1 Targeted quantification of phosphorylation sites identifies STRIPAK-dependent 2 phosphorylation of the Hippo pathway-related kinase SmKIN3

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12 **Running title: STRIPAK dependent phosphorylation of GCK SmKIN3**

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18 Abstract

19 We showed recently that the germinal centre
20 kinase III (GCKIII) SmKIN3 from the fungus
21 *Sordaria macrospora* is involved in sexual
22 development and hyphal septation. Our recent
23 extensive global proteome and
24 phosphoproteome analysis revealed that
25 SmKIN3 is a target of the striatin interacting
26 phosphatase and kinase (STRIPAK) multi-
27 subunit complex. Here, using protein samples
28 from wild type and three STRIPAK mutants, we
29 applied absolute quantification by parallel
30 reaction monitoring (PRM) to analyze
31 phosphorylation site occupancy in SmKIN3 and
32 other septation initiation network (SIN)
33 components, such as CDC7 and DBF2, as well
34 as BUD4, acting downstream of SIN. For
35 SmKIN3, we show that phosphorylation of
36 S668 and S686 is decreased in mutants lacking
37 distinct subunits of STRIPAK, while a third
38 phosphorylation site, S589, was not affected.
39 We constructed SmKIN3 mutants carrying
40 phospho-mimetic and phospho-deficient
41 codons for phosphorylation sites S589, S668
42 and S686. Investigation of hyphae in a
43 Δ SmKin3 strain complemented by the S668 and
44 S686 mutants showed a hyper-septation
45 phenotype, which was absent in the wild type,
46 the Δ SmKin3 strain complemented with wild
47 type gene, or the mutant S589. Furthermore,
48 localization studies with SmKIN3
49 phosphorylation variants and STRIPAK
50 mutants showed that SmKIN3 preferentially
51 localizes at the terminal septa, which is
52 distinctly different from the wild type strains.
53 We conclude that STRIPAK-dependent
54 phosphorylation of SmKIN3 has an impact on
55 controlled septum formation and on the time-
56 dependent localization of SmKIN3 on septa at
57 the hyphal tip. Thus, STRIPAK seems to
58 regulate SmKIN3, as well as DBF2 and BUD4
59 phosphorylation, affecting septum formation.

60 Introduction

61 The STRIPAK multi-subunit complex
62 functions as a macromolecular assembly
63 communicating through physical interactions
64 with other conserved signaling protein
65 complexes to constitute larger dynamic protein
66 networks. STRIPAK is involved in a broad
67 variety of developmental processes in higher
68 and lower eukaryotes. For example,
69 proliferation of several mammalian cancer cells
70 is correlated with dysfunctional STRIPAK
71 subunits (1-3), and in fungal microorganisms,

72 the lack of STRIPAK results in sexual
73 infertility, defects in hyphal fusion, and
74 impaired pathogenicity or symbiotic
75 interactions (4).

76 We are interested in identifying putative
77 phosphorylation and dephosphorylation targets
78 of STRIPAK in the filamentous fungus *S.*
79 *macrospora*, a filamentous ascomycete closely
80 related to *Neurospora crassa* (5). Techniques to
81 globally quantify the proteome and
82 phosphoproteome, such as label-free
83 quantification (LFQ) and label-based
84 approaches (isobaric Tags for Relative and
85 Absolute Quantitation, iTRAQ, tandem mass
86 tag, TMT), are indispensable for large-scale
87 detection of changes in phosphorylation of
88 peptides and the identification of potential
89 molecular targets of kinases and phosphatases
90 (6). We recently performed extensive isobaric
91 tagging for relative and absolute quantification-
92 based proteomic and phosphoproteomic
93 analyses to identify potential targets of
94 STRIPAK in *S. macrospora*. The proteome and
95 phosphoproteome of the wild type and three
96 different STRIPAK deletion mutants revealed a
97 total of 4,193 proteins and 2,489
98 phosphoproteins in all strains, where 1,727
99 proteins were present in both proteomes.
100 Among these, we identified 781
101 phosphoproteins that showed differential
102 phosphorylation in all mutants compared to the
103 wild type (7). However, the functional role of
104 the posttranslational protein modifications was
105 only characterized in a few cases (7,8).

106 Certain inherent limitations with these shotgun
107 methods, such as ratio compression and under
108 sampling, can only be overcome by the
109 complementary use of targeted mass
110 spectrometry (MS) approaches. These can be
111 employed as a means to validate a subset of the
112 results obtained by shotgun experiments. While
113 targeted MS is widely used for accurate protein
114 quantification, the high variability, increased
115 experimental effort, and need for validation has
116 limited the implementation of targeted
117 approaches in phosphoproteomics analysis (9).
118 A hallmark study showed that 25% of
119 differentially regulated phosphopeptides were
120 attributed to alterations at the protein level (10).
121 Thus, to accurately determine the
122 phosphorylation ratio of a given site, targeted
123 quantification of phosphorylation sites is highly
124 appropriate to quantify both the corresponding
125 phosphorylated and non-phosphorylated

126 peptides in order to obtain site-specific
127 phosphorylation ratios (11,12).

128 Among the 781 regulated proteins in *S.*
129 *macrospora* mentioned above (7), we found the
130 GCKIII SmKIN3, which however was absent in
131 the global proteome, and thus did not permit
132 quantitative measurement of phosphorylation.
133 Therefore, SmKIN3 phosphorylation was
134 determined by applying absolute quantification
135 by synthesis of stable isotope-labeled standard
136 (SIS) peptides combined with PRM, a method
137 which to the best of our knowledge has not yet
138 been applied to a fungal organism.

