

RESEARCH ARTICLE

# The role of *Aspergillus nidulans* polo-like kinase PlkA in microtubule-organizing center control

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## ABSTRACT

Centrosomes are important microtubule-organizing centers (MTOC) in animal cells. In addition, non-centrosomal MTOCs (ncMTOCs) have been described in many cell types. The functional analogs of centrosomes in fungi are the spindle pole bodies (SPBs). In *Aspergillus nidulans*, additional MTOCs have been discovered at septa (sMTOC). Although the core components are conserved in both MTOCs, their composition and organization are different and dynamic. Here, we show that the polo-like kinase PlkA binds the  $\gamma$ -tubulin ring complex ( $\gamma$ -TuRC) receptor protein ApsB and contributes to targeting ApsB to both MTOCs. PlkA coordinates the activities of the SPB outer plaque and the sMTOC. PlkA kinase activity was required for astral MT formation involving ApsB recruitment. PlkA also interacted with the  $\gamma$ -TuRC inner plaque receptor protein PcpA. Mitosis was delayed without PlkA, and the PlkA protein was required for proper mitotic spindle morphology, although this function was independent of its catalytic activity. Our results suggest that the polo-like kinase is a regulator of MTOC activities and acts as a scaffolding unit through interaction with  $\gamma$ -TuRC receptors.

**KEY WORDS:** *Aspergillus*, Polo like kinase, Microtubules, ApsB, SPB, sMTOC, PcpA,  $\gamma$ -TuRC,  $\gamma$ -TuCRs, Mto1, Mto2

## INTRODUCTION

Microtubules (MTs), along with actin and septins comprise the cell cytoskeleton in most eukaryotic cells. The cytoskeleton is essential in many cellular processes such as cell division, intracellular transport or polar growth. MTs are nucleated from specialized structures, called 'microtubule-organizing centers' (MTOCs). One century ago, the centrosome was identified as the primary MTOC in animals. It is composed of two centrioles and is surrounded by pericentriolar material (PCM) (Bornens, 2012; Wilson, 1925). Its functional analog in fungi, the spindle pole body (SPB), is a multilayered structure embedded in the nuclear envelope that nucleates MTs on both the cytoplasmic and the nuclear face (Jaspersen and Winey, 2004).

Despite fundamental structural differences between centrosomes and SPBs, they share many common molecular components and

regulators. The key nucleator  $\gamma$ -tubulin was discovered in the filamentous fungus *Aspergillus nidulans* and is conserved in all eukaryotes (Martin et al., 1997; Oakley et al., 1990; Oakley and Oakley, 1989; Prigozhina et al., 2004).  $\gamma$ -tubulin together with several  $\gamma$ -tubulin complex proteins (GCPs) assemble into a ring structure and initiate MT polymerization (Liu et al., 2020; Teixidó-Travesa et al., 2012; Zheng et al., 1995). Three components,  $\gamma$ -tubulin, GCP2 and GCP3 comprise a core structure, named the  $\gamma$ -tubulin small complex ( $\gamma$ -TuSC) (Kollman et al., 2010). This complex is found in SPBs of *Saccharomyces cerevisiae* (Knop and Schiebel, 1997). In the fission yeast *Schizosaccharomyces pombe* and higher eukaryotes, the complex is larger, includes additionally GCP4, GCP5, GCP6 and a recently discovered new component, MOZART, and is termed the  $\gamma$ -tubulin ring complex ( $\gamma$ -TuRC) (Cota et al., 2017; Masuda and Toda, 2016). A recent study of a MOZART1 ortholog in *A. nidulans* revealed an asymmetric composition of SPBs with  $\gamma$ -TuRCs at the inner and  $\gamma$ -TuSCs at the outer plaque (Gao et al., 2019).

$\gamma$ -tubulin complexes are regulated by various factors that recruit  $\gamma$ -tubulin complexes to different cellular sites and control MT nucleation activity. The recruiting proteins are often named  $\gamma$ -tubulin complex receptors ( $\gamma$ -TuCRs). The number of receptor proteins increases from yeast to human. Although lacking centrioles, yeasts share the PCM modules with animals, such as pericentrin and CDK5RAP2, which anchor  $\gamma$ -TuSC/ $\gamma$ -TuRC to the SPB/centrosome (Ito and Bettencourt-Dias, 2018). The *S. cerevisiae* SPB has two receptors, Spc110<sup>pericentrin</sup> and Spc72<sup>CDK5RAP2</sup> (mammalian homolog names indicated in superscript), targeting  $\gamma$ -TuSCs to the inner (nuclear) and outer (cytoplasmic) plaques of SPBs, respectively (Knop and Schiebel, 1998; Nguyen et al., 1998; Souès and Adams, 1998). Spc72 and its orthologs Mto1 in *S. pombe* and ApsB in *A. nidulans* belong to the CM1-MASC family proteins. They contain an N-terminal centrosomin motif 1 (CM1) that is essential for interacting with  $\gamma$ -TuSC, and a C-terminal MASC domain responsible for anchorage to MTOC sites (Lin et al., 2015; Samejima et al., 2010; Sawin et al., 2004). Spc110 (Pcp1 in *S. pombe* and PcpA in *A. nidulans*) belongs to the SPM-CM1-PACT family and contains two conserved motifs important for MT nucleation activation (SPM and CM1 domains are also present in human pericentrin) as well as a C-terminal MTOC-targeting PACT domain (Flory et al., 2002; Kollman et al., 2010; Lin et al., 2014). Interestingly, *S. pombe* and *A. nidulans* have two outer plaque receptors, ApsB<sup>Mto1</sup> and Spa18<sup>Mto2</sup>, and one inner plaque receptor, PcpA<sup>Pcp1</sup> (Suelmann et al., 1998; Zekert et al., 2010; Zhang et al., 2017).

The centrosome/SPB duplicates once per cell cycle to form two competent MTOCs for assembly of mitotic spindles to ensure proper nuclear division. It is known that increased numbers of centrosomes or mutations in centrosomal proteins can lead to cancer and microcephaly (Gönczy, 2015; Nigg and Raff, 2009). The timing

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of centriole/SPB duplication is tightly regulated by the cell cycle kinase cyclin-dependent kinase 1 (Cdk1) and polo-like kinases (Plks) both in animals and yeasts (Elserafy et al., 2014; Habedanck et al., 2005; Zitouni et al., 2016). Plks are known to play multiple roles in mitotic entry, progression and mitotic exit, DNA-damage checkpoint control and cytokinesis (Archambault and Glover, 2009; Glover, 2005; Liu and Maller, 2005; Smits et al., 2000; Takaki et al., 2008). The human genome encodes five Plks (Plk1–Plk5) that are classified into three subfamilies. Plk1, the most ancient member, is conserved in most eukaryotes except higher plants (Zitouni et al., 2014). Human Plk1 is involved in mitotic entry control, is required for cytokinesis and is essential for centrosome maturation through phosphorylating pericentrin (Lee and Rhee, 2011; Petronczki et al., 2008). *Drosophila* Polo (the Plk1 homolog) enables PCM recruitment to centrosomes via phosphorylation of the CDK5RAP2 homolog Cnn (Conduit et al., 2014) and Sas-4 (Ramani et al., 2018). Plk4 only exists in species with centrioles, where it is involved in centriole biogenesis via phosphorylating its binding partner STIL (Arquint et al., 2015; Moyer et al., 2015). Plk2, Plk3 and Plk5 belong to another subfamily that is less characterized and carries out fewer functions, mostly related to cell differentiation (Zitouni et al., 2014).

Budding and fission yeast have a single Plk1 ortholog, Cdc5 in budding yeast and Plo1 in fission yeast, that play similar roles at mitotic SPBs and cytokinesis sites. *S. cerevisiae* Cdc5 is essential and promotes mitotic exit and cytokinesis through MEN and FEAR networks (Hu et al., 2001; Rahal and Amon, 2008; Song and Lee, 2001). In addition, Cdc5 regulates SPB duplication and mitotic progression through G2/M transition (Avena et al., 2014; Elserafy et al., 2014; Sakchaisri et al., 2004). There is less evidence that Cdc5 drives the MT nucleation pathway directly (Lee et al., 2005). Instead, phosphorylation of Spc110 by the cell-cycle-regulating kinases Mps1 and Cdk is important for promoting Spc110 oligomerization (Lin et al., 2014). Spc72 is one of the Cdc5 substrates and can be phosphorylated *in vitro* (Snead et al., 2007). Similar to Cdc5, *S. pombe* Plo1 is essential for septation initiation network (SIN)-mediated cytokinesis (Tanaka et al., 2001). Additionally, Plo1 plays important roles in mitotic entry and SPB insertion into the nuclear envelope during mitosis (Fong et al., 2010; Wälde and King, 2014). *A. nidulans* encodes only one polo-like kinase, PlkA. It is the largest member of the family and is phylogenetically very distant from other Plk1s (Bachewich et al., 2005). By contrast, phylogenetic analysis places PlkA close to Plk4 (Mogilevsky et al., 2012). The PBD domain of PlkA still contains the conserved residues of Plk1 that are important for phospho-substrate recognition. As a big difference to yeasts, PlkA has lost its essential roles (Mogilevsky et al., 2012) in development and cytokinesis. *A. nidulans* PlkA, hence, could be an ancient protein combining more than one Plk functions that were separated during evolution to different proteins.

Although Plk1 plays important roles in cell cycle control and cytokinesis from yeast to human, the exact contribution to MT nucleation and localization of  $\gamma$ -tubulin complexes is not yet fully understood. Here, we studied the role of the sole polo-like kinase PlkA in MTOC regulation in *A. nidulans*. This organism is of particular interest, because at least two different MTOCs have been described, SPBs and MTOCs associated with septa (sMTOCs) (Konzack et al., 2005; Xiong and Oakley, 2009; Zhang et al., 2017). In addition, the SPB is composed of  $\gamma$ TuSC at the outer plaque with ApsB and Spa18 as receptors, and of  $\gamma$ TuRC at the inner plaque with PcpA as a receptor. Last but not least, the number of  $\gamma$ TuSC and  $\gamma$ TuRC units is very dynamic and changes throughout the cell cycle

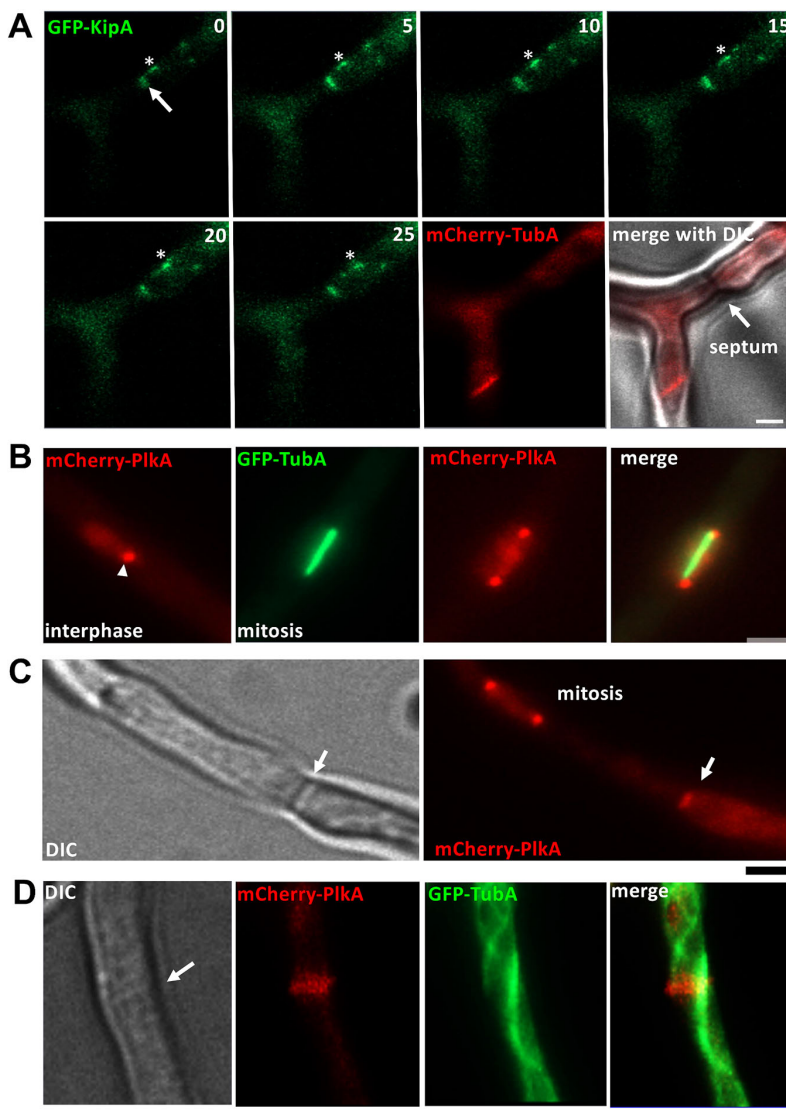
(Gao et al., 2019). These features raise the interesting questions how SPB outer and inner plaque complexes are oppositely regulated throughout the cell cycle and if sMTOC  $\gamma$ TuRCs are coordinately regulated with the outer or the inner plaque of SPBs or whether they are independent of cell cycle control. In this study, we present evidence that PlkA coordinates SPB and sMTOC activities via phosphorylating and recruiting  $\gamma$ TuCRs. Thus, this study adds new knowledge to the functions of polo-like kinases in lower eukaryotes and may provide novel aspects for the discussion of the origin and expansion of Plks in evolution.

## RESULTS

### sMTOC activity is regulated during the cell cycle and evidence for polo-like kinase as a potential regulator

MT generation in fungi at the outer and the inner plaque of SPBs is strictly coordinated with the cell cycle. At the onset of mitosis, in *A. nidulans* cytoplasmic MTs disassemble, and the mitotic spindles develop. As mitosis progresses to anaphase, cytoplasmic MTs (astral MTs) reassemble, followed by mitotic spindle disassembly in telophase. It has been suggested that MT catastrophe rates determine the number of cytoplasmic MTs (Szewczyk and Oakley, 2011). MT growth from nucleation sites is determined by a balance between assembly and disassembly rates. In addition, changes in MT nucleation abilities may contribute to the formation of the MT cytoskeleton. Indeed, changes of the numbers of cytoplasmic MTs are accompanied by changes of the number of  $\gamma$ -TuRC complexes at SPBs (Gao et al., 2019). However, much less is known about the dynamics of septal MTOCs. In order to analyze whether septal MTOCs are also regulated during the cell cycle and if their MT-polymerizing activity is synchronized with the activity of SPBs, we used the MT plus-end tracking kinesin KipA (Konzack et al., 2005). The GFP–kinesin fusion protein was co-expressed with mCherry-labeled  $\alpha$ -tubulin. We analyzed a hyphal compartment undergoing mitosis adjacent to a compartment in interphase and observed KipA comets moving only from septa towards the interphase (Fig. 1A) but not towards the mitotic spindle compartment. This indeed suggests co-regulation of sMTOCs and the outer plaque of the SPB. In contrast, the inner plaque of the SPB of the mitotic compartment was active and produced the spindle MTs. To further confirm the phenomenon, a *bimE7* (for ‘block in mitosis’) mutant was used to block the cell in mitosis (James et al., 1995). At the restrictive temperature most compartments were blocked in mitosis, as indicated by mitotic spindles, but some compartments escaped the block and were in interphase. Whereas MTs clearly emanated from the cytoplasmic side of the septa of the interphase compartment, the sMTOCs in the adjacent mitotic compartments appeared inactive, although some astral MTs were visible (Fig. S1A). The fact that, in the same cytoplasm, astral MTs appear to grow, whereas MTs from sMTOCs are still absent, suggests that sMTOC activity is blocked and responsible for the observed lack of MTs rather than a change of other parameters influencing MT formation and numbers.

If the outer SPB plaque and sMTOCs are co-regulated, the question arises as to which regulators could be involved. In budding yeast, nearly all SPB-associated proteins are modified through phosphorylation during the cell cycle, including the  $\gamma$ -TuSC and its receptors Spc72 and Spc110 (Keck et al., 2011; Lin et al., 2014; Pereira et al., 1998; Vogel et al., 2001). Anticipating that the same regulation takes place in *A. nidulans*, we considered three kinases to be involved in the regulation of the activity of sMTOCs. The kinase NIMA is essential for mitotic entry (Morris, 1975; Osmani et al., 1987; Shen et al., 2014), aurora A is essential for the spindle assembly checkpoint (De Souza et al., 2017) and the polo-like



**Fig. 1. Observation of sMTOC activities during mitosis and localization patterns of PlkA.** (A) The activities of sMTOCs are regulated during the cell cycle in *A. nidulans*. Observation of two hyphal compartments. Whereas the lower one performs mitosis, the upper right one is in interphase. sMTOCs are actively polymerizing MTs from the septum towards the cytoplasm in the interphase compartment. The MT plus-end tracking protein GFP-KipA was followed in a strain where MTs were labeled with mCherry. Strain SXW1 [*alcA(p)::GFP::kipA*; *alcA(p)::mCherry::tubA*] was grown in 8-well u-slides at 28°C overnight for long-term observation. Time-lapse images were taken every 5 s at room temperature. Arrows point to septa. The numbers indicate the time in seconds. The asterisks indicate the MT plus ends. (B,C) Localization of PlkA at SPBs and sMTOCs throughout the cell cycle. (B) Localization of PlkA to nuclei and SPBs at G2 and to mitotic spindles and SPBs at metaphase. The arrowhead indicates SPBs. Strains SXL158 [*alcA(p)::mCherry::plkA*] and SXL176 [*alcA(p)::mCherry::plkA*; *alcA(p)::GFP::tubA*] were incubated in MM (2% glycerol) at 28°C overnight and observed. (C) PlkA remains at septa during mitosis (arrows). Strain SXL158 or SXL176 was used. (D) PlkA localizes to the forming septum. Arrow indicates the position of a forming septum. Strain SXL176 were treated and imaged as above. Images are representative from three independent experiments. Scale bars: 2  $\mu$ m.

kinase PlkA is important, but not essential, for hyphal development (Mogilevsky et al., 2012). NIMA and Aurora A transiently localize at mitotic spindle zones and relocate to septa during specific phases, while PlkA is associated with SPBs throughout the whole cell cycle, with septal localization unknown (Bachewich et al., 2005; De Souza et al., 2017; Shen et al., 2014). In order to test which kinase could be involved in MTOC activity control, we performed preliminary interaction studies of the kinases with ApsB using the bimolecular fluorescence complementation (BiFC) system (data not shown and see below). Because this assay was positive for the combination of PlkA and ApsB, we focused on PlkA for further investigations.

