of 1E-6.

### 4.6 Construction of plasmids

All oligonucleotides and plasmids are listed in the supporting information (Tables S2 and S3). The knock-out plasmid pKO-8843 for the deletion of cla4 was generated by yeast recombination. For this purpose, the 5′- and 3′-flanking regions of cla4 were amplified by PCR from genomic DNA of S. macrospora with the primers...
8843-5fw/8843-5rv-hph and 8843-3fw/8843-3rv, respectively, and transformed together with an hph cassette cut with EcoRI out of plasmid pDrive-hph (Nowrousian & Cebula, 2005) and EcoRI/Xhol-linearized plasmid pRS426 (Christianson, Sikorski, Dante, Shero, & Hieter, 1992) in yeast.

Yeast recombination was also used to obtain the complementation plasmid pNA-8843. For this purpose, the cla4 gene and the corresponding 5′- and 3′-flanking regions containing the promoter and terminator of cla4 were amplified from genomic DNA of S. macrospora with the primers 8843-5fw and 8843-3rv, and recombined into the HindIII/Xhol-linearized plasmid pRSNat (Klix et al., 2010).

For construction of plasmids encoding phospho-mimetic and -deficient variants of CLA4 with the amino acid substitutions S685A or S685D, the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) was used according to the manufacturer’s protocol. For the PCR, pNA-8843 was used as template with the primers Cla4-S685A-2-fw/Cla4-S685-2-rv and Cla4-S685D-2-fw/Cla4-S685-2-rv, resulting in pNA-8843-S685A and pNA-8843-S685D respectively.

Plasmids encoding the phosphomimetic and -deficient variants of CLA4 with the amino acid substitutions S78A or S78D were generated by the yeast recombination system. PCRs with the primers 8843-5fw-Nhel/8843-S78A-rv, 8843-5fw-Nhel/8843-S78D-rv, 8843-S78A-fw/8843-3rv and 8843-S78D-fw/8843-3rv were performed using genomic DNA of S. macrospora as template. Using primers 8843-3fw/8843-3rv-Nhel, the 3′ end of cla4 was amplified. All PCR fragments, the EcoRI-linearized hph cassette, (pDrive-hph), together with the EcoRI/Xhol-linearized yeast vector pRS426, were transformed into yeast (P697-4a). The resulting plasmids carry the mutated cla4 gene and received the designations pCla4-S78A and pCla4-S78D. Plasmids pCla4-S78A and pCla4-S78D were hydrolyzed with Stul/Kspl, and fragments carrying the mutated cla4 gene were integrated into Stul/Kspl-linearized pNA-8843. The resulting plasmids were designated pNA-8843-S78A and pNA-8843-S78D respectively.

4.7 | Generation of Δcla4, and phosphomimetic and -deficient mutants

For generation of a Δcla4 strain, plasmid pKO-8843 was linearized with EcoRI and transformed into Δku70 (Pöggeler & Kück, 2006). The primary transformants were selected for hygromycin B resistance and verified by PCR analysis (data not shown). Ascospore isolates of the Cla4 strain with the wild-type genetic background (lacking the Δku70 mutation) were obtained by mating with spore color mutant fus as described previously (Kück, Pöggeler, Nowrousian, Nolting, & Engh, 2009; Nowrousian, Teichert, Masloff, & Kück, 2012). The recombinant strains were verified by PCR and Southern blot analysis (Figure S5). To restore the wild-type phenotype, plasmid pNA-8843 was transformed into the Δcla4 recipient. The corresponding transformant was designated Δcla4::cla4.

Transformation of pNA-8843-S78A, pNA-8843-S78D, pNA-8843-S685A and pNA-8843-S685D into the Δcla4 recipient strain resulted in cla4-S78A, cla4-S78D, cla4-S685A and cla4-S685D carrying the phosphomimetic and -deficient mutations in the cla4 gene. Primary transformants were used to generate single ascospore isolates and the corresponding point mutations of cla4 in diverse transformants were verified by PCR and DNA sequencing analysis.

4.8 | Microscopic investigations

Light microscopy of different developmental stages and hyphal fusion events was performed by differential interference contrast (DIC) microscopy using an AxioImager microscope (Zeiss) with a Photometrix Cool SnapHQ camera (Roper Scientific) and the software MetaMorph (version 7.7, Universal Imaging). For light microscopy of smashed perithecia and ascospores, the AxioPhot microscope (Zeiss) was used, and images were obtained with an AxioCam color and ZEN software (version 2.3, Zeiss). For microscopic investigation of different developmental stages, S. macrospora strains were grown on BMM-covered slides at 27°C and constant light for 2–7 days (Engh et al., 2007). For microscopic analysis of hyphal fusion events, strains were incubated on celophane-covered solid MMS medium at 27°C for 2–4 days (Rech, Engh, & Kück, 2007). For microscopic analysis of perithecia and ascospores, strains were grown at 27°C and constant light for 14 days. After transferring perithecia to slides, 0.96% NaCl solution was added and perithecia were smashed open.

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AUTHOR CONTRIBUTIONS

RM, BBL, ABR, AS, UK conceived and designed the study. RM and BBL acquired experimental data. RM, BL, AS and UK analyzed and interpreted the data. RM, BBL, ABR, AS, UK wrote the manuscript.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al., 2014) with the data set identifier PXD014858.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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