139 SmKIN3 is involved in septation of hyphae and
140 associated with the highly conserved SIN (13).
141 The SIN complex, homologous to Hippo
142 signaling in animals, comprises a STE-kinase, a
143 GCK and a nuclear DBF2-related (NDR) kinase
144 (14). For example, in *Neurospora crassa* the
145 STE-kinase CDC-7 phosphorylates GCK
146 SID-1, the homolog of SmKIN3, and activates
147 DBF-2 (15). The function of SIN is essential for
148 septation and cytokinesis, as demonstrated by
149 SIN-deletion strains (13,15,16). BUD4, an
150 anillin-related protein, acts further downstream
151 and specifies SIN-regulated septum formation
152 (17,18).

153 Here, we show that STRIPAK-directed
154 dephosphorylation of SmKIN3 has a significant
155 impact on proper hyphal septation and septal
156 localization. To the best of our knowledge, this
157 is the first report about STRIPAK-dependent
158 phosphorylation analyzed by targeted
159 quantification of phosphorylation sites, and will
160 have an impact on understanding the function of
161 mammalian homologs.

162

163 Results

164 Absolute quantification of phosphorylation 165 site occupancy by PRM of germinal centre 166 kinase SmKIN3

167 Previously, we showed that the GCKIII
168 SmKIN3 is associated with STRIPAK and
169 regulates fungal development (13). Sequence
170 comparison of the primary amino acid sequence
171 showed a homology of 92.43% with the
172 corresponding sequence from *N. crassa*; but
173 similarity with other homologues from
174 ascomycetes is low. Using the eukaryotic length
175 motif (ELM) database (19), we found a kinase
176 domain in the amino terminus, several LATS

177 kinase recognition motifs, and a binding motif
178 for forkhead-associated (FHA) domains. The
179 COILS sequence analysis program
180 ([http://www.ch.embnet.org/software/COILS_f](http://www.ch.embnet.org/software/COILS_form.html)
181 [orm.html](http://www.ch.embnet.org/software/COILS_form.html)), revealed two predicted putative
182 coiled-coil domains located next to each other
183 in a region between amino acids 688 and 788.
184 At the C-terminal end of SmKIN3 (amino acids
185 805-811), we detected a conserved sequence
186 motif, previously called T-motif (Fig. 1A). Such
187 T-motifs have only been found so far in a small
188 family of related fungal kinases (20). Database
189 research revealed that a motif occurs for
190 example in SmKIN3, its homologue Sid1p from
191 *Schizosaccharomyces pombe* and SID-1 from
192 *N. crassa*.

193 Recently, we determined the phosphoproteome
194 of *S. macrospora* wild type and STRIPAK
195 mutant strains (7,8), and detected three
196 phosphorylation sites S589, S668 and S686 in
197 the SmKIN3 sequence, which are conserved
198 between *S. macrospora* and *N. crassa* (Fig. 1B).
199 Due to its low abundance in the overall
200 proteome, we were unable to quantify the
201 STRIPAK-dependent phosphorylation of
202 SmKIN3 (7,8).

203 Here, we set out to determine the impact of
204 STRIPAK on SmKIN3 phosphorylation, by
205 applying targeted quantification of
206 phosphorylation site occupancy using PRM. As
207 described above, SmKIN3, together with CDC7
208 and DBF2 constitute the SIN complex, which is
209 associated with the downstream landmark
210 protein BUD4. Therefore, we included all these
211 components in our PRM analysis.

212 A targeted bipartite TiO₂-LC-PRM-based
213 workflow was established to quantify the site-
214 specific phosphorylation states of putative
215 STRIPAK targets. First, 70 phosphorylated
216 peptides and their corresponding non-
217 phosphorylated counterparts were selected and
218 SIS were synthesized. The median coefficient
219 of variation (CV) of the biological replicates
220 was calculated as 16.3% for all non-
221 phosphorylated, and 16.1% for all
222 phosphorylated peptides. To further prioritize
223 our approach, we selected 15 pairs of peptides,
224 representing phospho-sites from proteins
225 belonging to the SIN signaling pathway.
226 Dilution series were prepared to verify a linear
227 response and determine the limit of detection
228 (LOD) of each SIS peptide, as described in the
229 Methods section. LODs ranged from 1.7 amol
230 to 19.3 fmol per injection for phosphorylated

SIS peptides, and from 1.43 amol to 32.3 fmol per injection for their non-phosphorylated counterparts. The average lower limit of quantification (LLOQ) was 375 amol or 135 amol on-column for phosphorylated or non-phosphorylated peptides. The median CV throughout all dilution steps was calculated as 6.7% for phosphorylated, and 5.0% for non-phosphorylated peptides. These results are summarized in Supplementary data file S1.

S. macrospora wild type and the three STRIPAK deletion strains $\Delta pro11$, $\Delta pro22$ and $\Delta pp2Ac1$ were grown in triplicates prior to being subjected to lysis and protein extraction (Fig. 2). After tryptic digestion and quality control measurements, protein amounts were normalized and aliquots were spiked with either phosphorylated SIS peptides or their non-phosphorylated counterparts. The phosphopeptide aliquots were subjected to TiO_2 -based enrichment followed by LC-PRM measurement, while the fraction spiked with the non-phosphorylated peptides was measured directly. The targeted PRM measurements of SIS and endogenous peptides allowed the simultaneous quantification of both the phosphorylated and non-phosphorylated peptide counterparts, and thus enabled us to calculate the phosphorylation site occupancy.