#### **PlkA localizes at SPBs and sMTOCs throughout the cell cycle and is recruited to forming septa prior to ApsB**

To get first insights of PlkA functioning in MTOCs, we asked whether PlkA localizes at SPBs and septal MTOCs *in vivo*. PlkA was tagged with mCherry at the N-terminus, placed under the control of the *alcA*-promoter and introduced into a strain expressing

GFP-labeled TubA. mCherry-PlkA was always observed as dot-like structure at nuclei suggesting SPB association. During mitosis, both SPBs were labeled and, in addition, mitotic spindles were decorated, which is consistent with previous results (Fig. 1B) (Bachewich et al., 2005). As a new finding, PlkA was observed permanently at septa, even in mitotic cells (Fig. 1C). Thus, PlkA localizes at SPBs and sMTOCs throughout the whole cell cycle. We also generated a strain with GFP fused at the 3' end of PlkA under *alcA* promoter control and found that septal localization was lost (Fig. S1B). The C-terminus appears to be important for septal localization of PlkA. The strain did not have any obvious phenotype suggesting that the C-terminally tagged protein fulfilled its functions at SPBs. We also tried to view localization of GFP-PlkA when expressed from its own promoter, but the GFP signal was under the detection level of our microscopic setup. Nevertheless, the defects observed in the  $\Delta plkA$  deletion mutant (see below) are in perfect agreement with the observed localization pattern for the overexpressed protein.



Next, we studied the timing of PlkA recruitment to septa. PlkA localized at forming septa as a ring (Fig. 1D), but not as early as Spa10, which localized at the beginning of septation (Fig. S2A). Colocalization of ApsB and PlkA at a near-mature septum revealed that PlkA accumulated at forming septa earlier than ApsB (Fig. S2B). Since Spa10 acts as an anchor for sMTOCs (Zhang et al., 2017), we further checked whether localization of Spa10 was affected by PlkA. Spa10 signals at septa appeared to be normal in a  $\Delta plkA$  mutant (Fig. S2C).

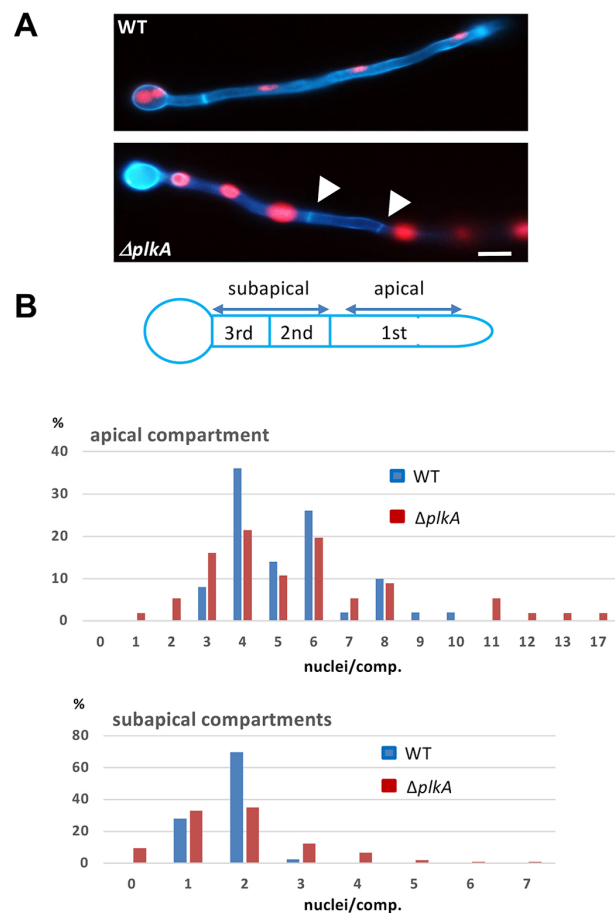
### PlkA is involved in polar growth, septation and nuclear distribution

In an early study *plkA* was suggested to be essential (Bachewich et al., 2005), but later it was shown that deletion was not lethal but caused a severe growth phenotype and affected nuclear distribution (Mogilevsky et al., 2012). In this study, we created a  $\Delta plkA$  deletion mutant via homologous recombination and compared the phenotype with *apsB* and *mztA* mutants. Colonies of the  $\Delta plkA$  deletion strain were very small and produced very few conidia, as was described before (Mogilevsky et al., 2012). The growth and sporulation phenotypes were more severe than in the *apsB* or the *mztA* mutants (Fig. S3A, left panel). All phenotypes were rescued by re-introducing an intact copy of the *plkA* open-reading frame (Fig. S3A, right panel). Surprisingly, more septa were observed in hyphae of the  $\Delta plkA$  deletion mutant than in wild type (WT), suggesting a role for PlkA in controlling septation (Fig. 2A). In agreement with this, overexpression of PlkA blocked septation (Bachewich et al., 2005). However, the effect is rather minor, as described before (Mogilevsky et al., 2012), which is in contrast to what is seen in other organisms, like *S. pombe*, where Plo1 is essential for septation (Mulvihill and Hyams, 2002; Tanaka et al., 2001). Nuclear distribution in the  $\Delta plkA$  mutant was abnormal with some anucleate subapical compartments (Fig. 2A). The variation of the number of nuclei in each compartment varied much more than in WT (Fig. 2B).

### PlkA colocalizes and interacts with the outer plaque receptor ApsB

Because ApsB was previously shown to play a role in nuclear distribution as well as in astral MT formation (Suelmann et al., 1998; Zhang et al., 2017), and because both proteins localize to MTOCs, we asked whether PlkA interacts with ApsB. First, we tested colocalization of the two proteins. We observed overlapping signals of mCherry–PlkA and GFP–ApsB at SPBs and mature septa both in interphase and during mitosis (Fig. 3A). Using the BiFC system, we tested for protein–protein interaction and detected fluorescent signals at interphase and mitotic SPBs and at mature septa (Fig. 3B). The interaction between PlkA and ApsB was confirmed via yeast two-hybrid (Y2H) analyses with the two combinations AD–PlkA with BD–ApsB, and BD–PlkA with AD–ApsB (Fig. 3C). In addition, we showed self-interaction of ApsB but not of PlkA. Thus, PlkA directly interacts with ApsB, suggesting it functions through regulating ApsB.

Owing to the physical association of PlkA and ApsB, we next asked whether PlkA plays a role in recruiting ApsB to MTOC sites. We introduced GFP–ApsB into a  $\Delta plkA$  deletion strain and compared the GFP signal intensities with those in a corresponding WT strain. GFP–ApsB signals were largely reduced at both SPBs and sMTOCs in the  $\Delta plkA$  strain (Fig. 4A, left panels). Quantification of signal intensities revealed significant reduction of the signals ( $P < 0.0001$ ) to only one-third of that in WT (Fig. 4A, right panel). However, weak ApsB signals were still detected at



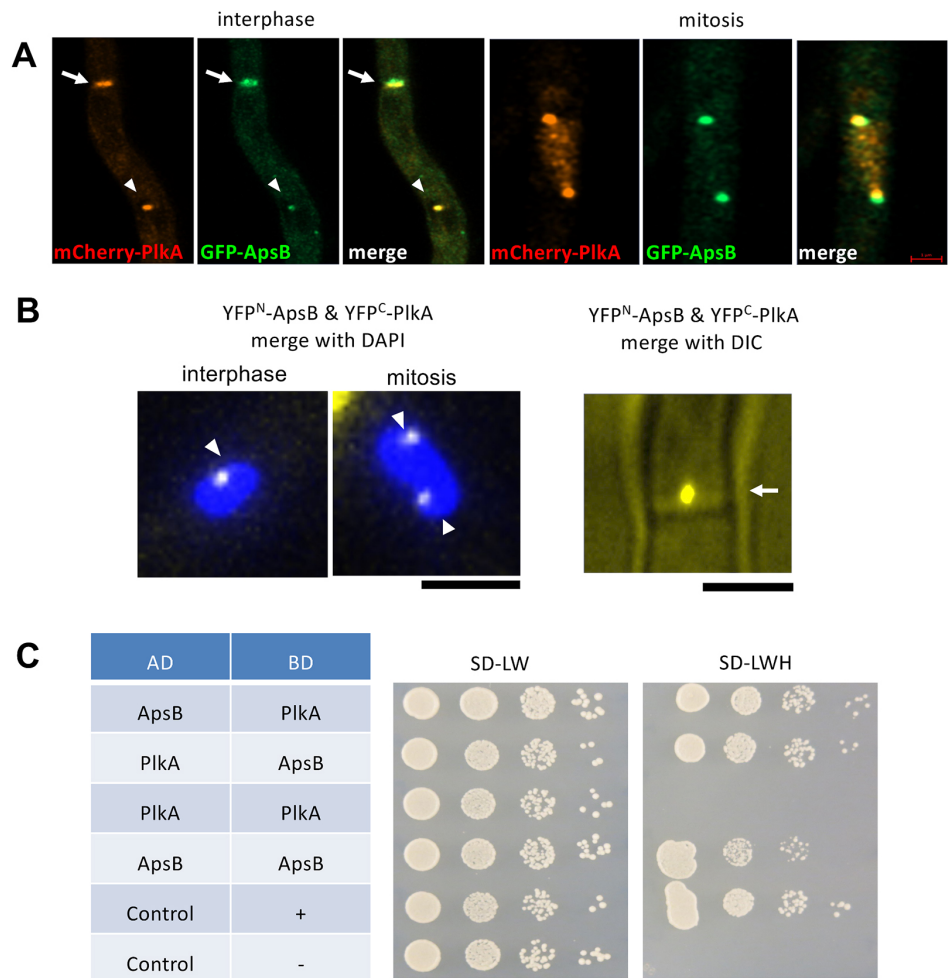
**Fig. 2. PlkA is involved in septation and nuclear distribution.** (A) Cell walls of WT and a  $\Delta plkA$  deletion strain were stained with Calcofluor White and nuclei visualized through mRFP–H1 expression. Arrowheads indicate two septa of a compartment without any nuclei. (B) Quantification of the percentage of nuclei per compartment in WT and the  $\Delta plkA$  deletion strain in apical ( $n=50$  and  $56$ , respectively) and subapical regions ( $n=79$  and  $106$ ) as defined in the scheme.

SPBs and at sMTOCs. The localization of PlkA appeared to be unaffected in the absence of ApsB (Fig. S3B). In the same way, we analyzed the concentration of two further  $\gamma$ -TuRC components, GcpC and MztA. Whereas GcpC is a component of the outer and the inner plaque, MztA is restricted to the inner plaque (Gao et al., 2019). As for ApsB, the concentration of GcpC and MztA at SPBs was largely reduced and, at septa, no signals were observed in the mutant (Fig. 4B,C). Because GcpC and MztA appeared to be absent at sMTOCs in the  $\Delta plkA$  mutant, we analyzed the sMTOC activity by observing the MT emergence from septa. In WT, MTs were nucleated from septa, whereas in the  $\Delta plkA$  mutant no MTs originated from septa, with MTs coming only occasionally from opposite directions (Movies 1 and 2). Hence PlkA improves recruitment of ApsB to both MTOCs and is essential for sMTOC activities. The effect of PlkA on MztA localization suggests that it has an additional role at the inner plaque.

### PlkA interacts with the inner plaque receptor PcpA

In order to investigate a role of PlkA at the inner plaque of SPBs, we first examined colocalization of PlkA and PcpA. Expressing the



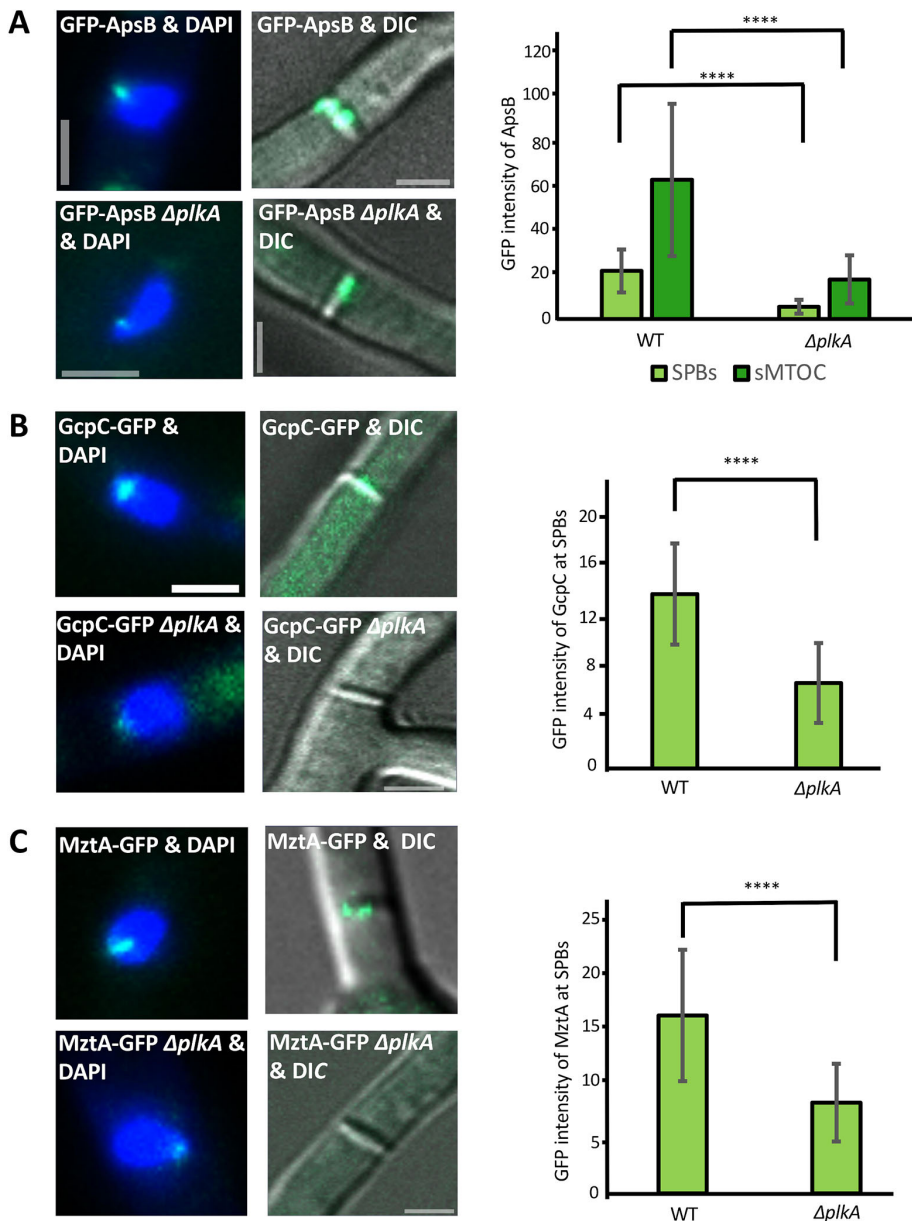


**Fig. 3. PlkA colocalizes and interacts with the outer plaque receptor ApsB.** (A) PlkA colocalizes with ApsB at sMTOCs and SPBs both in interphase and during mitosis. Strain SXL174 [*alcA(p)::GFP::apsB*; *alcA(p)::mCherry::plkA*] was incubated in MM (2% glycerol) at 28°C overnight. Arrows indicate septum positions and arrowheads indicate SPB positions. Pictures were taken with the LSM 980 Airyscan microscope. Scale bar: 1 µm. (B) PlkA interacts with ApsB at SPB and sMTOCs revealed with bimolecular fluorescence complementation (BiFC). Strain SXL124 [*alcA(p)::YFP<sup>N</sup>::apsB*; *alcA(p)::YFP<sup>C</sup>::plkA*] was incubated in MM (2% glycerol) at 28°C overnight and imaged. Nuclei were stained with DAPI. Arrowheads indicate YFP signals at SPBs and the arrow points to the YFP signal at a sMTOC. Scale bar: 2 µm. (C) Interaction of PlkA and ApsB confirmed with Y2H *S. cerevisiae* strain Gal4 AD–ApsB with BD–PlkA and AD–PlkA with BD–ApsB. Self-interaction of ApsB and PlkA were investigated with AD–ApsB with BD–ApsB, and AD–PlkA with BD–PlkA strains. Positive and negative controls were included according to the Matchmaker™ Gold yeast two-hybrid system by Clontech. Dilution series of respective strains were grown on SD–LW (selective dropout leucine and tryptophan) and SD–LWH (selective dropout leucine, tryptophan, histidine) agar plates at 30°C for 3 days. Images are representative from three independent experiments.

combination of the two tagged proteins appears to be difficult, because we were unable to generate a stable strain either by transformation or by crossing. Therefore, colocalization could only be observed in a heterokaryon (Fig. 5A). However, the results were confirmed by showing the interaction of the two proteins during interphase and during mitosis in the BiFC assay (Fig. 5B). For the combination of MztA and PlkA, no positive signals were observed (data not shown). PcpA, together with its orthologs (Spc110 in budding yeast and Pcp1 in fission yeast), belongs to the SPM-CM1-PACT receptor family (Chen et al., 2012; Lin et al., 2014; Zhang et al., 2017). The N-terminal SPM-CM1 motif defines the γ-TuRC-interacting motif, and the C-terminal PACT domain defines a MTOC-targeting motif. The central coiled-coil region of PcpA is composed of the Smc domain, which is involved in cell cycle control, cell division and chromosome partitioning. Y2H assays

revealed that PlkA interacts with the Smc domain of PcpA in the one combination of AD–PcpA-Smc with BD–PlkA. The other combination, AD–PlkA with BD–PcpA-Smc was negative, and did not result in yeast growth, which could be due to mis-folding of the proteins. No interaction was detected between GcpC and PlkA (Fig. 5C).