Using the proteomics workflow described above, we were able to distinguish three different outcomes. First, we determined the site occupancy for 37 phosphorylation sites on 4 proteins, where the phosphorylated and non-phosphorylated peptides were determined. Included is the phosphorylation site S686 from SmKIN3. For DBF2, we detected four phosphorylation sites of which three were quantified. Phosphorylation site S104 in DBF2 was significantly decreased when STRIPAK is non-functional, indicating that this site is STRIPAK dependent. Phosphorylation of sites S89 and S502 was not differentially regulated in STRIPAK deletion mutants (Dataset S2, Fig. S1-S3). We found five phosphorylation sites in BUD4 (T381, S367, S369, S742, S1373); all were dephosphorylated in STRIPAK deletion mutants (Dataset S2, Figs. S4-S7). Secondly, nine phosphorylation sites were detected on five proteins; however, the non-phosphorylated peptide was not detectable. The phosphorylation site occupancy for these peptides was indirectly calculated based on the concentration of the remaining peptide pairs of

the respective protein. Among these we found two sites (S668, S589) in the SmKIN3 protein. Finally, as a third option, we found seven phosphorylation sites on five proteins, where only the phosphorylated peptides were detected and no site occupancy value could be calculated (Dataset S2). However, changes in the abundance of selected phosphorylated peptides could still be used for quantification, since the corresponding proteins were stably expressed in wild type and STRIPAK deletion mutants in previous experiments. An example is the CDC7 protein, which was recently detected in a phosphoproteomic analysis (7)

Overall, 19 phosphorylation sites showed significantly different site occupancy between the wild type and at least one of the STRIPAK mutant strains (student's t-test p-value < 0.05; the results are summarized in Supplementary data file S2). This quantification of site occupancy helped us to prioritize our functional analyses of phosphorylation sites from SmKIN3, the main objective of this investigation. It was also the source for constructing a mechanistic model of phosphorylation-dependent protein regulation. The number of peptides where both phosphorylation sites S668 and S686 are dephosphorylated in STRIPAK deletion mutants is higher than in wild type, i.e. peptides containing phosphorylated site S668 and S686 are less abundant in STRIPAK deletion mutants than in the wild type (Fig. 3). In contrast, we detected only a slightly higher number of peptides with phosphorylation at site S589 (Fig. S8).

Phospho-mimetic and phospho-deficient SmKIN3 mutants display a hyper-septation phenotype

Our quantitative analysis of the phosphosite occupancy in SmKIN3 indicated that SmKIN3 is a target of STRIPAK. To analyze the function of phosphorylated SmKIN3, we generated six different phospho-deficient plus phospho-mimetic mutants, by subjecting the triplets encoding S589, S668 and S686 to *in vitro* mutagenesis, substituting the serine triplets by either alanine (no phosphorylation) or glutamic acid (mimics phosphorylation) triplets, as described in the Material and Methods section (Fig. S9). Phosphorylation of S589 is apparently STRIPAK independent (Fig. S8),

while S668 and S686 seem to depend on STRIPAK (Fig. 3).

For functional analysis of SmKIN3 and its variants, we used a previously described *Smkin3* deletion strain for complementation analysis (13). Recombinant plasmids (Table 2) encoding SmKIN3-GFP phospho-variants were transformed into Δ SmKin3, and primary transformants were used to isolate homokaryotic ascospores. The expression of SmKIN3-GFP wild type and phospho-variants was verified by Western blot analysis indicating the expression of a 118 kDa protein (Fig. S10). When the wild type tagged *Smkin3* gene was used for complementation, we obtained fully fertile strains with wild-type mycelial growth, indicating that GFP-labeled SmKIN3 is fully functional. We then investigated three homokaryotic ascospore isolates of each phospho-mimetic strain, S589E, S668E, S686E, and phospho-deficient strain, S589A, S668A and S686A. Fluorescence microscopy showed that phospho-mutated SmKIN3 still localizes to septa and close to nuclei and thus resembles the wild type situation (Fig. S11-S17). However, we found that both S668A and S668E showed an increased number of septa in hyphal branches, as detected by GFP fluorescence of the SmKIN3-phospho variants. The identity of septa was further confirmed by Calcofluor White (CFW) staining. We investigated at least 200 single hyphal branches for each strain. In these cases, 600 hyphal branches were counted for each recombinant strain (Fig. 4A-C, Dataset S3). We counted all septa within 20 μ m behind hyphal branches. Double, triple, quadruple and quintuple septa were considered when they were located within a distance of maximum 12 μ m. These are referred to as hyper-septation phenotype.

In the case of the hyper-septation, the value for the *S. macrospora* wild type was $1.84\% \pm 0.50$. The hyper-septation value for the complemented strains and phospho-mutants were as follows: WT::*Smkin3-gfp* $1.00\% \pm 0.69$, Δ SmKin3::*Smkin3-gfp* $3.08\% \pm 2.90$, phospho-mutants S589A $1.04\% \pm 0.64$, S589E $1.44\% \pm 0.63$, S668A $5.80\% \pm 1.69$, S668E $6.96\% \pm 2.53$, S686A $4.00\% \pm 3.80$, S686E $3.41\% \pm 2.50$.

Obviously, mutations of S668 and S686 have an effect on hyphal septation. To verify their effect further, we constructed phospho-mimetic (S668ES686E) and phospho-deficient

(S668AS686A) double mutants and analyzed derived ascospore isolates. The strains were fully fertile and the phospho-mutated SmKIN3 proteins are still found at septa and at nuclei in young hyphae (Fig. S18-19). We determined the corresponding values for hyper-septations: S668AS686A $7.00\% \pm 2.99$, S668ES686E $6.84\% \pm 2.14$. Interestingly, we detected triple, quadruple and quintuple septa only in the phosphomutants S668 and S686, and the double mutants S668S686, but they were lacking in wild type, strains complemented with the wild type gene, or phospho-mutants S589A and S589E.