**PlkA is required for mitotic progression and acts by affecting chromosome condensation**  
Since PlkA interacted with both receptor proteins ApsB and PcpA, we studied a role in mitosis progression and chromosome segregation. Initial experiments had already shown that *plkA* deletion does have some effect on mitotic progression (Bachewich et al., 2005; Mogilevsky et al., 2012). Therefore, we aimed at analyzing the effect by high-resolution microscopy, and labeled

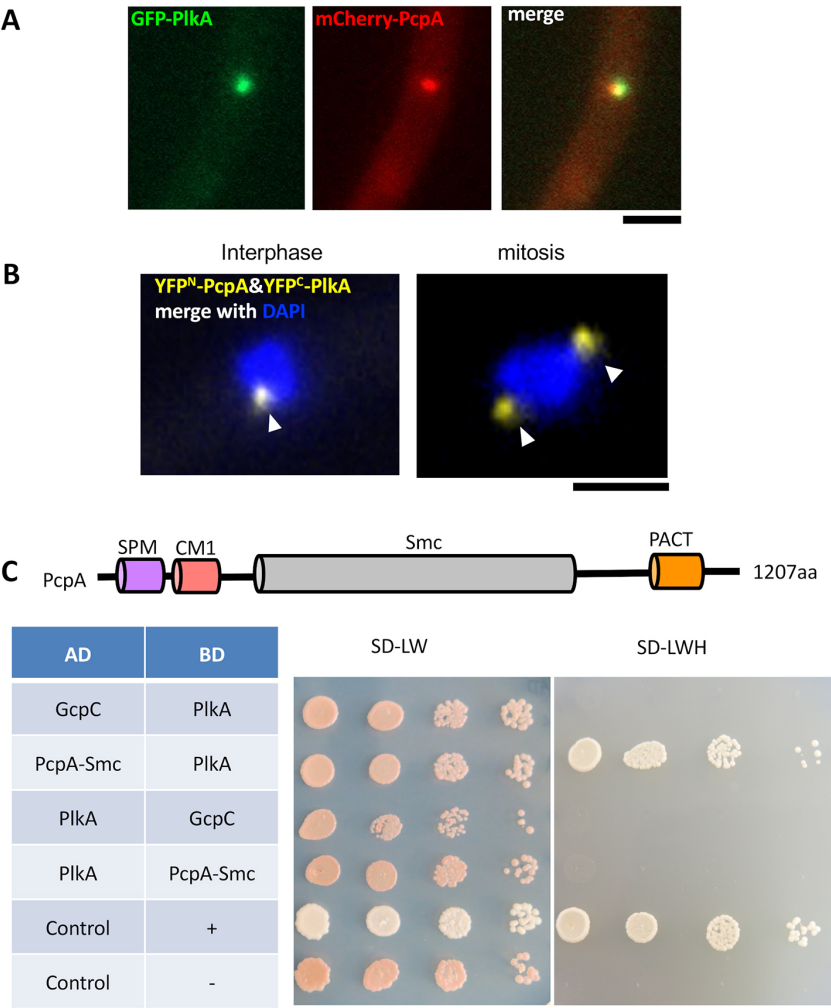


**Fig. 4. ApsB, GcpC and MztA localization to SPBs and sMTOCs depend on PlkA.** (A) Analysis of the role of PlkA for ApsB recruitment to SPBs and sMTOCs. Strains SY22 [*alcA(p)::GFP::apsB*] and SXL135 [ $\Delta plkA$ ; *alcA(p)::GFP::apsB*] were incubated in MM (2% glycerol) at 28°C overnight and imaged. Nuclei were stained with DAPI. Scale bars: 2  $\mu$ m. Images of 10–15 sections were taken along the Z-axis at 0.25- $\mu$ m increments. Maximum projection images were obtained and maximum fluorescence intensities over the background were used for statistical analysis. The exposure time and shutter level were kept identical. 40–50 SPBs and 35–40 septa were checked for each strain. (B) Analysis of the role of PlkA for GcpC recruitment to SPBs and sMTOCs. Strains were SNZ-SH84 (*gcpC::GFP*), SXL131 ( $\Delta plkA$ ; *gcpC::GFP*). Growth and imaging were performed as in A. No GFP signal was observed at septa in the  $\Delta plkA$  deletion strain. 50–65 SPBs were analyzed for each strain. (C) Analysis of the role of PlkA for MztA recruitment. Strains were SMS4 (*mztA::GFP*) and SXL129 ( $\Delta plkA$ ; *mztA::GFP*). Growth and imaging were performed as in A. At septa no GFP signal was observed in the  $\Delta plkA$  deletion strain. 41–47 SPBs were analyzed. Error bars show the s.d. \*\*\*\* $P < 0.0001$  compared to WT (Mann–Whitney U test was performed with GraphPad Prism 8).

MTs with GFP-TubA and the chromosomes with mRFP-H1 (histone) in WT and the  $\Delta plkA$  deletion strain to simultaneously visualize mitotic spindles and chromosomes. Time-lapse analyses of mitosis revealed that mitosis was delayed in the mutant as compared to WT, and chromosomes appeared to take longer to condense early during mitosis (Fig. 6A; Movies 3 and 4). We also noticed that the GFP signal intensity in mitotic spindles in the  $\Delta plkA$  deletion strain is reduced in comparison to that in WT (Fig. 6B). PlkA appears to be involved in ensuring adequate numbers of spindle MTs, probably through interacting with PcpA. However, MT numbers may also be determined by other MT dynamics parameters (Szewczyk and Oakley, 2011). However, we did not find significant differences in the polymerization, pausing or depolymerization kinetics between WT and mutant (Fig. 6C,D; Movies 5 and 6).

#### Kinase activity of PlkA is required for recruitment of ApsB to MTOC sites and astral MT formation but not for spindle morphology

Since PlkA interacts and helps to recruit ApsB to SPBs and sMTOCs, we further asked whether recruitment of ApsB requires the kinase activity of PlkA. To achieve this, we generated a kinase-dead form of PlkA by mutating a conserved catalytic lysine 95 (K) to arginine (R) which should render the protein inactive (PlkA<sup>95K/R</sup>; Fig. 7A) (Berlanga et al., 1998). Whether the modified protein lacks all kinase activity, or whether the activity is only reduced, was not tested. Endogenous PlkA was N-terminally labeled with mCherry and the mutation introduced by homologous integration. mCherry allowed us to analyze the localization of the mutated protein. PlkA<sup>95K/R</sup> localization at SPBs and sMTOCs appeared not to be affected by the point mutation (Fig. 7B, left),



**Fig. 5. PlkA interacts with the inner plaque receptor PcpA.** (A) PlkA colocalizes with PcpA at SPBs. Strain SSH172 [*alcA(p)::GFP::plkA*; *alcA(p)::mCherry::pcpA*] is a heterokaryon obtained after the fusion of strains SXL137 and SXL80. Scale bar: 2  $\mu$ m. (B) Interaction analysis of PlkA and PcpA using bimolecular fluorescence complementation (BiFC). Strain SXW7 [*alcA(p)::YFP<sup>N</sup>::pcpA*; *alcA(p)::YFP<sup>C</sup>::plkA*] was incubated in MM (2% glycerol) at 28°C overnight and imaged. Nuclei were stained with DAPI. The arrowheads mark the SPB positions during interphase and mitosis. Scale bar: 2  $\mu$ m. (C) Interaction of PlkA and PcpA confirmed with Y2H analysis. The domains of the protein are indicated in the scheme above the experimental data. The Smc domain was analyzed in the Y2H. *S. cerevisiae* strain Gal4 AD–PcpA<sup>Smc</sup> with BD–PlkA showed positive interaction while neither AD–GcpC with BD–PlkA nor AD–PlkA with BD–GcpC were positive. Positive and negative controls were included according to the Matchmaker™ Gold Yeast Two-Hybrid System by Clontech. Dilution series of respective strains were grown on SD–LW (selective dropout leucine and tryptophan) and SD–LWH (selective dropout leucine, tryptophan, histidine) agar plates at 30°C for 3 days. Images are representative from three independent experiments.

indicating that the kinase activity is not required for correct localization. This is consistent with the notion that localization of polo-like kinases can be attributed to the PBD domain. We then introduced GFP–ApsB into mCherry–PlkA<sup>95K/R</sup> and compared the signal intensities with those of the WT mCherry–PlkA. In mitotic spindles, we observed an obvious reduction of GFP–ApsB signals at both spindle poles in the catalytic mutant (Fig. 7B, right). GFP–ApsB signals at septa and at SPBs were also reduced during interphase (Fig. S4A).

In WT, mitotic spindles are very compact and condensed during metaphase, and abnormal spindles are very rare. In our experiment, all spindles appeared normal in wild type ( $n=152$ ), whereas the deletion mutant showed ~20% and the catalytic mutant ~4% abnormal spindles (defined as thin, fusiform, D-form or curved) (Fig. 7C,D). The spindle morphology phenotype in the  $\Delta plkA$  deletion strain is in agreement with previous findings (Mogilevsky et al., 2012). The fact that the deletion mutant displayed a more-severe phenotype than the catalytic mutant, suggests that there are additional non-catalytic roles of PlkA.

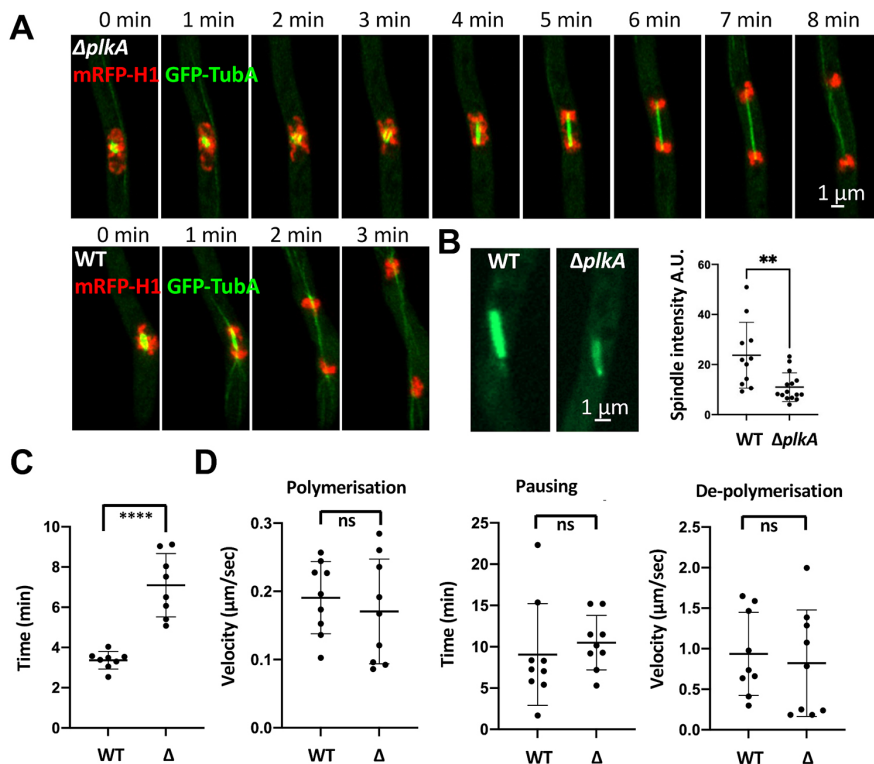
Because ApsB is required for astral MT formation during anaphase, and the fact that PlkA kinase activity supports ApsB recruitment to SPBs, we asked whether the catalytic activity is required for astral MT formation. Nearly 95% of the nuclei in WT

nucleated more than three astral MTs at anaphase as compared to 4% in the  $\Delta plkA$  deletion or the catalytic  $plkA$  mutant strain (Fig. 7E). Thus, we suggest that PlkA regulates astral MT formation via recruiting ApsB to the outer plaques of SPBs in a phosphorylation-dependent manner. Furthermore, PlkA plays additional roles in spindle morphology that are independent of its phosphorylation activity.

**PlkA functions as a kinase and as a scaffolding protein**

To gain insights of the role of the catalytic activity of PlkA in vegetative growth, we compared the phenotypes of a  $\Delta plkA$  deletion strain with those of the deletion strain expressing mCherry–PlkA or mCherry–PlkA<sup>95K/R</sup>. Both constructs were driven by the *alcA* promoter, which is repressed during growth on glucose and de-repressed during growth on glycerol. Compact colonies were observed with the  $\Delta plkA$  deletion strain and the  $plkA^{95K/R}$  mutant under repressing conditions (Fig. S4B), indicating that kinase function is important for polar growth. The fact that the deletion mutant displayed a more-severe phenotype shows that the *alcA* promoter is not 100% repressed on agar plates with 2% glucose. However, with the low expression levels, we noticed that sporulation in the  $plkA^{95K/R}$  mutant colonies appeared higher than that in the  $\Delta plkA$  deletion strain when grown on glucose (Fig. S4B).





**Fig. 6. The role of PlkA in mitosis.**

(A) Visualization of mitosis in WT and a  $\Delta plkA$  deletion strain using GFP-TubA (green) and mRFP-H1 (red). Pictures were taken with the LSM 980 Airyscan microscope. (B) Quantification (A.U., arbitrary units) of mitotic spindle intensities in WT ( $n=15$ ) and the  $\Delta plkA$  deletion strain ( $n=11$ ). Images were taken as Z-stack with maximum projection. The intensities of the spindle area were multiplied by the size and the cytosolic background subtracted. Scale bar: 1  $\mu m$ . (C) The duration of mitotic events was quantified in each strain ( $n=8$ ). (D) Determination of MT dynamics ( $n=9$ ) in WT and  $\Delta plkA$  ( $\Delta$ ) strains. Error bars are s.d. \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ ; ns, not significant compared to WT (Mann-Whitney U test was performed with GraphPad Prism 8).

Therefore, we quantified the number of conidia produced in each strain ( $n=16$ ) and found for WT the number was  $30 \times 10^6/cm^2$ , for the  $plkA^{95K/R}$  mutant this was  $41 \times 10^6/cm^2$  and this was only  $1.8 \times 10^6/cm^2$  for the  $\Delta plkA$  deletion strain. These results indicate that there are additional roles of PlkA besides the catalytic kinase activity.

To identify putative interacting partners of PlkA and phosphorylation sites in ApsB, we performed pulldown experiments using the GFP-trap technology followed by mass spectrometry. Western blot analysis of the precipitate showed successful pulldown of endogenous PlkA and ApsB (data not shown). Using mass spectrometry, we found all tubulin proteins. TubA and TubB ( $\alpha$ -tubulins), BenA, TubC ( $\beta$ -tubulins) were among the putative binding partners for PlkA (Fig. S5A, Table S1). In combination with the fact that PlkA localizes along mitotic spindles, this may explain the abnormal morphology of spindles in the absence of PlkA.

Mass spectrometry of pulled-down ApsB revealed three phosphorylation sites in two peptides, two of them prior to the CM motif and one at S1414 located in front of the C-terminal MASC domain (Fig. S5). For details for identifying the sites see Figs S5–S7. However, in the  $\Delta plkA$  deletion mutant, those phosphorylated peptides were not detected. Because of the incomplete coverage of ApsB with the identified peptides, currently we cannot exclude there could be additional phosphosites. To gain insights into the function of the phosphorylation sites, we mutated the three serine residues into alanine residues (non-phosphorylatable) or aspartic acid (a phosphomimetic) individually. However, ApsB protein with single changes of either serine to alanine (S/A) or aspartic acid (S/D) rescued all the  $\Delta apsB$  deletion phenotypes (Fig. S8A), and the localization of ApsB to SPBs and sMTOCs appeared not to be affected. We then created an ApsB allele with a triple mutation of the three serine residues to alanine or

aspartic acid (denoted AAA or DDD) and introduced it into the  $\Delta apsB$  deletion strain. The sporulation deficiency of the  $\Delta apsB$  deletion strain was not fully rescued indicating that those sites are important for development (Fig. S8B). Mitotic spindle morphology or functioning was not affected in the strains, suggesting additional residues are required in this process (Fig. S8C).

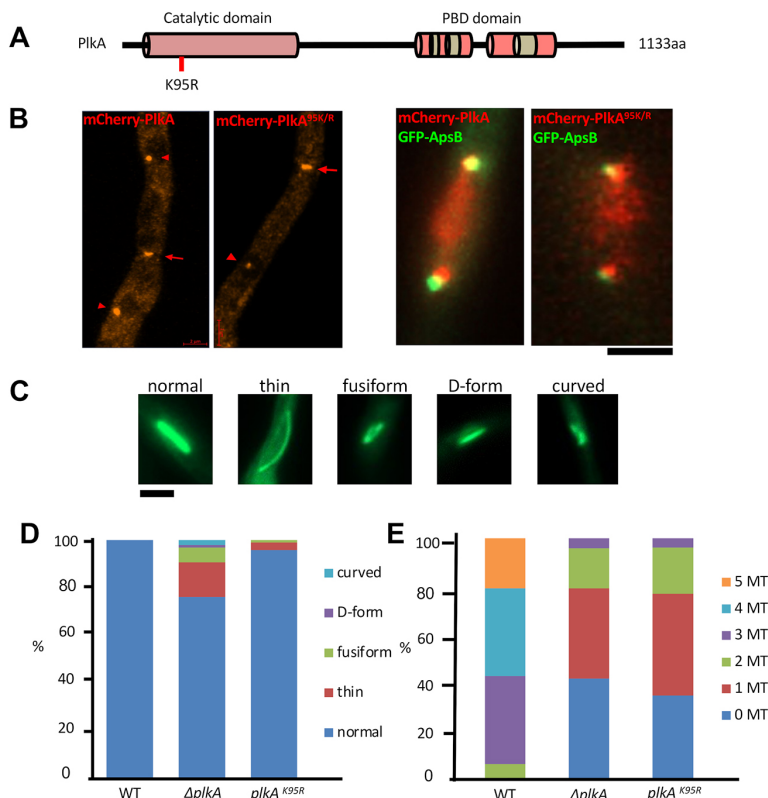
Taken together, we show that PlkA interacts and recruits ApsB in a phosphorylation-dependent manner, which is important for astral MT formation and sMTOC activities. In addition, PlkA interacts with the inner plaque receptor PcpA and mitotic spindles to stabilize the mitotic spindles, which function as a scaffolding unit inside the nuclei.

## DISCUSSION

The study of the role of PlkA in MTOC functioning in *A. nidulans* revealed several interesting aspects. Our results suggest that PlkA is involved in the coordination of the activity of SPB outer plaques and septal MTOCs (Fig. 8). In addition, the protein is required at the inner plaque of the SPB. However, unlike what is found in other organisms, PlkA is not essential. This is even more surprising in the light that PlkA is the only polo-like kinase in *A. nidulans*. Besides its catalytic function as a kinase, PlkA appears to fulfill scaffold functions. This could be an ancient function, because the catalytic and the non-catalytic functions appear to have diverged during evolution.

### PlkA of *A. nidulans* serves catalytic and non-catalytic functions

We generated a  $plkA$  gene deletion strain and a strain with a PlkA kinase-dead version. They both were impaired in MT formation from SPB outer plaques and sMTOCs. This can be explained through the action on the receptor protein, ApsB, which needs to be



**Fig. 7. Effects of the replacement of PlkA lysine 95 with arginine on ApsB recruitment to SPBs, on spindle morphology and on astral MT formation.** (A,B) Analysis of the catalytic role of PlkA in ApsB recruitment to MTOCs. (A) Scheme of the domain organization of PlkA and indication of the critical lysine residue 95. (B) Mutation of the catalytic residue PlkA<sup>K95K/R</sup> does not affect PlkA localization but reduces ApsB recruitment to mitotic spindles. Strains SXL158 [*alcA(p)::mCherry::plkA*], SXL159 [*alcA(p)::mCherry::plkA<sup>K95K/R</sup>*], SXL174 [*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA*] and SXL175 [*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA<sup>K95K/R</sup>*] were incubated in MM (2% glycerol) at 28°C overnight and imaged. Left, the arrows indicate the positions of septa and the arrowheads indicate the positions of SPBs. Right, merged images of mCherry-PlkA with GFP-ApsB and mCherry<sup>K95R</sup> with GFP-ApsB at the mitotic SPBs. Pictures were taken with the LSM 980 Airyscan microscope. Images are representative from at least three independent experiments. Scale bar: 2 μm. (C) Shape of mitotic spindles in WT, the  $\Delta plkA$  deletion and the catalytic mutant. Strains SXL158 [*alcA(p)::mCherry::plkA*], SXL159 [*alcA(p)::mCherry::plkA<sup>K95K/R</sup>*], SXL174 [*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA*] and SXL175 [*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA<sup>K95K/R</sup>*] were incubated in MM (2% glycerol) at 28°C overnight and imaged. Left, the arrows indicate the positions of septa and the arrowheads indicate the positions of SPBs. Right, merged images of mCherry-PlkA with GFP-ApsB and mCherry<sup>K95R</sup> with GFP-ApsB at the mitotic SPBs. Pictures were taken with the LSM 980 Airyscan microscope. Images are representative from at least three independent experiments. Scale bar: 2 μm. (D) Quantification of the spindle shapes. 152 spindles in WT, 74 spindles in the  $\Delta plkA$  deletion and 92 spindles in the  $plkA^{K95R}$  mutant strain were counted. (E) Quantification of astral MTs. The same strains as in C were grown in 8-well u-slides at 28°C overnight. 19 anaphase spindles in WT, 24 in the  $\Delta plkA$ -deletion and 26 in the  $plkA^{K95R}$ -mutant strain were analyzed.

phosphorylated by PlkA in order to recruit the  $\gamma$ -TuSC to the outer plaque of the SPB and the  $\gamma$ -TuRCs to the sMTOCs (Fig. 8). PlkA appears to be dispensable for mitotic spindle formation. However, whereas spindle morphology was affected in the  $\Delta plkA$  deletion mutant, it was normal in the kinase-dead mutant strain. Given the fact that PlkA interacts with the inner plaque receptor PcpA, it might facilitate the recruitment of  $\gamma$ -TuRCs to inner plaques, thereby ensuring adequate numbers of spindle MTs during mitosis (Fig. 8). In addition, PlkA localized inside the mitotic spindle and interacted with tubulins (Fig. 1B; Fig. S5A). This could be important for proper spindle formation and morphology. We noticed that without PlkA, chromosomes did not condense as fast as in WT. However, further studies are required to unravel the specific function of PlkA in this process. The localization and functioning of PlkA at septal MTOCs define a novel function for a polo-like kinase at non-centrosomal MTOCs. Thus, we propose that PlkA in *A. nidulans* plays dual roles as a kinase at the outer plaques of SPBs and sMTOCs, and as a scaffolding protein inside mitotic nuclei (Fig. 8). There is already some evidence for non-catalytic roles for Plk2, Plk4 and Plk5. In human, Plk5 is an inactive kinase and Plk2 has synaptic functions that are independent of its kinase activity (de Cárcer et al., 2011; Evers et al., 2010). Furthermore, *Caenorhabditis elegans* ZYG-1 (the homolog of Plk4) has been shown to have a kinase-independent function in SAS-6 recruitment to the mother centriole (Lettman et al., 2013). *A. nidulans* PlkA hence could be an ancient protein combining the function of several Plks, which were separated during evolution to different proteins. Likewise, phylogenetic analysis placed PlkA closest to Plk4 (Mogilevsky et al., 2012).

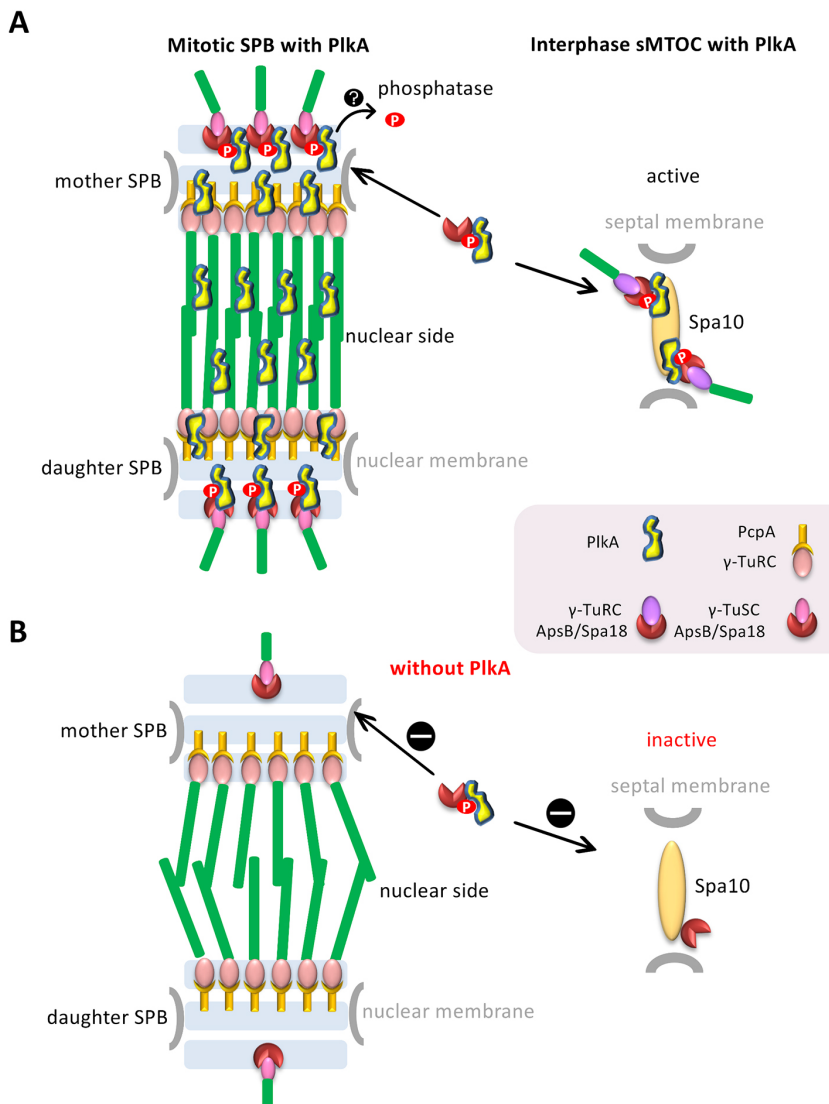
There is a second line of evidence that PlkA serves functions independently of the kinase activity. We observed that a kinase-dead version of PlkA was sufficient to allow sporulation, whereas in the

absence of PlkA sporulation was strongly impaired. The exact role of the PlkA protein during sporulation presently remains unknown.

The next question concerns the targeting mechanism of PlkA. In Cdc5/Plk1 the Polo box domain (PBD) domain, specifically a phosphopeptide-binding motif inside the PBD domain, is essential for subcellular localization and function (Lee et al., 1998; Song et al., 2000). The PBD domain binds to a phosphopeptide in a so-called priming substrate (Elia et al., 2003). In addition, the PBD domain in Plk1 regulates its kinase activity via an auto-inhibitory mechanism (Cheng et al., 2003; Jang et al., 2002). Although the PBD domain of *A. nidulans* PlkA is divergent from Plk1, it contains the conserved residues that are important for phospho-peptide recognition (Mogilevsky et al., 2012). Other studies have revealed that Plk1 can bind also to unphosphorylated substrates (García-Álvarez et al., 2007). Therefore, it is possible that both the phosphopeptide recognition mechanism and another binding mechanism regulate PlkA localization. Although, the priming substrate and its upstream kinase are not known yet for PlkA, we can exclude ApsB as the priming substrate, because PlkA also localized to MTOCs in the absence of ApsB.

#### The conserved and divergent roles of PlkA compared to other Plk1 proteins

In this study, we present evidence that PlkA binds and either indirectly or directly phosphorylates the  $\gamma$ -TuCR protein ApsB at the outer plaque of SPBs and sMTOCs, which are essential for astral MT (mitosis) and septal MT (interphase) nucleation. In addition, PlkA binds to the inner plaque receptor PcpA, which is indispensable for mitotic spindle assembly. In humans, Plk1 is essential for driving centrosome maturation through phosphorylating the PCM protein pericentrin during mitosis (Lee and Rhee, 2011).



**Fig. 8. Proposed model of PlkA in regulating mitotic SPBs and sMTOC activities in *A. nidulans*.** PlkA coordinates the activity of SPB outer plaques and the sMTOC activity via phosphorylating and recruiting ApsB, thus promoting astral and septal MT formation. PlkA also binds to PcpA at SPB inner plaques and interacts with spindle microtubules as a scaffolding unit. For further explanation, see the Discussion.

Similarly, in *D. melanogaster*, Polo (the Plk1 homolog) initiates the phosphorylation-dependent assembly of a Cnn (the CDK5RAP2 homolog) scaffold that is essential for centrosome maturation (Conduit et al., 2014). *A. nidulans* PlkA shares its roles in phosphorylating PCM-related components at centrosomes/SPBs to increase MT formation with metazoan Plk1. However, at the inner plaque, it only binds to PcpA and guarantees morphologically and functionally normal mitotic spindles, probably without phosphorylation of the binding partner. Mitotic spindles, although often misshapen and probably containing less MTs than in WT, were produced in the PlkA kinase-dead and in the  $\Delta plkA$  deletion strain. Furthermore, we present a novel function for the polo-like kinase PlkA in regulating non-centrosomal MTOC activities.

Another difference concerns the essential roles of other Plk1 orthologs for mitotic entry and cytokinesis. In *S. cerevisiae* and in *S. pombe*, Cdc5/Plp1 function as essential upstream regulators of the MEN network and the SIN networks (Song and Lee, 2001; Tanaka et al., 2001). Both, mitotic entry and cytokinesis appear to occur in

the  $\Delta plkA$  deletion strain. By contrast, NIMA kinase in *A. nidulans* is essential for mitotic entry and its degradation is required for mitotic exit (Osmani et al., 1991; Pu and Osmani, 1995).

Although Plks are traditionally implicated in cell cycle progression, there is increasing evidence for roles of Plks outside cell division, especially for Polo, Plk2 and Plk3 functioning in meiosis, asymmetric cell fate determination and synaptic plasticity (Archambault and Glover, 2009). As mentioned before, the *A. nidulans* PlkA sequence is related to that of Plk4. Plk4 plays a role in centriole-independent MTOC formation, and promotes MT nucleation in the acentriolar mouse embryo (Coelho et al., 2013). The non-centrosomal functions of PlkA may be attributed to this similarity with Plk4.

## MATERIALS AND METHODS

### Strains, plasmids and culture conditions

Supplemented minimal (MM) and complete medium (YAG) for *A. nidulans* were prepared as previously described, and standard strain construction procedures were used (Hill and Käfer, 2001). Expression of tagged genes



under the control of the *alcA* promoter was regulated by the carbon source with repression on 2% glucose and de-repression on glycerol (2%) (Waring et al., 1989). Lists of *A. nidulans* strains and yeast strains used in this study are given in Tables S2 and S3. Standard laboratory *Escherichia coli* strains (Top 10 F<sup>+</sup>) were used. Plasmids are listed in Table S4.

### Molecular techniques

Standard DNA transformation procedures were used for *A. nidulans* (Yelton et al., 1984) and *Escherichia coli* (Sambrook and Russell, 1999). For PCR experiments, standard protocols were applied using a Biometra Personal Cycler (Biometra, Göttingen, Germany) for the reaction cycles. Phusion polymerase was used (expressed in *E. coli* and purified). Denaturation was achieved at 98°C, annealing temperatures were chosen according to the corresponding DNA oligonucleotides, and the polymerization temperature was 72°C. All DNA oligonucleotides used in this study are listed in Table S5. DNA sequencing was performed commercially (MWG Biotech, Ebersberg, Germany). Total DNA was extracted from *A. nidulans* according to Zekert et al. (2010).

### Construction of the $\Delta$ *plkA* deletion strain and re-complementation

The  $\Delta$ *plkA* deletion strain was created by protoplast transformation and homologous integration of a fusion PCR-derived knockout cassette. The flanking regions of *plkA* were amplified by PCR with genomic DNA as template. Primer pair *plkA*-LB\_fwd/*plkA*-LB-pyruA linker\_rev (Table S5) was used for the upstream region of *plkA* and *plkA*-RB-pyruA linker\_fwd/*plkA*-RB\_rev for the downstream region. *pyruA* was amplified with *pyruA*\_fwd/*pyruA*\_rev. Then the left border (LB), *pyruA* and right border (RB) were fused together by fusion PCR with nested primers *plkA*-LB-N\_fwd and *plkA*-RB-N\_rev. The construct was ligated into pJET1.2/blunt, yielding vector pXL79. After transformation of pXL79 to TN02A3 and selection by diagnostic PCR (Table S5; primers, *plkA*-dele\_check\_fwd/*plkA*-RB\_rev), the  $\Delta$ *plkA* deletion strain SXL122 was selected.

For re-complementation, we constructed a plasmid with the whole open reading frame of *plkA* and an auxotrophic marker *AfpyrG* and introduced it into the deletion mutant. The natural promoter of *plkA*, open reading frame (ORF), terminator and full-length *AfpyrG* was fused into pJET with NEBuilder HiFi DNA Assembly Master Mix (NEB) in one step. The ORF of *plkA* was amplified using primers: *plkA*-promo\_builder\_fwd/*plkA*-termi\_pyrG linker\_rev; and *AfpyrG* was amplified using primers *pyrG*\_fwd/*pyrG* builder\_rev. The two fragments were ligated to pJET1.2/blunt resulting in plasmid pXL81. After transformation into the  $\Delta$ *plkA* deletion strain SXL122, all phenotypes were rescued (SXL126).

### Protein tagging in the $\Delta$ *plkA* deletion strain

To check ApsB localization in a  $\Delta$ *plkA* deletion strain, we crossed the two strains SXL122 ( $\Delta$ *plkA*) and SYZ2 (GFP–ApsB) (Todd et al., 2007). After microscopic and phenotypic screening, the recombinant strain SXL135 was selected. For checking GcpC and MztA localization in the absence of PlkA, we introduced a C-terminal tagging cassette of GcpC/MztA into the  $\Delta$ *plkA* deletion strain, resulting in strains SXL129 ( $\Delta$ *plkA*; *mztA*-GFP) and SXL131 ( $\Delta$ *plkA*; *gcpC*-GFP). The homologous integration events of GcpC and MztA were confirmed with primers *pyrG* check\_fwd/*gcpC* C-termi check\_rev and *pyrG* check\_fwd/*mztA* C-termi check\_rev (Table S5).

### C-terminal tagging of PlkA with GFP under natural promoter control

PlkA was tagged with GFP at the C-terminus expressed from the natural promoter. A 1 kb region from the 3'-end of the *plkA* gene was amplified with the primer pair *PlkA*-C-termi\_fwd/*PlkA*-C-termi\_GA linker\_rev, and 1 kb of the terminator region of the gene was amplified with the primer pair *PlkA*-RB-pyrG linker\_fwd/*PlkA*-RB\_rev. The fragment of a *GFP::pyrG* cassette was amplified from pFNO3 (Yang et al., 2004) using primers GA linker\_fwd/*pyrG* overhang\_rev. Subsequently, the three fragments were fused together by fusion PCR with nested primers *PlkA*-C-termi-N\_fwd/*PlkA*-RB-N\_rev (Table S5). The construct was ligated to pJET1.2/blunt,

resulting in plasmid pXL82 (Table S4). The plasmid was transformed into TN02A3, and the strain SXL137 (Table S2), in which the construct had replaced the endogenous 3'-end of the gene, was checked with primers *pyrG* check\_fwd/*PlkA* C-termi check\_rev. The strain expresses PlkA–GFP from the natural promoter and PlkA–GFP is the only source of PlkA.

### N-terminal tagging of PlkA with mCherry under the control of the *alcA* promoter

Besides C-terminal tagging with GFP, we also created two N-terminally tagged strains. PlkA was fused with either GFP or mCherry at the N-terminus under *alcA* promoter control. N-terminally, 1.0 kb of *plkA* starting from the start codon was amplified with primers *plkA*\_AscI\_fwd and *plkA* 1kb\_PacI\_rev (*AscI* and *PacI* restriction sites are used and are in italics in Table S5), cloned into the pMCB17apx vector, resulting in pXL83 (*alcA*-mCherry-*plkA*) and pXL84 (*alcA*-GFP-*plkA*) (Table S4). The plasmids were subsequently transformed into TN02A3. After homologous recombination, this yields the N-terminally fused protein expressed under the control of the *alcA* promoter and a truncated 5'-end fused to the natural promoter. The resulting strains are SXL158 (mCherry–PlkA) and SXL164 (GFP–PlkA) (Table S2). Using primers *alcA* check\_fwd/*PlkA* 1 kb Int check\_rev (outside of the 1 kb ORF), homologous recombination was confirmed.

### Colocalization of two proteins with GFP and mCherry

To observe the sMTOC activities during mitosis, we created a strain in which the MT plus-end tracking protein KipA was tagged with GFP while mitotic spindles were labeled with mCherry. The plasmid PSK82 *pMCB17apx-GFP-kipA* was transformed into strain SSH127 (mCherry-tubA) resulting in strain SXW1.

To investigate colocalization of PlkA with ApsB, strain SYZ2 (GFP–ApsB) was crossed with strain SXL158 (mCherry–PlkA) according to protocol from Todd et al. (2007). After microscopic screening, the recombinant strain SXL174 was selected.

To investigate the timing of the recruitment of PlkA at forming septa, it was colocalized with Spa10. PlkA was tagged with mCherry at the N-terminus in a strain where Spa10 was C-terminally tagged with GFP. Strain SXL158 (mCherry–PlkA) was crossed with SRM212 (Spa10–GFP) yielding strain SXL179.

To investigate colocalization of PlkA and  $\alpha$ -tubulin TubA, the N-terminal tagging plasmid of PlkA was introduced into the GFP–TubA strain. The plasmid pXL83 *pMCB17apx-mCherry-plkA* was transformed into strain 65 resulting in strain SXL176. The homologous integration event for PlkA was confirmed with primers *alcA* check\_fwd/*plkA* 1 kb Int check\_rev (Table S5).

To investigate simultaneous localization of chromosomes and tubulin, we created a strain (SSH167) harboring mRFP–H1 and GFP–TubA by crossing CDS445 (kindly provided by Steven Osmani, Ohio State University, OH, USA) with SJW65. SSH167 was also crossed to SXL122 ( $\Delta$ *plkA*) to generate strain SSH169, which harbors mRFP–H1 and GFP–TubA in a  $\Delta$ *plkA* background.

### Generation of a GFP-labeled MT strain with a temperature-sensitive block in mitosis

To block the cells in mitosis, we combined a temperature-sensitive mutation *bimE7* with GFP labeling of TubA. The original *bimE7* mutant SO4 was crossed with SJW02, and, after temperature screening and microscopic analysis, the recombinant strain SXL136 was selected. The resulting strain allowed us to block the hyphae in mitosis at restrictive temperature of 42°C and monitor sMTOC activities.