From our quantitative results we conclude that the phospho-mimetic and phospho-deficient SmKIN3 mutations at S686 and S668 are responsible for the hyper-septation phenotype, while phosphorylation site S589 has no effect on septum formation. Interestingly, the investigated double mutants resemble mostly to mutants S668. To analyze the impact of STRIPAK on septum formation, we examined three STRIPAK deletion strains lacking either the regulatory subunit of the STRIPAK phosphatase (*Pro11*), the catalytic subunit of the STRIPAK phosphatase (*App2Ac1*), or the STRIP1/2 homologue (*Pro22*). We found no hyper-septation in *Pro22* and *App2Ac1* and only a very low number of double septa in *Pro11* ($0.8\% \pm 1.1$) (Fig. 4C, Fig. S20). We conclude that septum formation is reduced in STRIPAK mutants, and thus is probably positively regulated by STRIPAK.

Localization of SmKIN3 at septa is dependent on phosphorylation and an intact STRIPAK complex

Next, we investigated the effect of phosphorylation of SmKIN3 on its cellular localization at septa. For fluorescence and differential interference microscopy we used all eight phosphorylation variants as well as the wild type and two deletion strains lacking genes for STRIPAK subunits. We selected the four terminal septa at the hyphal tip for our detailed localization analysis. We counted the first terminal septa at hyphal tips, where GFP fluorescence indicated SmKIN3 localization (representative images in Fig. 5). As an example, more than 50% of wild type septa showed fluorescence at the 3rd terminal septum (Table 1). Similar values were obtained for the

445 Δ SmKin3 strain complemented with the wild
446 type *Smkin3* gene. However, Δ SmKin3 strains,
447 carrying the mutated codon S589 were
448 different; most of the localization was observed
449 at the 2nd and 3rd septum. A significantly
450 different result was observed with all mutated
451 codons S668, S686 as well as with the double
452 mutants S668S686. Here, we found preferential
453 localization at the first or second septum (Fig.
454 S23).

455 To further investigate the role of STRIPAK, we
456 analyzed the localization of SmKIN3 in two
457 STRIPAK deletion mutants, Δ *pro11* and
458 Δ *pp2Ac1*. In both deletion strains, SmKIN3
459 mainly localized at the first or second septum,
460 similar to the phospho-variants of S668 and
461 S686, and unlike in the wild type strain. This
462 suggests that temporally controlled
463 phosphorylation on sites S668 and S686 is
464 required for the localization of SmKIN3 to early
465 septation sites. The microscopic study was
466 further extended by demonstrating that
467 SmKIN3 also localized to septa in mature
468 hyphae (Figs. S11-S17, S22-S24).

469

470 Discussion

471 Here, we provide analytical molecular evidence
472 that phosphorylation of SmKIN3 at distinct
473 sites is STRIPAK dependent. Tightly regulated
474 phosphorylation of SmKIN3 is important for its
475 function. Not only did we discover that
476 deregulation of SmKIN3 phosphorylation leads
477 to a hyper-septation phenotype, but also that
478 temporal phosphorylation of SmKIN3 regulates
479 its septal localization.

480 We accurately quantified 53 phosphorylated
481 peptides, and for 37 of those directly determined
482 the site occupancy of the phosphorylation sites
483 by quantifying the corresponding non-
484 phosphorylated peptide. To date, accurate
485 targeted quantification and determination of
486 phosphorylation site occupancy has only been
487 performed in several studies, and these are
488 mostly limited to just a handful of sites (11,21-
489 24), or only used crude SIS peptides (25). Since
490 our targeted phosphorylated peptides were
491 found at concentrations lower than 30 amol per
492 μ g of protein lysate, to the best of our
493 knowledge this result confirms that we have
494 used a most sensitive assay to determine
495 phosphorylation site occupancy in a non-human
496 model organism.

497 STRIPAK regulates SmKIN3 498 phosphorylation indirectly

499 In mammalian cells, Hippo comprises two
500 kinases, GCK MST1/2 and NDR kinase
501 LATS1/2 (26,27). Recently, it was shown that
502 STRIPAK integrates upstream signals to
503 control the activity of the SmKIN3 homolog
504 MST1/2 for initiating Hippo signaling. Deletion
505 of STRIP1/2, a homolog of *S. macrospora*
506 PRO22, results in upregulation of MST1/2,
507 which led to the conclusion that STRIPAK
508 regulates MST1/2 (26,28,29).

509 In fungi, the Hippo homologous kinase cascade
510 SIN comprises three kinases. For example, in
511 *N. crassa* the Ste20 kinase CDC-7 acts
512 upstream of GCK SID-1 and NDRK DBF-2
513 (30). SID-1, the middle component of SIN, is
514 the homolog of SmKIN3 and MST1/2. Our
515 PRM analysis revealed that the phosphosite
516 occupancy, i.e. phosphorylation in two out of
517 three sites in SmKIN3 is decreased in
518 STRIPAK deletion mutants. This finding is
519 intriguing, since STRIPAK is a
520 dephosphorylating complex. However,
521 comparable results in fission yeast showed that
522 SIN is negatively regulated by the SIN
523 inhibitory PP2A (SIP) complex, the STRIPAK
524 homolog (31). SIP dephosphorylates the
525 upstream Ste20 kinase Cdc7p, which leads to
526 assembly of SIN. Cdc7p itself phosphorylates
527 SID1p, the homolog of SmKIN3. Dysfunction
528 of SIP prevents assembly of SIN and thus
529 abolishes phosphorylation of SID1p (31).
530 Sid1p, SID-1 and SmKIN3 not only resemble
531 each other in their posttranslational
532 modifications, but also in their functions.
533 Similar to Sid1p in *S. pombe*, SmKIN3 and SID-
534 1 in *N. crassa* are required for proper septum
535 formation (15,31).

536 The SIN component SmKIN3 is a positive 537 regulator of septum formation

538 In filamentous ascomycetes, such as
539 *S. macrospora*, hyphae are compartmentalized
540 by the formation of septa, which are assembled
541 at an actomyosin-based cortical ring (CR),
542 followed by CR constriction. The CR protein
543 complex includes structural proteins, molecular
544 motors, and signaling enzymes (17). Since
545 SmKIN3 localizes at the centre of the hyphal
546 septum around the pore, SmKIN3 may be
547 involved in the function of CR signaling
548 enzymes to activate or inhibit targets via
549 posttranslational modifications, such as

550 phosphorylation. In particular, the presence of
551 SmKIN3 at mature septa suggests additional
552 functions besides septum formation, as was
553 hypothesized for BUD4 (32).

554 Our PRM approach provides evidence that
555 SmKIN3 is a dephosphorylation target of
556 STRIPAK. The analysis of phospho-mimetic
557 and phospho-deficient SmKIN3 expressing
558 strains revealed a hyper-septation phenotype.
559 Since phosphorylation at distinct SmKIN3 sites
560 is regulated by STRIPAK, we hypothesize that
561 the phosphorylation status of S668 and S686 is
562 important for the temporal and spatial fine
563 tuning of the septation process, and the correct
564 formation of septa.

565 In *A. nidulans*, SEPH, the homolog of CDC7,
566 has been identified as a central component for
567 the initiation of septation prior to actin ring
568 formation. SEPH is the upstream kinase of
569 SEPL, which is the homolog of SmKIN3,
570 followed by SIDB (homolog of DBF2) (Bruno
571 et al. 2001; Kim et al. 2006). SIN and its
572 downstream effectors are involved in forming
573 the CR, which is responsible for initiating the
574 formation of septa. To specify the location for
575 septum formation, axial landmark proteins such
576 as BUD3 and BUD4 are needed, which recruit
577 septin AspB to the CR (17). Downregulation of
578 SepH abolishes septation, whereas hyper-
579 activation results in the formation of multiple
580 septa (Bruno et al. 2001). This indicates that
581 SEPH acts as a positive regulator of SIN, which
582 triggers cytokinesis in *A. nidulans* (33).
583 Comparable phenotypes were observed in
584 *S. macrospora*, where the loss of *Smkin3* or
585 mutation in the ATP-binding site results in the
586 reduction of septa (13). Phosphorylation
587 mutants of SmKIN3, as reported here, show a
588 hyper-septation phenotype that was similarly
589 observed in *A. nidulans* mutants with hyper-
590 activation of SEPH. Our results are consistent
591 with findings for *A. nidulans* and *N. crassa*,
592 which showed that homologs of CDC7 and
593 SmKIN3 act in the same pathway as positive
594 regulators of SIN.

595 **The phosphorylation state of SmKIN3 affects** 596 **its localization**

597 Here, we demonstrate that SmKIN3's septal
598 localization is altered in mutated phospho-
599 deficient strains, and STRIPAK deletion
600 mutants, indicating that this process is also
601 mediated by STRIPAK. Furthermore, the
602 STRIPAK-dependent phosphorylation state of

603 SmKIN3 affects its affinity for septal proteins
604 and thus its localization. These findings are
605 consistent with results obtained in fission yeast.
606 There, it was shown that SIN and STRIPAK
607 affect each other by phosphorylation and
608 dephosphorylation (31). SIN phosphorylates
609 GEFs and GTPases, which in turn
610 phosphorylate the formin SepA. The
611 phosphorylation of SepA is important for the
612 assembly of actin to form the CR. The NDR
613 kinase Sid2p is necessary to phosphorylate the
614 septin Cdc12 and other proteins to form the CR
615 (34). Such phosphorylation at the CR must be
616 tightly regulated to form the final septum.

617 To summarize our findings, we have designed a
618 schematic mechanistic model, as depicted in
619 Fig. 6. The SIN complex acts downstream of
620 STRIPAK, which dephosphorylates CDC7
621 directly (31). However, STRIPAK acts
622 indirectly on phosphorylation sites S668 and
623 S686 of SmKIN3, while phosphorylation of
624 S589 is likely regulated by an unknown
625 phosphatase and kinase. Furthermore,
626 STRIPAK also indirectly phosphorylates S104
627 in DBF2, while S89 and S502 are not STRIPAK
628 dependent. Finally, from our PRM analysis, we
629 propose that phosphorylation of all sites from
630 BUD4 depends on STRIPAK.

631 We hypothesize further that deletion of
632 STRIPAK subunits results in higher
633 phosphorylation of CDC7, which prevents
634 assembly of SIN (Singh et al. 2011). This results
635 in a lower level of phosphorylated SmKIN3,
636 which consequently is unable to phosphorylate
637 the downstream kinase DBF2. Subsequently,
638 DBF2 does not phosphorylate the anillin-like
639 protein BUD4 to form the CR, leading to lower
640 septation. Indeed, our quantitative
641 phosphorylation data provide evidence that four
642 phosphorylation sites of BUD4 and the
643 phosphorylation site S104 of DBF2 are
644 dephosphorylated in STRIPAK deletion
645 mutants compared to wild type. The
646 phosphorylation site S502 of DBF2, which is
647 the homolog to the phosphorylation site S499 in
648 *N. crassa*, is not regulated by STRIPAK
649 (Supplementary data S2). This is consistent
650 with results obtained in *A. nidulans*. There,
651 localization of septin AspB, which is the
652 homolog of CDC12 in *S. macrospora*, is
653 dependent on the formin SepA (SMAC_04496),
654 the SIN kinase SepH (CDC7), and on its own
655 phosphorylation state.