### Bimolecular fluorescence complementation assay

To analyze the interaction of PlkA with ApsB and PlkA with PcpA, a bimolecular fluorescence complementation assay (BiFC or Split YFP) was performed. In these analyses, PlkA was tagged with YFP<sup>C</sup> at the N-terminus under the control of the *alcA* promoter while ApsB or PcpA were tagged with YFP<sup>N</sup> at the N-terminus. Similar to the generation of the GFP-tagging plasmid, 1 kb of *plkA* was amplified with primers *plkA*\_AscI\_fwd and *plkA*\_PacI\_rev, and was cloned into the pMCB17apx-YFP<sup>C</sup> vector, which

contained *YFP<sup>C</sup>* instead of *GFP*, yielding pXW8. The N-terminal YFP<sup>N</sup> tagging plasmid of ApsB (pYZ59) and PcpA (pXL58) were created previously. Then pXW8 and pYZ59 were co-transformed into TN02A3; pXW8 and pXL58 were co-transformed into TN02A3 as well. After screening, the resulting strains SXL124 and SXL125 were examined for YFP signals. All the YFP strains were checked for homologous integration events by PCR.

### Yeast two-hybrid assay

The Matchmaker™ Gold Yeast Two-Hybrid System (Clontech Laboratories, Inc.) was used in this work following the user manual. Full-length *plkA* and *gcpC* cDNA fragments were amplified by annealing three or two coding sequences (CDS) together with genomic (g)DNA as template due to the large size of cDNAs. Primer pair *plkA*-CDS1\_builder\_fwd/*plkA*-CDS1\_CDS2 linker\_rev was used for amplifying PlkA CDS1, primer pair *plkA*-CDS2\_fwd/*plkA*-CDS2\_rev for PlkA CDS2 and primer pair *plkA*-CDS3\_CDS2 linker\_fwd/*plkA*-CDS3\_builder\_rev for PlkA CDS3. Then the three CDS were fused into pJET with NEBuilder HiFi DNA Assembly Master Mix (NEB) in a one-step ligation. The resulting plasmid is pXL102 with the *plkA* exon in pJET. Then primer pair *plkA* *NdeI* AD/BD\_fwd/*plkA* *XmaI* AD/BD\_rev was used for amplifying from pXL102 as cDNA template. After digestion of the PCR products with *NdeI* and *XmaI* restriction enzymes, and respective ligation into digested pGBKT7 and pGADT7-Rec, two plasmids pXL111 (BD-PlkA) and pXL112 (AD-PlkA) were generated. GcpC plasmids were created similarly, resulting in plasmid pJA5 (AD-GcpC) and pJA6 (BD-GcpC). Primer pair *pcpA*<sup>S<sub>mc</sub></sup>*NdeI* AD/BD\_fwd/*pcpA*<sup>S<sub>mc</sub></sup>*XmaI* AD/BD\_rev was used to amplify S<sub>mc</sub> domain of PcpA with gDNA as template. After digestion, the PCR product was ligated into digested pGBKT7 and pGADT7-Rec, resulting in two plasmids pXL125 (AD-PcpA<sup>S<sub>mc</sub></sup>), pXL126 (BD-PcpA<sup>S<sub>mc</sub></sup>). The yeast strain AH109 was used for transformation of the AD vectors and strain Y187 was used for transformation of the BD vectors. The lithium chloride method was used for transformation and transformants were selected on selective synthetic dropout medium as described in the Matchmaker™ GAL4 two-hybrid system manual. The resulting transformed strains of AH109 and Y187 were then mated yielding Y2H strains used for interaction detection. All Y2H strains are listed in Table S3. To analyze the interaction of the proteins, a 5 ml overnight culture of Y2H strain in SD medium was incubated overnight at 30°C at 230 rpm. The culture was diluted to an optical density at 600 nm (OD<sub>600</sub>) of 1, as well as a dilution series (1:10, 1:100, 1:1000). Then 5 µl of each suspension (1:1–1:1000) was dropped onto the SD–LW and SD–LWH agar plates respectively with positive and negative controls. We exclude the possibility of self-activation in BD or AD vector alone via the negative interaction result between GcpC and PlkA.

### Modification of specific amino acids in PlkA and ApsB by site-directed mutagenesis

To generate a kinase-dead form of PlkA, the Q5 site-directed mutagenesis kit was used for mutating lysine 95 (K) to arginine (R) by designing a mutagenesis primer pair. Primer pair *plkA*\_95K/R\_fwd/*plkA*\_95K/R\_rev was designed with NEBase Changer tool including the annealing temperature. The template plasmid used was pXL83 (*alcA*-mCherry-*plkA*). Following the procedures in the kit, the mutated plasmid was sequenced to make sure only the targeted base pairs were mutated using primer *plkA*\_95 seq\_fwd (Table S5). The resulting plasmid was pXL114. After transformation into TN02A3, the resulting strain SXL159 (Table S2), harboring the homologous integration and mutated site, was selected after sequencing.

To generate the single mutation of ApsB phosphorylation sites, the same method was applied. The used primer pairs designed with NEBase Changer tool are *apsB*-321S/A\_fwd/*apsB*-321S/A\_rev; *apsB*-327S/A\_fwd/*apsB*-327S/A\_rev and *apsB*-1414S/A\_fwd/*apsB*-1414S/A\_rev for mutating serine sites at 321, 327 and 1414 to alanine, respectively. Primer pairs *apsB*-321S/D\_fwd/*apsB*-321S/D\_rev, *apsB*-327S/D\_fwd/*apsB*-327S/D\_rev and *apsB*-1414S/D\_fwd/*apsB*-1414S/D\_rev were used for mutating the three sites to aspartic acid, respectively (Table S5). The used template plasmid was pXL106. After sequencing with primers *apsB*-

321,327 seq\_fwd and *apsB*-1414 seq\_fwd, the resulting plasmids which harbor the respective single mutation and maintain the integrity of ORF are pXL107(1414A), pXL108(1414D), pXL109(321A), pXL110(321D), pXL117(327A) and pXL118(327D). Then the plasmids were transformed into *ΔapsB* deletion strain, resulting in strains SXL160, SXL161, SXL162, SXL163, SXL168 and SXL169, respectively (Table S2).

To generate the triple mutation of ApsB phosphorylation sites, a second round of site-directed mutagenesis was performed based on the 1414-site mutation plasmids. Primer pair *apsB*-321S/A\_327S/A\_fwd/*apsB*-321S/A\_327S/A\_rev was used for mutating both the 321 and 327 sites into alanine at the same time. Plasmid pXL107 was used as template. Primer pair *apsB*-321S/D\_327S/D\_fwd/*apsB*-321S/D\_327S/D\_rev was used for mutating the 321 and 327 sites into aspartic acids. Plasmid pXL108 was used as template. After sequencing, the resulting plasmids pXL123(AAA) and pXL124(DDD), which harbor the triple mutation, were generated. Then the plasmids were transformed into the *ΔapsB* deletion strain, resulting in strains SXL180 and SXL181, respectively (Table S2).

### Mass spectrometry after pulldown with PlkA and ApsB

Strains SXL137 (PlkA-GFP), SYZ2 (GFP-ApsB), SXL135 (*ΔplkA*; GFP-ApsB) as well as control strains only expressing GFP were incubated in 500 ml MM liquid medium (2% glycerol and 0.2% glucose) with appropriate auxotrophic markers at 37°C for 24 h. After that, the hyphae were collected, washed and performed with GFP-trap according to procedures from Chromotek (gta-20 agarose beads). PlkA and ApsB were pulled down with anti-GFP conjugated beads followed by elution with 2 × SD loading dye and detected with anti-GFP N-terminal mouse monoclonal antibody (Sigma-Aldrich) in western blots. The immunoprecipitated proteins were loaded onto an SDS-PAGE gel and just run into the separating gel. Then the polyacrylamide gels were cut into pieces and subsequently subjected to tryptic in-gel digestion (Shevchenko et al., 1996). The used trypsin was sequencing grade modified from Promega. Dried peptide pellets were stored at 4°C until liquid chromatography-mass spectrometry (LC-MS) analysis.

Peptides were purified using C18 Stop and go extraction (Stage) tips (Rappsilber et al., 2003, 2007). Dried peptides were dissolved in 20 µl sample buffer (2% acetonitrile, 0.1% formic acid) whereof 2 µl were subjected to LC-MS analysis. Peptides were separated on Acclaim PepMap RSLC columns (Thermo Fisher Scientific) at nano-flow rates (300 nl/min) by running water-acetonitrile gradients on an Ultimate 3000 LC system online coupled to either an Orbitrap Velos Pro mass spectrometer or an Q Exactive HF mass spectrometer (instruments from Thermo Fisher Scientific). Peptides were on-line ionized by nano-electrospray ionization (nESI) using the Nanospray Flex Ion Source (Thermo Fisher Scientific) at 2.4 kV (Orbitrap Velos Pro) or 1.5 kV (Q Exactive HF) and continuously transferred into the respective mass spectrometer. Orbitrap Velos Pro: Full scans within the range of 300–1850 *m/z* were recorded with the Orbitrap-FT analyzer at a resolution of 30,000. In parallel, data-dependent top-ten fragmentation spectra (MS2) were acquired by collision-induced dissociation (CID) in the linear Velos ion trap. For Q Exactive HF, full scans were taken in a mass range of 300–1650 *m/z* and recorded at a resolution of 30,000 followed by data-dependent top 10 HCD fragmentation at a resolution of 15,000. The XCalibur software versions 2.2 and 4.0 (Thermo Fisher Scientific) were used for LC-MS method programming and data acquisition. MS/MS2 raw data processing for protein identification and analysis was carried out with the Proteome Discoverer 2.4 software (Thermo Fisher Scientific) using the SequestHT and Mascot search engines. An *A. nidulans*-specific protein database was used for the database search considering a maximum of two missed cleavage sites. Oxidation at methionine, N-terminal protein acetylation and phosphorylation of serine, threonine, and tyrosine were considered as variable modifications. Carbamidomethylation at cysteine was set as fixed modification. Phospho-site localization was evaluated using phosphoRS (Olsen et al., 2006; Olsen and Mann, 2004).

### Light and fluorescence microscopy

For live-cell imaging, fresh spores were inoculated in 0.5 ml MM (2% glycerol for *alcA* promoter induction) with appropriate selection markers on

18×18 mm cover slip (Roth, Karlsruhe). The samples were incubated at 28°C overnight, followed by 2 h incubation at room temperature before microscopy. VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) was used for staining nuclei in cells. Light and fluorescence images were taken with the Zeiss Microscope “AxioImager Z1” (Carl Zeiss, Jena, Germany) and the software ZEN pro 2012, using the Planapochromatic 63× oil immersion objective lens, and the Zeiss AxioCam MR camera. Image and video processing were undertaken with ZEN pro 2012, Adobe Photoshop or ImageJ (National Institutes of Health, MD, USA).

Alternatively, for *in vivo* time-lapse microscopy, cells were incubated in u-slide 8 well glass bottom dishes from Ibidi (cells in focus) in 0.5 ml MM plus 2% glycerol and appropriate supplements, and an additional 2 ml of medium added after overnight incubation. Movies were taken at a time-lapse interval of 5 s (for activity analysis) or 1 min (for mitosis progression), and maximum projections of a deconvolved Z-stack image were applied. For quantification of fluorescence intensity, images of 15–20 sections were taken along the Z-axis at 0.25-μm increments. After deconvolution, projection images of maximum intensity were obtained, and maximum fluorescence intensities over the background intensity were used for statistical data analysis. A 5×5-pixel region of interest (ROI) was selected and two 20×20-pixel regions around the ROI were used for background subtraction.

Calcofluor White staining was performed with M2R (fluorescent brightener 28 [F3543]; Sigma-Aldrich, Munich, Germany) at a 1:1000 dilution for 5 min immediately before microscopy).

### High-resolution microscopy

For confocal imaging, samples were observed using an Axio Observer Z.1/LSM900 confocal microscope (Carl ZEISS microscopy) equipped with an oil-immersion objective (Plan Apochromat 63×/1.4; Carl ZEISS). Excitation laser wavelengths were 488 for EGFP and 561 nm for mCherry. Fluorescence signals were detected using the Airy scan detector with super resolution (SR) mode ([https://www.embl.de/services/core\\_facilities/almf/events\\_ext/2017/EN\\_wp\\_LSM-880\\_Basic-Principle-Airyscan.pdf](https://www.embl.de/services/core_facilities/almf/events_ext/2017/EN_wp_LSM-880_Basic-Principle-Airyscan.pdf)). Images were collected using the ZEN blue software (ZEISS). MT dynamics at hyphal tips were imaged for at least 1 min using the Airy scan detector in Multiplex 4Y mode.

### Image analysis

Prior to time analysis, time-lapse movies of mitotic progression were bleached corrected using the bleach correction plugin (Correction Method: Simple Ratio) of ImageJ/Fiji. To analyze MT dynamics, time-lapse movies were converted into kymographs using the ZEN blue software (Zeiss). Quantification of MT dynamics was performed in ImageJ/Fiji using the Velocity measurement tool macro ([http://dev.mri.cnr.fr/projects/imagej-macros/wiki/Velocity\\_Measurement\\_Tool#Velocity-Measurement-Tool](http://dev.mri.cnr.fr/projects/imagej-macros/wiki/Velocity_Measurement_Tool#Velocity-Measurement-Tool)).

### Statistical analysis

Significant differences were calculated using the two-tailed Mann–Whitney U test with GraphPad 8. *P*-values are denoted as \**P*<0.05; \*\**P*<0.01; \*\*\*\**P*<0.0001; ns, not significant.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: R.F., X.G.; Methodology: X.G.; Validation: O.V.; Investigation: X.G., S.H., V.W., O.V.; Resources: S.E.; Data curation: X.G.; Writing - original draft: X.G.; Writing - review & editing: R.F.; Supervision: G.H.B., R.F.; Project administration: R.F.; Funding acquisition: R.F.

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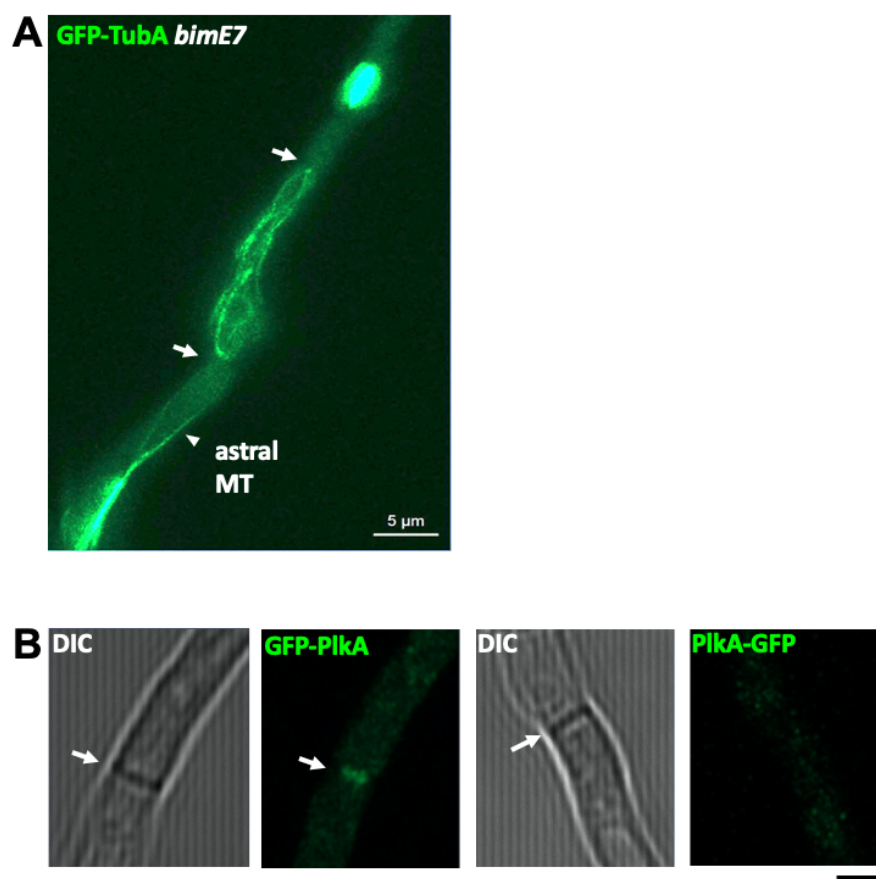
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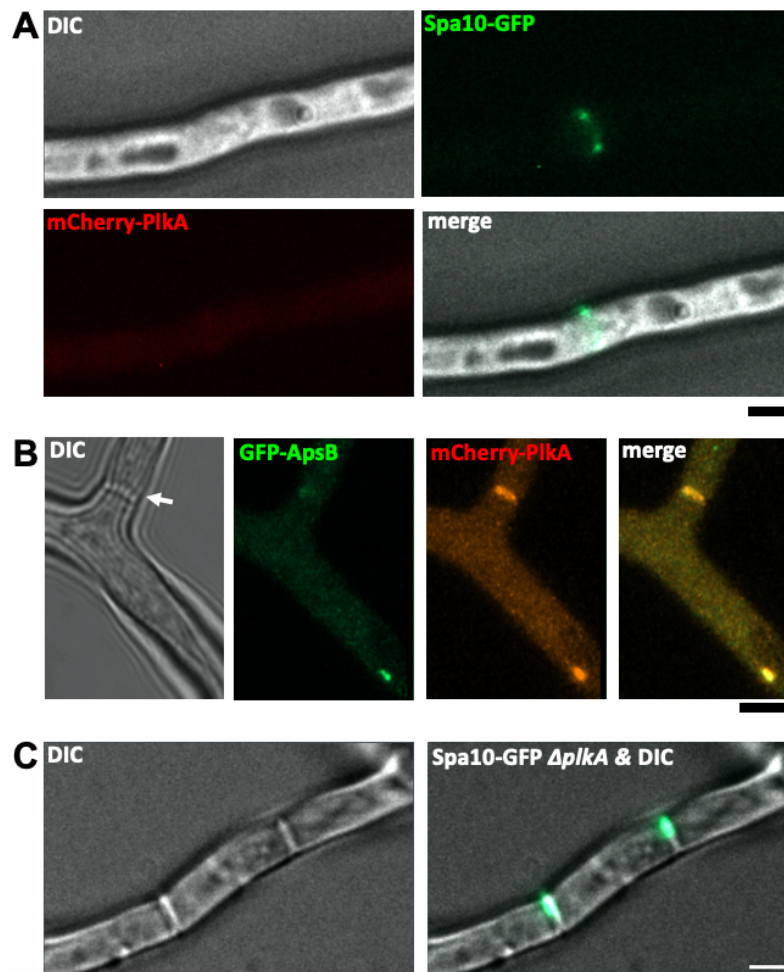
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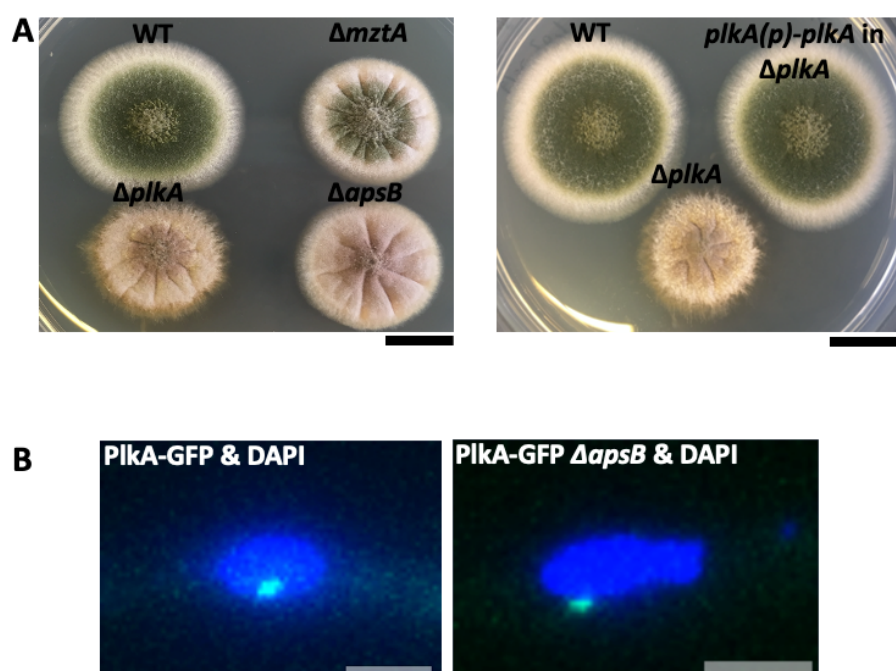


**Fig. S1. (A)** Study of sMTOC activities in interphase and in two compartments blocked in mitosis (left picture). Strains SXL136 (*alcA(p)::GFP::tubA*; *bimE7*) was incubated in MM (2% glycerol with auxotrophic markers) at 28°C overnight followed by 42°C treatment for 4h to block mitosis. Arrows indicate septa. Scale bar, 5 $\mu\text{m}$ . **(B)** GFP fusion at the C-terminus of PlkA abolished the septa localization. Strains SXL164 (*alcA(p)::GFP::plkA*) and SXL137 (*plkA::GFP*) were incubated in MM (2% glycerol) with supplements at 28°C overnight. Arrows indicate the septa positions. Scale bar, 2  $\mu\text{m}$ .