Dephosphorylation of conserved threonine 68 in SepH was shown to be critical for timing of septation and localization (35,36). In *N. crassa*, phosphorylation analysis revealed that phosphorylation of DBF-2 is stimulated by SID-1. Interestingly, phospho-deficient and phospho-mimetic mutants of DBF-2 phosphorylation site S499 in *N. crassa* are nonfunctional *in vivo* and reduce the kinase activity of DBF-2 *in vitro* (15). However, our data indicate that S499 (S502 in *S. macrospora*) seems not to be regulated by STRIPAK, but is essential in septum formation.

The interaction between components of the SIN cascade is decreased when their dephosphorylation is diminished due to a non-functional STRIPAK complex. Moreover, the lack of SIN phosphorylation prevents the recruitment of SIN components to septal pore proteins at the hyphal tip (37). Thus, the interaction of SmKIN3 with SIN and STRIPAK has to be strongly regulated to form septa in a wild type-like manner, as suggested by our localization experiments with wild type and mutant strains.

SmKIN3 homologs are conserved from yeast to humans, and SIN-like pathways have been mentioned in this discussion. We propose that the mechanism of SmKIN3 function described in this study may be applicable to that of homologous networks in other organisms.

Experimental procedures

Strains and growth conditions

Electro-competent *E. coli* cells XL1 Blue MRF⁺ were used for cloning and propagation of recombinant plasmids (38) under standard laboratory conditions (39). All *S. macrospora* strains used in this study are listed in Table S1 and were grown under standard conditions (40,41). For analysis of distribution, SmKIN3 strains were grown for 24 h on solid biomalt-cornmeal medium (BMM)-coated glass. Isogenic and homokaryotic strains were generated by genetic crossing and ascospore isolation (40). To obtain phospho-mutants, the mutated plasmids (Table S2) were transformed into a Δ *Smkin3* strain. Phospho-mutations in the generated strains were verified by PCR analysis and DNA sequencing (Eurofins Genomics; Ebersberg, Germany). The number of ectopically integrated recombinant DNA

fragments was verified by Southern hybridization analysis (Fig. S24).

In vitro recombinant techniques

Plasmids used in this study are listed in Table S2. pIG1783-*Smkin3-gfp* was created by restricting the plasmid pIG1783 with *Nco*I. *Smkin3* was amplified via PCR with overhangs at the 5' and 3' ends containing recognition sites for *Nco*I. Ligation was performed with T4 DNA ligase. For phospho-mimetic and phospho-deficient strains, plasmid pIG1783-*Smkin3-gfp* was used for Q5 mutagenesis (NEB biolabs). With specific primers (Table S3), we generated eight plasmids, containing phospho-mimetic and phospho-deficient mutations (Fig. S3)

Microscopic investigations. Microscopic experiments were performed using an AxioImager microscope (Zeiss [Carl Zeiss], Thornwood, NY) coupled with a CoolSnap HQ camera (Roper Scientific) and a SpectraX LED lamp (Lumencor) at room temperature. Images were acquired and edited with MetaMorph (version 7.7.0.0; Universal Imaging). Strains were grown on glass slides covered with solid BMM and incubated for 12 to 24 hours. Co-localization of proteins was obtained by inoculating two different strains on the same BMM-coated glass slides in Petri dishes for 1 to 2 days. Hyphal fusion of both strains enabled the formation of heterokaryons by exchanging nuclei. GFP fluorescence and mRFP fluorescence were analyzed using filter sets (Chroma Technology Corp.) 49002 (GFP, excitation filter HQ470/40, emission filter HQ525/50, beamsplitter T495LPXR) and 49008 (mRFP, excitation filter HQ560/40, emission filter ET630/75m, beamsplitter T585lp). Septa in vegetative hyphae and ascogonial coils were stained using Calcofluor White M2R (CFW; Sigma [Sigma Chemical], St. Louis, MO) with a concentration of 1 μ g/ml CFW stock solution diluted 1:400 in A. dest solution. CFW fluorescence was analyzed using Chroma filter set 31000v2 (excitation filter D350/50, emission filter D460/50, beam splitter 400dclp; Chroma Technology Corp., Bellows Falls, VT, USA).

To analyze the distribution of SmKIN3 on septa, 50 to 100 hyphal tips of the growing front were observed in 10 to 15 independent samples per strain in three different independent strains. Septal distances in hyphae were measured using MetaMorph (version 6.3.1; Universal Imaging). For analysis of hyper-septation, e.g. double and

triple septa, we investigated at least 100 single hyphal branches for each strain. In the case of recombinant strains, three different single ascospore isolates were investigated to exclude side effects from random integration of recombinant DNA. In these cases, 600 hyphal branches were counted for each recombinant strain.

Peptide selection for targeted quantification of phosphorylation sites

Based on the results of the global ITRAQ-based (phospho-)proteomic analyses previously performed (7,8), as well as unpublished work, phosphorylated peptides that showed differential regulation in STRIPAK mutant strains compared to wild type were selected and identified as putative dephosphorylation targets of the STRIPAK complex. In addition, the corresponding non-phosphorylated peptides were selected for quantification, thus enabling calculation of a site-occupancy value for the respective phosphorylation sites. As controls, phosphopeptides representing cell division control protein 48 (CDC48; SMAC_00109) and heat shock protein 90 (HSP90; SMAC_04445) were included (Figs. S25-S28), since they showed no regulation in the global experiments, and to our knowledge are not functionally connected to the STRIPAK complex.

Synthesis, purification and quantification of SIS peptides

Synthesis of all SIS peptides was performed in-house using a Syro I synthesis unit (MultiSynTech, Witten, Germany) and Fmoc chemistry. Synthesis and subsequent purification were performed as described previously (42). Heavy-labeled lysine ($^{13}\text{C}_6$ $^{15}\text{N}_2$) and arginine ($^{13}\text{C}_6$ $^{15}\text{N}_4$) were incorporated at the C-terminus, and amino acid analysis was applied to determine peptide concentrations (43). A five-point calibration curve of derivatized amino acids, ranging from 5-25 pmol/ μL , was used for quantification.

Protein extraction, digestion and normalization and phosphopeptide enrichment were performed as recently reported (7).

nano-LC-MS/MS for PRM of unphosphorylated peptides

Samples were analyzed on an Ultimate 3000 nanoRSLC HPLC system coupled to a Q Exactive HF mass spectrometer (MS, both Thermo Scientific). The HPLC was equipped with a trapping column (100 μm x 2 cm C18, PepMap RSLC, Thermo Scientific) for pre-concentration, and an analytical column (75 μm x 50 cm C18, PepMap RSLC, Thermo Scientific) for separation of the peptides. Pre-concentration was performed for 5 min at a flow rate of 20 $\mu\text{L}/\text{min}$ using 0.1% TFA, and separation was performed at a flow rate of 250 nL/min. An optimized binary gradient of solvent A (0.1% FA) and solvent B (84% acetonitrile, 0.1% FA) was used with the following steps: 0 min – 2%B; 5 min – 2%B; 10 min – 5%B; 50 min – 9%B; 73 min – 15%B; 100 min – 21%B; 115 min – 45%B, followed by two washing steps for 5 min at 95%B and 20 min of equilibration at 2%B. The MS was operated in PRM mode at a resolution of 60,000 (at 200 m/z) with a fixed first mass of 150 m/z. The AGC target was set to 1×10^6 and a maximum injection time of 118 ms. Targeted precursors were isolated with a quadrupole isolation width of 0.4 m/z and fragmented with a normalized collision energy of 27. PRM acquisition was scheduled with a retention-time window of 2 min per target.

nano-LC-MS/MS for PRM of phosphorylated peptides

Enriched phosphorylated peptides were analyzed with the same instrumentation and settings as described above. The LC gradient was optimized to suit the targeted phosphopeptides and the steps were modified as follows: 0 min – 3%B; 5 min – 3%B; 15 min – 7%B; 37 min – 10%B; 90 min – 20%B; 110 min – 27%B; 120 min – 45%B, followed by the same washing and equilibration steps as above.

PRM development and analysis

To verify the linearity of response and determine the limit of blank (LOB), LOD, and LLOQ response curves of both phosphorylated and non-phosphorylated SIS peptides were acquired. In both cases, a background matrix was generated by pooling aliquots of all individual samples. SIS peptides were spiked in at 8 different concentrations, covering a range of three orders of magnitude. Based on prior determination of individual SIS response factors, the highest concentrations for non-phosphorylated SIS peptides varied between

54 fmol and 2.73 pmol on-column, while the lowest concentrations were between 13 and 667 amol on-column. For the analysis of the individual samples, a total amount of non-phosphorylated SIS peptides ranging from 0.05 fmol to 60 fmol was spiked into 3 µg of total protein digest, depending on the expected endogenous concentration (all concentrations are given in Supplementary data file S1). For phosphorylated SIS peptide calibration curve measurements, a total of 8 dilution points were generated with SIS peptide concentrations ranging from 600 amol to 2.4 pmol in 200 µg of background matrix for the best responding peptides, as detailed in supplementary data file S1. In addition, six phosphoisomeric standard isotope-labeled (SIL) peptides were included in the assay, but no endogenous peptide was detected. These peptides could be further used to rule out the presence of these isomers through knowledge of their retention time and diagnostic transitions. The median CV of the biological replicates was calculated as 16.3% for all non-phosphorylated and 16.1% for all phosphorylated peptides. The lowest average site occupancy was detected at 0.06% on S369 in the HSP 90 protein SMAC_04445. This site was included as negative control and its occupancy showed no significant difference between the wild type and any of the deletion strains. The phosphorylation site S2349 in the phosphatidylinositol 3-kinase TOR2 (SMAC_03322) exhibited the highest site occupancy with 92.2% measured in the wild type.

All calibration curve samples were subjected to TiO₂-based phosphopeptide enrichment and nano-LC-MS/MS measurement in technical

triplicates (16.7% of eluate per replicate) as detailed above. Additionally, two replicates without SIS spike-in (DS0) were processed in parallel to appropriately determine background signal levels. For the final analysis of individual samples, a total amount of 410 amol to 4.1 fmol was spiked into 450 µg of protein digest, followed by phosphopeptide enrichment and nano-LC-MS/MS measurement of 25% of the eluates after enrichment.

Skyline software (version 4.1, (44)) was used to analyze all of the PRM data. The top 3 most suitable transitions of every light and SIS peptide pair were chosen. All data were manually inspected for correct peak detection, retention time and integration, and peak areas were exported. R software (45) (version 3.5.3) was used for data analysis and calibration curve measurements, and LOB and LOD were calculated using the MSSStats package (46). The L/H peak area ratios were used to determine the concentration (c) of phosphorylated and non-phosphorylated peptide per sample. The phosphorylation site occupancy was calculated using the following formula:

$$\frac{c(\text{phosphorylated peptide})}{c(\text{phosphorylated peptide}) + c(\text{non-phosphorylated peptide})} * 100 = \text{site occupancy (\%)}$$

Statistical comparison of the phosphorylation site occupancy between wild type and knock-out strains was performed using a two-sided student's t-test.