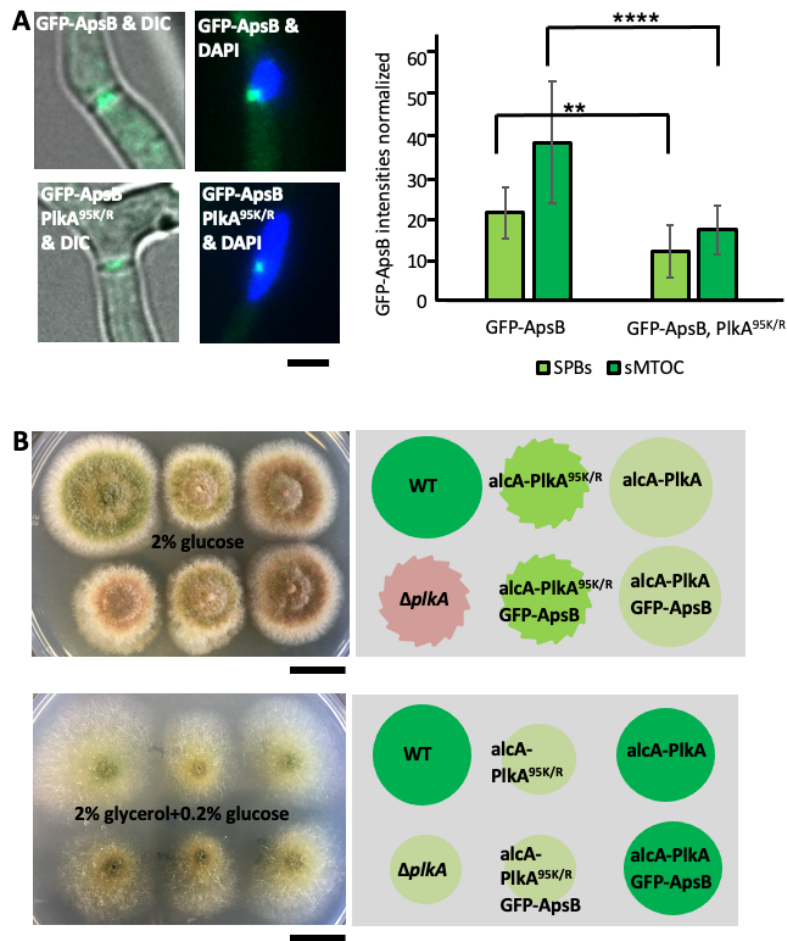




**Fig. S2. (A)** Analysis of PlkA and Spa10 localizations at septa. Spa10 localizes to forming septa earlier than PlkA. At the beginning of septation, where Spa10 localizes as a constriction ring, PlkA is not yet appeared. Strain SXL179 (*alcA(p)::mCherry::plkA; spa10::GFP*) was incubated in MM (2% glycerol) at 28°C overnight and observed. Scale bar, 2  $\mu$ m. **(B)** PlkA localizes to forming septa earlier than ApsB. Arrows indicate the position of a forming septum. Strains SXL174 (*alcA(p)::mCherry::plkA; alcA(p)::GFP::apsB*) was incubated as above. Pictures were taken with the LSM 980 Airyscan microscope. Scale bar, 2  $\mu$ m. **(C)** Spa10 localization to mature septa was unaffected in the absence of PlkA. Strain SXL182 ( $\Delta plkA; spa10::GFP$ ) was incubated as above and observed. Scale bar, 2  $\mu$ m.

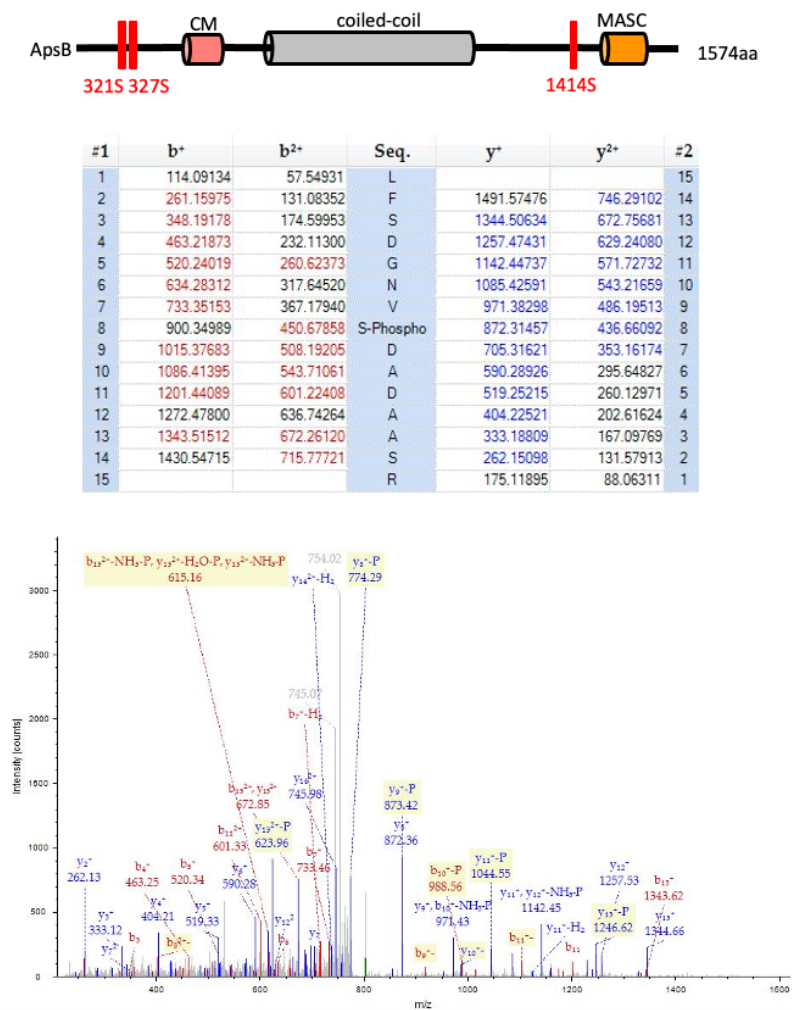


**Fig. S3. (A)** Phenotype of a *plkA*-deletion strain. Comparison of colonies in WT,  $\Delta mztA$ ,  $\Delta apsB$ , and  $\Delta plkA$  (left) and re-complementation of  $\Delta plkA$  (right). Strains TN02A3 (WT), SXL49 ( $\Delta mztA$ ), SYZ3 ( $\Delta apsB$ ) and SXL122 ( $\Delta plkA$ ) were incubated in MM plate with supplements for three days. Scale bar 1cm. **(B)** Localization of PlkA was not affected in the absence of ApsB. Strains SXL137 (*plkA::GFP*) and SXL138 (*plkA::GFP*;  $\Delta apsB$ ) were incubated in MM (2% glycerol) with supplements at 28°C overnight. Nuclei were stained with DAPI.



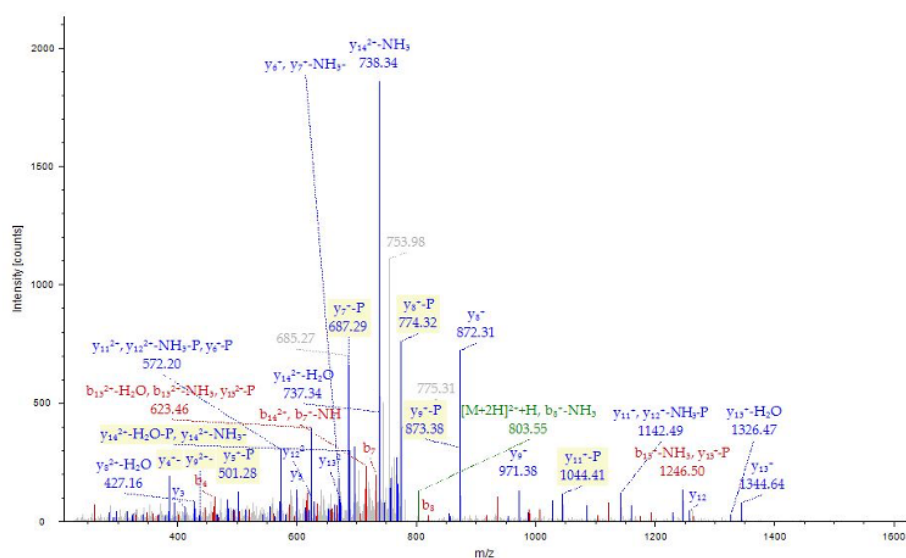
**Fig. S4. Analyzing the catalytic role of PlkA.** (A) Images of GFP-ApsB signal intensities at sMTOCs and SPBs in WT PlkA and mutated PlkA<sup>95K/R</sup>. Strains SXL174 (*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA*) and SXL175 (*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA<sup>95R</sup>*) were incubated in MM (2% glycerol) at 28°C overnight and imaged. Nuclei were stained with DAPI. Scale bar, 2 μm. Localization of ApsB at SPBs and sMTOCs are significantly reduced in catalytic mutant of *plkA*. Totally 30 SPBs and 15 sMTOCs were calculated for each strain. The vertical lines mark the region of the SD. Mann-Whitney U tests were performed with GraphPad Prism 8. The asterisks \*\*\*\* above the graph indicate significant differences compared to wild type ( $p < 0.0001$ ). (B) Comparison of the phenotype in WT, Δ*plkA* and *plkA<sup>95R</sup>* in repressing (upper) and de-repressing (lower) plates. Strains TN02A3 (WT), SXL122 (Δ*plkA*), SXL158 (*alcA(p)::mCherry::plkA*), SXL159 (*alcA(p)::mCherry::plkA<sup>95R</sup>*), SXL174 (*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA*) and SXL175 (*alcA(p)::GFP::apsB; alcA(p)::mCherry::plkA<sup>95R</sup>*) were incubated in MM plates (2% glucose for repressing *alcA* promoter and 2% glycerol for de-repressing) with supplements for three days. Scale bar 1cm.





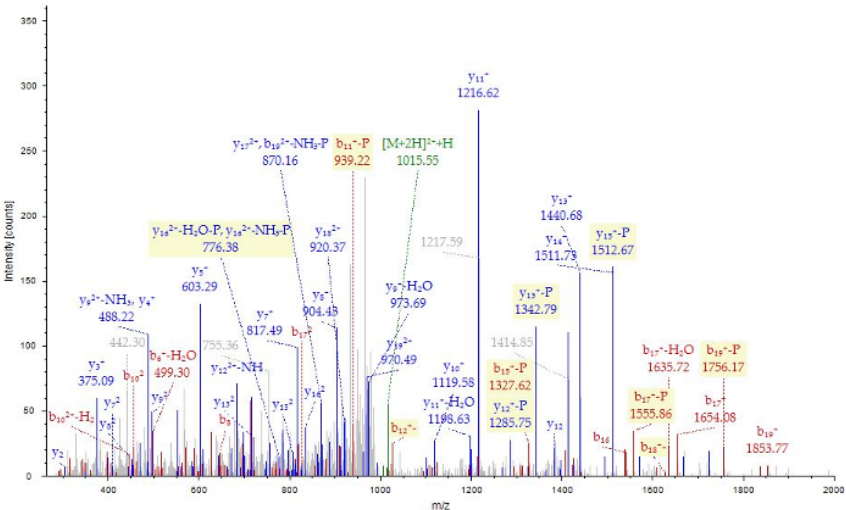
**Fig. S5. Annotated fragmentation spectrum and ion series for the peptide LFSDGNVSDADAASR phosphorylated at S321 (34% isolation interference; Proteome Discoverer 2.2).**

#1	b <sup>+</sup>	b <sup>2+</sup>	Seq.	y <sup>+</sup>	y <sup>2+</sup>	#2
1	114.09134	57.54931	L			15
2	261.15975	131.08352	F	1491.57476	746.29102	14
3	348.19178	174.59953	S	1344.50634	672.75681	13
4	463.21873	232.11300	D	1257.47431	629.24080	12
5	520.24019	260.62373	G	1142.44737	571.72732	11
6	634.28312	317.64520	N	1085.42591	543.21659	10
7	733.35153	367.17940	V	971.38298	486.19513	9
8	820.38356	410.69542	S	872.31457	436.66092	8
9	935.41050	468.20889	D	785.28254	393.14491	7
10	1006.44762	503.72745	A	670.25560	335.63144	6
11	1121.47456	561.24092	D	599.21848	300.11288	5
12	1192.51167	596.75947	A	484.19154	242.59941	4
13	1263.54879	632.27803	A	413.15443	207.08085	3
14	1430.54715	715.77721	S-Phospho	342.11731	171.56229	2
15			R	175.11895	88.06311	1



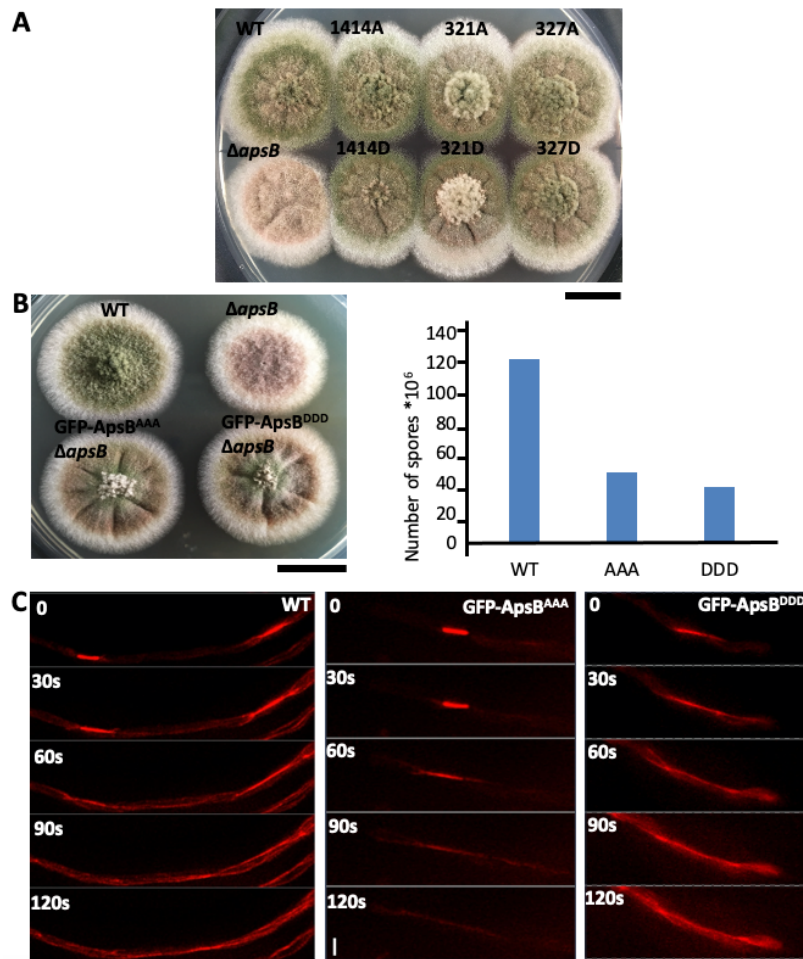
**Fig. S6. Annotated fragmentation spectrum and ion series for the peptide LFSDGNVSDADAASR phosphorylated at S327 (15% isolation interference; Proteome Discoverer 2.2).**

#1	b <sup>+</sup>	b <sup>2+</sup>	Seq.	y <sup>+</sup>	y <sup>2+</sup>	#2
1	88.03930	44.52329	S			20
2	189.08698	95.04713	T	1940.89609	970.95168	19
3	290.13466	145.57097	T	1839.84841	920.42784	18
4	361.17178	181.08953	A	1738.80073	869.90401	17
5	418.19324	209.60026	G	1667.76362	834.38545	16
6	517.26165	259.13446	V	1610.74216	805.87472	15
7	588.29877	294.65302	A	1511.67374	756.34051	14
8	645.32023	323.16375	G	1440.63663	720.82195	13
9	812.31859	406.66293	S-Phospho	1383.61517	692.31122	12
10	909.37135	455.18932	P	1216.61681	608.81204	11
11	1037.42993	519.21860	Q	1119.56404	560.28566	10
12	1124.46196	562.73462	S	991.50546	496.25637	9
13	1211.49399	606.25063	S	904.47344	452.74036	8
14	1312.54167	656.77447	T	817.44141	409.22434	7
15	1425.62573	713.31650	I	716.39373	358.70050	6
16	1540.65267	770.82997	D	603.30967	302.15847	5
17	1653.73674	827.37201	L	488.28272	244.64500	4
18	1724.77385	862.89056	A	375.19866	188.10297	3
19	1853.81644	927.41186	E	304.16155	152.58441	2
20			R	175.11895	88.06311	1



**Fig. S7. Annotated fragmentation spectrum and ion series for the peptide STTAGVAGSPQSSTIDLAER phosphorylated at S1414 (7% isolation interference; Proteome Discoverer 2.2).**





**Fig. S8. (A)** Single mutation of ApsB phosphorylation sites to alanine or to aspartic acid does not affect its function and localization. Strains SYZ2 (*alcA(p)::GFP::apsB<sup>WT</sup>*), SYZ3 ( $\Delta$ apsB), SXL160 ( $\Delta$ apsB; *alcA(p)::GFP::apsB<sup>1414A</sup>*), SXL161 ( $\Delta$ apsB; *alcA(p)::GFP::apsB<sup>1414D</sup>*), SXL162 ( $\Delta$ apsB; *alcA(p)::GFP::apsB<sup>321A</sup>*), SXL163 ( $\Delta$ apsB; *alcA(p)::GFP::apsB<sup>321D</sup>*), SXL168 ( $\Delta$ apsB; *alcA(p)::GFP::apsB<sup>327A</sup>*) and SXL169 ( $\Delta$ apsB; *alcA(p)::GFP::apsB<sup>327D</sup>*) were grown on MM plate with 2% glucose for three days at 37°C. Scale bar, 1 cm. **(B)** Triple mutation of ApsB phosphorylation sites to A or to D partially rescues the deletion phenotype. Colony phenotype of triple mutations shows sporulation defects. Strains TN02A3 (WT), SYZ3 ( $\Delta$ apsB), SXL180 ( $\Delta$ apsB; *alcA(p)::GFP::apsB<sup>AAA</sup>;mCherry::tubA*), SXL181 ( $\Delta$ apsB;*alcA(p)::GFP::apsB<sup>DDD</sup>; mCherry::tubA*) were grown on MM plate with 2% glucose for three days and washed for spore counting. Scale bar, 1 cm. The spore numbers in triple mutants are highly decreased ( $47.5 \times 10^6$ ,  $37.5 \times 10^6$ ) compared to wild type ( $122.5 \times 10^6$ ). **(C)** Mitotic process of triple mutants behaves normally. The same strains as in **B** were grown in 8 well u-slides at 28°C overnight for long term observation. Time lapse images were taken every 30s at room temperature. Scale bar, 2  $\mu$ m.

**Table S1. Protein identification details for PIkA-GFP capture.** The experiment was performed in duplicate using a strain expressing free GFP as negative control. The Table lists the number of peptides and number of peptide spectrum matches (PSMs, SequestHT and Mascot) for the bait protein PIkA and the four tubulin proteins. In brackets, the total number of proteins identified with high FDR confidence is provided.

	Sample							
Protein	PIkA-GFP replicate 1 (313 proteins identified)		PIkA-GFP replicate 2 (229 proteins identified)		GFP control replicate 1 (61 proteins identified)		GFP control replicate 2 (37 proteins identified)	
	# peptides	# PSMs	# peptides	# PSMs	# peptides	# PSMs	# peptides	# PSMs
PIkA	29	116	21	56	0	0	0	0
tubA ( $\alpha$ tubulin)	10	24	5	12	1	2	0	0
tubB ( $\alpha$ tubulin)	12	32	6	11	2	4	0	0
benA ( $\beta$ tubulin)	10	29	7	19	3	9	0	0
tubC ( $\beta$ tubulin)	7	24	4	13	2	7	0	0

**Table S2. List of *A. nidulans* strains used in this study**

Strain	Genotype	Source
<b>TN02A3</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; veA1</i>	(Nayak et al., 2006)
<b>SJW65</b>	<i>wA3; pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::GFP::tubA::pyr4</i>	J. Warmboldt, Marburg
<b>SJW02</b>	<i>wA3; pyrG89; pyroA4; ΔargB::trpCΔB; alcA(p)::GFP::tubA::pyr4</i>	J. Warmboldt, Marburg
<b>SSH62</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::tubA::pyroA</i>	(Takeshita et al., 2013)
<b>SSH27</b>	<i>wA3, alcA(p)::GFP::kipA::pyr4; ΔargB::trpCΔB</i>	(Herrero et al., 2011)
<b>SNZ-SH80</b>	<i>fwA1; pyrG89; chaA1, sE15, wA3; argB2; ΔnkuA::argB, pyroA4; nirA14; gcpC(p)::gcpC::GFP::AfpYrG;</i>	(Zekert et al., 2010)
<b>SYZ2</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::GFP::apsB::pyr4</i>	(Zhang et al., 2017)
<b>SYZ3</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA</i>	(Zhang et al., 2017)
<b>SXL49</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔmztA::pyroA</i>	(Gao et al., 2019)
<b>SMS4</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; mztA::GFP::AfpYrG</i>	(Gao et al., 2019)
<b>Only GFP</b>	<i>yA2; pabaA1; gpdA(p)::GFP</i>	B. Joehnk, Göttingen
<b>SO4</b>	<i>bimE7; wA2; pabaA1</i>	(Osmani et al., 1991)



<b>SO1191</b>	<i>An-nup2-GFP::AfpYrG; bimE7; riboA2; nicB8; yA2</i>	SA. Osmani, USA
<b>SRM212</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; spa10::GFP::AfpYrG</i>	(Zhang et al., 2017)
<b>SXW1</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::tubA::pyroA; alcA(p)::GFP::kipA::pyr4</i> (SSH transformed with pSK82)	This work
<b>SXW7</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::YFP<sup>N</sup>::pcpA::pyroA; alcA(p)::YFP<sup>C</sup>::plkA::pyr4</i> (TN02A3 transformed with pXL58 and pXW8, homologous integration)	This work
<b>SXL122</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔplkA::pyroA</i> (TN02A3 transformed with pXL79 homologous integration)	This work
<b>SXL124</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::YFP<sup>N</sup>::apsB::pyroA; alcA(p)::YFP<sup>C</sup>::plkA::pyr4</i> (TN02A3 transformed with pYZ59 and pXW8, homologous integration)	This work
<b>SXL125</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::YFP<sup>N</sup>::pcpA::pyroA; alcA(p)::YFP<sup>C</sup>::plkA::pyr4</i> (TN02A3 transformed with pXL58 and pXW8, homologous integration)	This work
<b>SXL126</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔplkA::pyroA; plkA(p)::plkA::AfpYrG</i> (SXL122 transformed with pXL81)	This work
<b>SXL129</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔplkA::pyroA mztA(p)::mztA::GFP::AfpYrG</i> (SXL122 transformed with pXL23, homologous integration)	This work
<b>SXL131</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔplkA::pyroA gcpC(p)::gcpC::GFP::AfpYrG</i> (SXL122 transformed with <i>gcpC(C-terminal)::GFP::AfpYrG::gcpC(RB)</i> cassette, homologous integration)	This work
<b>SXL133</b>	<i>An-nup2-GFP::AfpYrG; bimE7</i> (SO1191 cross with 65)	This work
<b>SXL134</b>	<i>An-nup2-GFP::AfpYrG; bimE7; ΔplkA::pyroA</i> (SO1191 cross with SXL122)	This work

<b>SXL135</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔplkA::pyroA alcA(p)::GFP::apsB::pyr4</i> (SYZ2 cross with SXL122)	This work
<b>SXL136</b>	<i>wA3; argB2; alcA(p)::GFP::tubA::pyr4; bimE7</i> (SO4 cross with SJW02)	This work
<b>SXL137</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; plkA(p)::plkA::GFP::AfpYrG</i> (TN02A3 transformed with pXL82, homologous integration)	This work
<b>SXL138</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA; plkA(p)::plkA::GFP::AfpYrG</i> (SYZ3 transformed with pXL82, homologous integration)	This work
<b>SXL156</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>full</sup>::pyr4</i> (SYZ3 transformed with pXL106)	This work
<b>SXL158</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::plkA::pyroA</i> (TN02A3 transformed with pXL83 homologous integration)	This work
<b>SXL159</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::plkA<sup>95 K to R</sup>::pyroA</i> (TN02A3 transformed with pXL114 homologous integration)	This work
<b>SXL160</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>1414S to A</sup>::pyr4</i> (SYZ3 transformed with pXL107)	This work
<b>SXL161</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>1414S to D</sup>::pyr4</i> (SYZ3 transformed with pXL108)	This work
<b>SXL162</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>321S to A</sup>::pyr4</i> (SYZ3 transformed with pXL109)	This work
<b>SXL163</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>321S to D</sup>::pyr4</i> (SYZ3 transformed with pXL110)	This work
<b>SXL164</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB;</i>	This work

	<i>alcA(p)::GFP::plkA::pyr4</i> (TN02A3 transformed with pXL84)	
<b>SXL168</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>327S to A</sup>::pyr4</i> (SYZ3 transformed with pXL117)	This work
<b>SXL169</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>327S to D</sup>::pyr4</i> (SYZ3 transformed with pXL118)	This work
<b>SXL174</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::plkA::pyroA; alcA(p)::GFP::apsB::pyr4</i> (SYZ2 crossed with SXL158)	This work
<b>SXL175</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::plkA<sup>95K to R</sup>::pyroA; alcA(p)::GFP::apsB::pyr4</i> (SYZ2 crossed with SXL159)	This work
<b>SXL176</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::plkA::pyroA; alcA(p)::GFP::tubA::pyr4</i> (65 transformed with pXL83, homologous integration)	This work
<b>SXL177</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔplkA::pyroA; alcA(p)::GFP::tubA::pyr4</i> (65 transformed with plkA deletion cassette, homologous integration)	This work
<b>SXL178</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::plkA<sup>95R</sup>::pyroA; alcA(p)::GFP::tubA::pyr4</i> (SXL159 transformed with pXL32, homologous integration)	This work
<b>SXL179</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; alcA(p)::mCherry::plkA::pyroA; spa10(p)::spa10::GFP::Afp<sub>pyrG</sub></i> (SRM212 crossed with SXL158)	This work
<b>SXL180</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>321S to A; 327 S to A; 1414S to A</sup>::pyr4; alcA(p)::mCherry::tubA::pyroA</i> (SYZ3 co-transformed with pXL123 and pSH44)	This work
<b>SXL181</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔapsB::pyroA, alcA(p)::GFP::apsB<sup>321S to D; 327 S to D; 1414S to D</sup>::pyr4; alcA(p)::mCherry::tubA::pyroA</i> (SYZ3 co-transformed with pXL124 and pSH44)	This work



<b>SXL182</b>	<i>pyrG89; argB2; pyroA4, ΔnkuA::argB; ΔplkA::pyroA; spa10(p)::spa10::GFP::Afp<sub>pyrG</sub></i> (SRM212 transformed with pXL79, homologous integration)	This work
<b>CDS449</b>	<i>wA3, AnNup49-GFP::pyrGAf; histone H1-mRFP1::pyrG; pabaA1; fwA1</i>	Stephen Osmani (Ohio State Univ.)
<b>SSH167</b>	<i>CDS449 crossed to 65; wA3; alcA(p)::GFP-tubA; AnNup49-GFP; histone H1-mRFP1; pabA1</i>	This work
<b>SSH168</b>	<i>SSH167 crossed to SXL122; alcA(p)::GFP-tubA; AnNup49-GFP; histone H1-mRFP1; □plkA</i>	This work
<b>SSH169</b>	<i>CDS449 crossed to 65; wA3; alcA(p)::GFP-tubA; histone H1-mRFP1; pyroA4</i>	This work
<b>SSH171</b>	<i>pyrG89; plkA(p)::GFP-plkA::pyr-4 argB2; pyroA4, ΔnkuA::argB; veA1</i> (TN02A3 transformed with pSH108)	
<b>SSH172</b>	<i>Heterokaryon SXL80 x SXL137; pyrG89; argB2; pyroA4, ΔnkuA::argB; plkA(p)::plkA::GFP::Afp<sub>pyrG</sub>; alcA(p)::mCherry::pcpA::pyroA</i>	This work

**Table S3. List of yeast strains used in this study**

Strain	Genotype	Source
<b>AH109</b>	<i>MATa</i> ; <i>trp1-901</i> ; <i>leu2-3, 112</i> ; <i>ura3-52</i> ; <i>his3-200</i> ; <i>gal4Δ</i> ; <i>gal80Δ</i> ; <i>LYS2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3</i> ; <i>GAL2<sub>UAS</sub>-GAL2<sub>TATA</sub>-ADE2</i> ; <i>URA3::MEL1<sub>UAS</sub>-MEL1<sub>TATA</sub>-lacZ</i>	(James et al., 1996)
<b>Y187</b>	<i>MATα</i> ; <i>ura3-52</i> ; <i>his3-200</i> ; <i>ade2-101</i> ; <i>trp1-901</i> ; <i>leu2-3, 112</i> ; <i>gal4Δ</i> ; <i>met-</i> ; <i>gal80Δ</i> ; <i>URA3::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-lacZ</i>	(Harper et al., 1993)
<b>AH109_AD-ApsB</b>	AH109 transformed with pRS88; Gal4 AD-ApsB	This work
<b>Y187_BD-PikA</b>	Y187 transformed with pXL111; Gal4 BD-PikA	This work
<b>AH109_AD-PikA</b>	AH109 transformed with pXL112; Gal4 AD-PikA	This work
<b>Y187_BD-ApsB</b>	Y187 transformed with pRS89; Gal4 BD-ApsB	This work
<b>Y2H_AD-ApsB/BD-PikA</b>	AH109 mated with Y187; Gal4 AD-ApsB; Gal4 BD-PikA	This work
<b>Y2H_AD-PikA/BD-ApsB</b>	AH109 mated with Y187; Gal4 AD-PikA; Gal4 BD-ApsB	This work
<b>Y2H_AD-PikA/BD-PikA</b>	AH109 mated with Y187; Gal4 AD-PikA; Gal4 BD-PikA	This work
<b>Y2H_AD-ApsB/BD-ApsB</b>	AH109 mated with Y187; Gal4 AD-ApsB; Gal4 BD-ApsB	This work
<b>AH109_AD-GcpC</b>	AH109 transformed with pJA5; Gal4 AD-GcpC	This work

<b>Y187_ BD-GcpC</b>	Y187 transformed with pJA6; Gal4 BD-GcpC	This work
<b>AH109_ AD-PcpA<sup>Smc</sup></b>	AH109 transformed with pXL125; Gal4 AD-PcpA <sup>Smc</sup>	This work
<b>Y187_ BD-PcpA<sup>Smc</sup></b>	Y187 transformed with pXL126; Gal4 BD-PcpA <sup>Smc</sup>	This work
<b>Y2H_AD- GcpC/BD- PlkA</b>	AH109 mated with Y187; Gal4 AD-GcpC; Gal4 BD-PlkA	This work
<b>Y2H_AD- PlkA/BD- GcpC</b>	AH109 mated with Y187; Gal4 AD-PlkA; Gal4 BD-GcpC	This work
<b>Y2H_AD- PcpA<sup>Smc</sup>/BD- PlkA</b>	AH109 mated with Y187; Gal4 AD-PcpA <sup>Smc</sup> ; Gal4 BD-PlkA	This work
<b>Y2H_AD- PlkA/BD- PcpA<sup>Smc</sup></b>	AH109 mated with Y187; Gal4 AD-PlkA; Gal4 BD-PcpA <sup>Smc</sup>	This work
<b>Positive control</b>	AH109 transformed with pGADT7-T and pGBKT7-53	This work
<b>Negative control</b>	AH109 transformed with pGADT7-T and pGBKT7-Lam	This work

**Table S4. List of plasmids used in this study**

Plasmid	Genotype	Source
<b>pJET1.2/blunt</b>	Blunt end cloning vector	Fermentas
<b>pMCB17apx</b>	<i>alcA(p)::GFP</i> , for N-terminal fusion of GFP to proteins of interest; contains <i>N. crassa pyr4</i>	(Efimov et al., 2006)
<b>pFNO3</b>	<i>(GA)<sub>5</sub>::GFP::AfpyrG</i> , Kan <sup>r</sup> , Amp <sup>r</sup>	Fungal Genetic Stock Center, MO
<b>pGADT7-Rec</b>	Gal4 DNA-activation domain (AD); Amp <sup>r</sup>	Clontech
<b>pGBKT7</b>	Gal4 DNA-binding domain (BD); Kan <sup>r</sup>	Clontech
<b>pSH44</b>	<i>alcA(p)::mCherry::alpha tubulin</i> , <i>pyroA</i> , Amp <sup>r</sup> (pMCB17apx with 3.1 kb <i>alpha tubulin</i> )	(Manck et al., 2015)
<b>pSK82</b>	<i>alcA(p)::GFP::kipA</i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with 1 kb <i>kipA</i> )	(Konzack et al., 2005)
<b>pYZ59</b>	<i>alcA(p)::YFP<sup>N</sup>::apsB</i> , <i>pyroA</i> , Amp <sup>r</sup> (pMCB17apx with full length <i>apsB</i> )	(Zhang et al., 2017)
<b>pXL23</b>	pJET1.2/blunt with <i>mztA</i> (C-terminal)::GFP::AfpyrG::mztA(RB)	(Gao et al., 2019)
<b>pXL32</b>	<i>alcA(p)::GFP::tubA</i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with 3.1kb <i>tubA</i> )	(Gao et al., 2019)
<b>pXL58</b>	<i>alcA(p)::YFP<sup>N</sup>::pcpA</i> , <i>pyroA</i> , Amp <sup>r</sup> (pMCB17apx with 1kb <i>pcpA</i> )	(Gao et al., 2019)
<b>pRS89</b>	pGBKT-7 with <i>apsB</i> cDNA, Kan <sup>r</sup>	R. Suelmann, Marburg