Data availability

All targeted proteomics data and raw files are available through the Panorama repository (47) with the dataset identifier PXD023130 and via <https://panoramaweb.org/SmKIN3.url>

942

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951 **Conflict of Interest:** The authors declare no conflicts of interest in regards to this manuscript.

952 **Abbreviations**-The abbreviations used are: BMM, biomalt-cornmeal medium; CFW, Calcofluor White;
953 CR, cortical ring; CV, coefficient of variation; ELM, eukaryotic length motif; FHA, forkhead-
954 associated; GCK, germinal centre kinase; iTRAQ, isobaric Tags for Relative and Absolute Quantitation;
955 LFQ, label-free quantification; LLOQ, lower limit of quantification; LOB, limit of blank; LOD, limit of
956 detection; NDR, nuclear DBF2-related; PRM, parallel reaction monitoring; SIL, standard isotope
957 labeled; SIN, septation initiation network; SIP, SIN inhibitory PP2A; SIS, stable isotope-labeled
958 standards; STRIPAK, striatin-interacting phosphatase and kinase; TMT, tandem mass tag

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Table 1: Localization of SmKIN3, and its phosphorylation variants in wild type and STRIPAK deletion strains. In wild type, SmKIN3 is preferentially seen at the third terminal septum, while in the three variants S668, S686 and S668S686, and both STRIPAK mutants $\Delta pp2Ac1$ and $\Delta pro11$, localization is observed mostly at the first or second septum. $N \geq 50$ per strain. Localization of SmKIN3 in the complemented $\Delta SmKin3$ strain and in both phospho-variants of S589 resembles the wild type.

	1 st septum	2 nd septum	3 rd septum	4 th septum
WT::kin3-gfp*	-	30.93%	51.55%	13.40%
$\Delta kin3::kin3$ -gfp	-	22.00%	64.00%	14.00%
S589A	3.96%	47.52%	46.53%	1.98%
S589E	-	43.84%	46.58%	9.59%
S668A	53.48%	46.51%	-	-
S668E	27.42%	67.74%	4.83%	-
S686A	52.63%	47.37%	-	-
S686E	44.78%	47.76%	7.46%	-
S668AS686A	26.79%	64.29%	8.92%	-
S668ES686E	39.29%	53.57%	7.14%	-
$\Delta pro11::kin3$ -gfp*	68.00%	32.00%	-	-
$\Delta pp2Ac1::kin3$ -gfp*	84.00%	16.00%	-	-

* strains are isogenic

Figure 1: Linear structure of SmKIN3 and identified protein domains. Depicted are a serine/threonine kinase domain (aa 10-279, red), large tumor suppressor kinase 1 (LATS1) kinase phosphorylation motifs (aa 721-727; 740-746; 794-800, green), a coiled-coil domain (aa 688-788, grey), a T-motif (aa 801-806, blue), and a phospho-threonine motif, binding a subset of FHA domains (aa 805-811, yellow). Asterisks below indicate phosphorylation sites S589, S668, and S686.

Figure 2: Targeted quantification of phosphorylation site occupancy. Graphical representation of the proteomics workflow including (1) extraction and digestion of proteins from *S. macrospora* strains grown in triplicates, (2) spike-in of phosphorylated and unphosphorylated SIS peptides, (3) TiO₂-based enrichment of phosphorylated peptides, (4) targeted analysis using a RP-nHPLC-nESI-PRM setup, and (5) data analysis and calculation of phosphorylation site occupancy.

Figure 3: Quantitative analysis of the non-phosphorylated and phosphorylated peptides containing phosphorylation site S668 and S686 of SmKIN3 in three different STIPAK deletion mutants. (A, C) The y-axis on the left gives the amount of the peptides EPQLLQQHsAPSLQK or ENPPPLQQQLsHPVLQQEK in amol as non-phosphorylated (green) and phosphorylated variants (orange). The y-axis on the right shows the quantity of phosphosite occupancy in percent, plotted as red

1000 dots in the bar chart. Error bars indicate standard deviation. Lower case letters in the peptides indicate
 1001 the phosphosite. (B, D) Alignment of sequences of SmKIN3 from *S. macrospora* (*S.m.*), and SID-1 from
 1002 *Neurospora crassa* (*N.c.*).
 1003

Fig. 4: Hyper-septation phenotype in wild type and phospho-mutants. (A) Fluorescence microscopic investigation of septation at hyphal branches. Mycelia were stained with CFW, and SmKIN3 is labeled with GFP. (B) Quantitative investigation of hyper-septation. All values are given in percent. We investigated at least 100 hyphal branches for each STRIAPK mutant strain. In the case of recombinant strains, three different single ascospore isolates were investigated to exclude side effects from random integration of recombinant DNA into genomic DNA. In these cases, 600 hyphal branches were counted for each recombinant strain. As a control, 600 hyphae from ΔSmKin3 complemented with wild type, and ΔSmKin3 strains were investigated in 3 technical replicates. Percentage of hyperseptation (2-5 septa) are given in dark blue, no septa are indicated in light blue. Error bars indicate standard deviations.

Fig. 5: STRIPAK-dependent localization of SmKIN3-GFP at hyphal septa. The strains shown here are indicated on the left. The first three septa from the hyphal tip (on the left) were numbered. Arrowheads label green fluorescing SmKIN3 (GFP), or CFW stained septa.

Figure 6: Mechanistic model of STRIPAK-dependent phosphorylation of SmKIN3, as part of the SIN complex. Relevant phosphorylation sites in SIN components and the related BUD4 protein are indicated around the proteins. STRIPAK subunits are colored in blue, SIN components are colored in orange. Grey dashed arrows indicate STRIPAK regulated phosphorylation. Red arrows indicate direct phosphorylation. This model is based on data from this study, and from a recent publication (7)

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