<b>pRS88</b>	pGADT-7 with <i>apsB</i> cDNA, Amp <sup>r</sup>	R. Suelmann, Marburg
<b>pXW8</b>	<i>alcA(p)::YFP<sup>C</sup>::plkA</i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with 1 kb <i>plkA</i> , <i>YFP<sup>C</sup></i> instead of <i>GFP</i> )	This work
<b>pXL79</b>	pJET1.2/blunt with <i>plkA(LB)::pyroA::plkA(RB)</i> deletion cassette	This work
<b>pXL81</b>	pJET1.2/blunt with <i>plkA(p)::plkA::Afp<sub>pyrG</sub></i> complementation cassette	This work
<b>pXL82</b>	pJET1.2/blunt with <i>plkA(C-ter)::GFP::Afp<sub>pyrG</sub>::plkA(RB)</i> cassette	This work
<b>pXL83</b>	<i>alcA(p)::mCherry::plkA</i> , <i>pyroA</i> , Amp <sup>r</sup> (pMCB17apx with 1kb <i>plkA</i> )	This work
<b>pXL84</b>	<i>alcA(p)::GFP::plkA</i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with 1kb <i>plkA</i> )	This work
<b>pXL102</b>	pJET1.2/blunt with <i>plkA</i> exon cassette	This work
<b>pXL106</b>	<i>alcA(p)::GFP::apsB</i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with full length of <i>ApsB</i> )	This work
<b>pXL107</b>	<i>alcA(p)::GFP::apsB<sup>1414S to A</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of <i>ApsB</i> )	This work
<b>pXL108</b>	<i>alcA(p)::GFP::apsB<sup>1414S to D</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of <i>ApsB</i> )	This work
<b>pXL109</b>	<i>alcA(p)::GFP::apsB<sup>321S to A</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of <i>ApsB</i> )	This work
<b>pXL110</b>	<i>alcA(p)::GFP::apsB<sup>321S to D</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of <i>ApsB</i> )	This work
<b>pXL111</b>	pGBKT-7 with full length of <i>plkA</i> cDNA, Kan <sup>r</sup>	This work

<b>pXL112</b>	pGADT-7 with full length of <i>plkA</i> cDNA, Amp <sup>r</sup>	This work
<b>pJA5</b>	pGADT-7 with <i>gcpC</i> cDNA, Amp <sup>r</sup>	This work
<b>pJA6</b>	pGBKT-7 with <i>gcpC</i> cDNA, Kan <sup>r</sup>	This work
<b>pXL114</b>	<i>alcA(p)::mCherry::plkA<sup>95 K to R</sup></i> , <i>pyroA</i> , Amp <sup>r</sup> (pMCB17apx with 1kb mutated <i>plkA</i> )	This work
<b>pXL117</b>	<i>alcA(p)::GFP::apsB<sup>327S to A</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of ApsB)	This work
<b>pXL118</b>	<i>alcA(p)::GFP::apsB<sup>327S to D</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of ApsB)	This work
<b>pXL123</b>	<i>alcA(p)::GFP::apsB<sup>321S to A; 327S to A; 1414S to A</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of ApsB)	This work
<b>pXL124</b>	<i>alcA(p)::GFP::apsB<sup>321S to D; 327S to D; 1414S to D</sup></i> , <i>pyr4</i> , Amp <sup>r</sup> (pMCB17apx with mutated full length of ApsB)	This work
<b>pXL125</b>	pGADT-7 with Smc domain of <i>PcpA</i> , Amp <sup>r</sup>	This work
<b>pXL126</b>	pGBKT-7 with Smc domain of <i>PcpA</i> , Kan <sup>r</sup>	This work
<b>pSH108</b>	<i>plkA(p)::GFP-plkA</i> full length in pMBC17apx	This work

**Table S5. Oligonucleotides used in this work.**

Name	Sequence 5'-3'
<b><i>plkA</i> deletion</b>	
plkA-LB_fwd	CGTCATCCCTTCGGTTATTCT
plkA-LB-pyroA linker_rev	ATGGTCTCGAACTGACCTTACGGTTCTCGAACAAATGC GG
pyroA_fwd	GTAAGGTCAGTTCGAGACCA
pyroA_rev	TGCATCAAAGAACGCTATATCAAC
plkA-RB-pyroA linker_fwd	GTTGATATAGCGTTCTTTGATGCAGACTGATGACCTTGG GTTGT
plkA-RB_rev	ACAGGCAATGCAAAGAACTG
plkA-LB-N-fwd	GATGAAAGTTGCGCCTGATT
plkA-RB-N_rev	GAGAGGAAAACAGATCGGCT
<b>PlkA C-terminal localization</b>	
plkA-C-termi_fwd	TAACTTGGTTCCTGGTTCGG
plkA-C-termi_GA linker_rev	CAGCGCCTGCACCAGCTCCTAAGCCCGCTAATCGCAG
GA linker_fwd	GGAGCTGGTGCAGGCGCTG
pyrG overhang_rev	CTGTCTGAGAGGAGGCACTGAT
plkA-RB-pyrG linker_fwd	ATCAGTGCCTCCTCTCAGACAGGACTGATGACCTTGGG TTGT
plkA-RB_rev	ACAGGCAATGCAAAGAACTG
plkA-C-termi-N_fwd	CATGCGTATGTCATCAAGTGG
plkA-RB-N_rev	GAGAGGAAAACAGATCGGCT

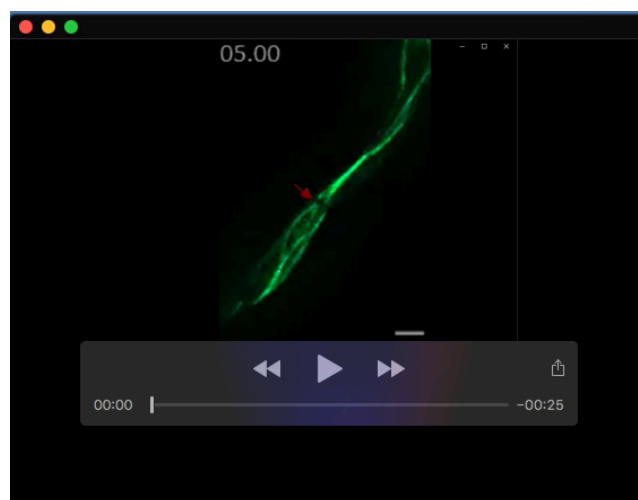
<b>PlkA N-terminal localization</b>	
plkA_AscI_fwd	AGGCGCGCCAATGGAGAGACACCTCCAAC
plkA_PacI_rev	CCTTAATTAAGTCAAATCTAGGCCATGGC
<b>plkA re-complementation</b>	
plkA-promo_builder_fwd	CTCGAGTTTTTCAGCAAGATAATCATCCTTATTCGTTGAC CAAG
plkA-termi_pyrG linker_rev	GAAGAGCATTGTTTGAGGCGTGGGATCGAGAATTGGGG
pyrG_fwd	CGCCTCAAACAATGCTCTTC
pyrG builder_rev	AGGAGATCTTCTAGAAAGATCTGTCTGAGAGGAGGCAC TGAT
<b>PlkA cDNA for Y2H</b>	
plkA-CDS1_builder_fwd	CTCGAGTTTTTCAGCAAGATATGGAGAGACACCTCCAAC
plkA-CDS1_CDS2 linker_rev	TCTGCAGCTCTGTTCGAAACTTCTCTTGCATCTTTTTCT GGC
plkA-CDS2_fwd	TTTCGAACAGAGCTGCAGA
plkA-CDS2_rev	CAGATCACTCCCAGGGAC
plkA-CDS3_CDS2 linker_fwd	GTCCCTGGGAGTGATCTGTTTTGCAATGCTTACGGGATA T
plkA-CDS3_builder_rev	AGGAGATCTTCTAGAAAGATTAAGCCCGCTAATCGCAG
plkA NdeI AD/BD_fwd	GGAATTCCATATGATGGAGAGACACCTCCAAC
plkA XmaI AD/BD_rev	TCCCCCGGGTAAGCCCGCTAAACGCAG
<b>plkA catalytic mutation</b>	
plkA_95K/R_fwd	CTTTGCAATGAGAGTCGTAAAGTCTGACATG
plkA_95K/R_rev	ACACGACCGTTGCGCAAC



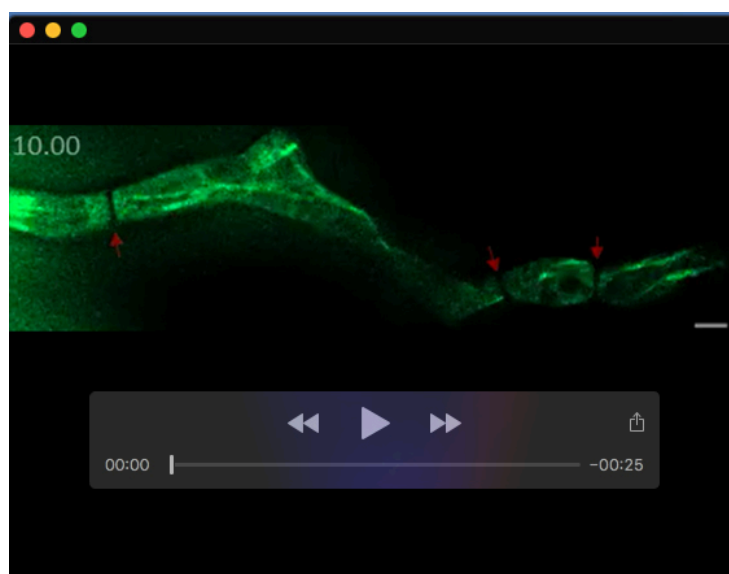
plkA_95 seq_fwd	ATGGAGAGACACCTCCAAC
<b>PcpA<sup>Smc</sup> cDNA for Y2H</b>	
pcpA <sup>Smc</sup> _NdeI AD/BD_fwd	GGAATTCCATATGGACCAGCGCGTGAAAGAA
pcpA <sup>Smc</sup> _XmaI AD/BD_rev	TCCCCCGGGTTTCTTGATCGTGGCATCGT
<b>GcpC cDNA for Y2H</b>	
gcpC-CDS1_builder_fwd	CTCGAGTTTTTCAGCAAGATATGTCAAGTCGGCACCAT
gcpC-CDS1_CDS2 linker_rev	TAATTGACCTCCTTTCTTGCTCTGAGCCTCCTCAACAAT T
gcpC-CDS2_fwd	GCAAGAAAGGAGGTCAATTAGTG
gcpC-CDS2_buildDCer_rev	AGGAGATCTTCTAGAAAGATTTGCTCAGTCGAATCCTTC TT
gcpC_XmaI_BD_fwd	TCCCCCGGGATGTCAAGTCGGCACCAT
gcpC_NotI_BD_rev	ATAGTTTAGCGGCCGCTTGCTCAGTCGAATCCTTCTT
gcpC_SMARTII_AD_fwd	ACGCAGAGTGGCCATTATGGATGTCAAGTCGGCACCAT
gcpC_CDSIII_AD_rev	AGGCCGAGGCGGCCGACATGTTGCTCAGTCGAATCCTT CT
<b>ApsB phosphor-mutant</b>	
apsB-321S/A_fwd	TGGCAATGTCGCCGATGCGGATG
apsB-321S/A_rev	TCACTAAATAGTTTGGTGTG
apsB-327S/A_fwd	GGATGCCGCAGCTCGCAGAGGTA
apsB-327S/A_rev	GCATCGCTGACATTGCCATCACTAAATAG
apsB-1414S/A_fwd	CGTGGCAGGCGCGCCGAGTCAT
apsB-1414S/A_rev	CCAGCTGTCGTGCTGCGGC

apsB-321S/A,327S/A_fwd	TGCCGCAGCTCGCAGAGGTAGCCGAAGG
apsB-321S/A,327S/A_rev	TCCGCATCGGCGACATTGCCATCACTAAATAGTTTGG
apsB-321S/D_fwd	TGGCAATGTCGACGATGCGGATG
apsB-321S/D_rev	TCACTAAATAGTTTGGTGTG
apsB-327S/D_fwd	GGATGCCGCAGATCGCAGAGGTAG
apsB-327S/D_rev	GCATCGCTGACATTGCCA
apsB-1414S/D_fwd	CGTGGCAGGCGACCCGCAGTCATCCAC
apsB-1414S/D_rev	CCAGCTGTCTGTGCTGCGG
apsB-321S/D,327S/D_fwd	TGCCGCAGATCGCAGAGGTAGCCGAAGG
apsB-321S/D,327S/D_rev	TCCGCATCGTCGACATTGCCATCACTAAATAGTTTGG
apsB-321,327 seq_fwd	CACGCCTTAGACTTCACAGA
apsB-1414 seq_fwd	ACCAGCCTTGAGCAACAG
<b>confirmation of <i>plkA</i> deletion</b>	
plkA-dele_check_fwd	CGTCATCCCTTCGGTTATTCT
plkA-ORF_rev	ATTTTCGCGCAGTTCCATC
plkA-RB_rev	ACAGGCAATGCAAAGAACTG
<b>confirmation of N-terminal homologous integration</b>	
alcA check_fwd	AGACGGAGCACTTTCTGG
apsB 1kb Int check_rev	CCAGATGCCATACTTCCCAA
plkA 1kb Int check_rev	ATTTTCGCGCAGTTCCATC
pcpA 1kb Int check_rev	CCTCCAGCTGTTCTTTTGC
<b>confirmation of C-terminal</b>	

<b>homologous integration</b>	
pyrG check_fwd	CGCCTCAAACAATGCTCTTC
plkA C-termi check_rev	ACAGGCAATGCAAAGAACTG
mztA C-termi check_rev	TCACGAATCACGGGAAGG
gcpC C-termi check_rev	ACCGTCATGGCAGAAACGAAG

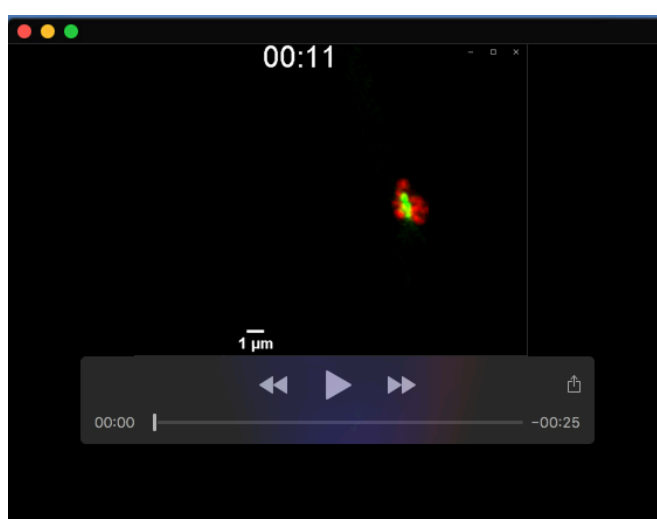


**Movie 1. GFP-TubA interphase at septa in wild type.** Strain SJW02 (*alcA(p)::GFP::tubA*) was cultured in 8 well u-slides at 28°C overnight. Arrows marked septum positions. Time-lapse interval was 5s in the 30-frames movie. The length of the movie was 6s and the speed 5fps. Scale bar, 2μm.

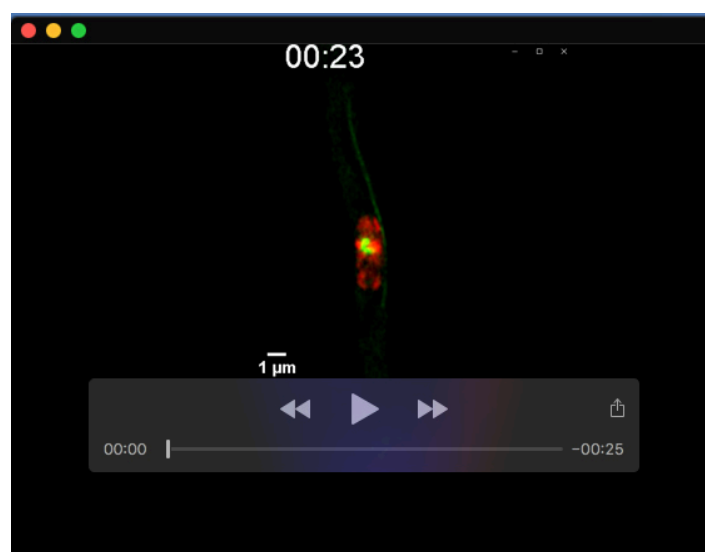


**Movie 2. GFP-TubA interphase at septa in the  $\Delta pIkA$ -deletion mutant.** SXL177 ( $\Delta pIkA$ , *alcA(p)::GFP::tubA*) was cultured in 8 well u-slides at 28°C overnight. Arrows marked septum positions. Time-lapse interval was 10s in the 12-frames movie. The length of the movie was 4s and the speed 3fps. Scale bar, 2μm.





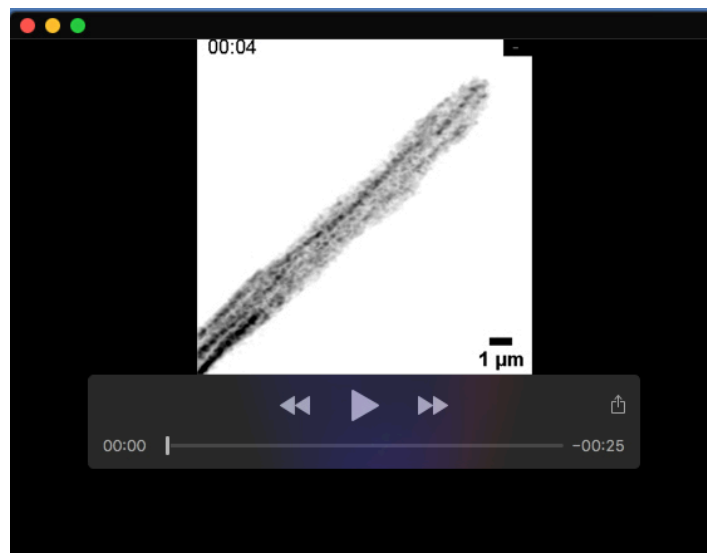
**Movie 3. Visualization of mitosis in WT.** Strain: (*alcA(p)::mRFP::H1, alcA(p)::GFP::tubA*). Excitation laser wavelengths were 488 for GFP and 561 nm for mRFP. Fluorescence signals were detected using the Airy scan detector with SR (super resolution) mode (Weisschart, 2014 *ZEISS Technology Note*). Images were collected using the ZEN blue software (ZEISS).



**Movie 4. Visualization of mitosis in the  $\Delta plkA$ -deletion strain.** Strain: ( $\Delta plkA$ , *alcA(p)::mRFP::H1, alcA(p)::GFP::tubA*). Excitation laser wavelengths were 488 for GFP and 561 nm for mRFP. Fluorescence signals were detected using the Airy scan detector with SR (super resolution) mode (Weisschart, 2014 *ZEISS Technology Note*). Images were collected using the ZEN blue software (ZEISS).



**Movie 5.** MT dynamics in WT. Imaging for 1:34 min (WT) and 1 min ( $\Delta p/kA$ -deletion mutant) using the Airy scan detector in Multiplex 4Y mode.



**Movie 6.** MT dynamics in the  $\Delta p/kA$ -deletion strain. Imaging for 1:34 min (WT) and 1 min ( $\Delta p/kA$ -deletion mutant) using the Airy scan detector in Multiplex 4Y mode.