A Novel Basal Body Protein That Is a Polo-like Kinase Substrate Is Required for Basal Body Segregation and Flagellum Adhesion in *Trypanosoma brucei*

Huiqing Hu, Qing Zhou, and Ziyin Li

From the Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston, Texas 77030

**Background:** The substrates of Polo-like kinase in *Trypanosoma brucei* are mostly not identified.

**Results:** A basal body protein was identified as a substrate of Polo-like kinase and is required for basal body segregation and flagellum adhesion.

**Conclusion:** Polo-like kinase regulates a basal body protein.

**Significance:** Dissecting the Polo-like kinase pathway is crucial for understanding its cellular functions.

The Polo-like kinase (PLK) in *Trypanosoma brucei* plays multiple roles in basal body segregation, flagellum attachment, and cytokinesis. However, the mechanistic role of TbPLK remains elusive, mainly because most of its substrates are not known. Here, we report a new substrate of TbPLK, SPBB1, and its essential roles in *T. brucei*. SPBB1 was identified through yeast two-hybrid screening with the kinase-dead TbPLK as the bait. It interacts with TbPLK *in vitro* and *in vivo*, and is phosphorylated by TbPLK *in vitro*. SPBB1 localizes to both the mature basal body and the probasal body throughout the cell cycle, and co-localizes with TbPLK at the basal body during early cell cycle stages. RNAi against SPBB1 in procyclic trypanosomes inhibited basal body segregation, disrupted the new flagellum attachment zone filament, detached the new flagellum, and caused defective cytokinesis. Moreover, RNAi of SPBB1 confined TbPLK at the basal body and the bilobe structure, resulting in constitutive phosphorylation of TbCentrin2 at the bilobe. Altogether, these results identified a basal body protein as a TbPLK substrate and its essential role in promoting basal body segregation and flagellum attachment zone filament assembly for flagellum adhesion and cytokinesis initiation.

Polo-like kinases (PLKs) are evolutionarily conserved serine/threonine protein kinases, which are characterized by the C-terminal Polo box domain (PBD) and play essential roles in mitosis and cytokinesis in eukaryotes (1, 2). The localization of PLK to various subcellular structures is known to be mediated by the PBD via association with different substrate proteins located at specific subcellular structures (3–6).

*Trypanosoma brucei*, an early branching unicellular eukaryote and the causative agent of human sleeping sickness, expresses a single PLK homolog, TbPLK, which displays a subcellular localization pattern different from its yeast and animal homologs (7–9). Early in the cell cycle, TbPLK is localized to the flagellar basal body and the bilobe structure adjacent to the proximal region of the flagellum attachment zone (FAZ) filament. During G2 and mitotic phases, TbPLK is enriched at the flagellar basal body and the bilobe structure and appears to be required to maintain flagellum adhesion. Phosphorylation of TbCentrin2 by TbPLK occurs only on the bilobe structure and appears to be required to maintain flagellum attachment (16). However, RNAi of TbCentrin2 does not cause flagellum detachment (17). To better understand the function of TbPLK in regulating basal body segregation, flagellum attachment, and cytokinesis, we sought to identify additional substrates of TbPLK and characterize their function. Through yeast two-hybrid screening using TbPLK-K70R, a kinase-dead mutant of TbPLK (14), and the PBD as baits, we identified 12 proteins as potential TbPLK-associated proteins and substrates. In this study, we report the functional characterization of one of these proteins, SPBB1, and its interplay with TbPLK. Through biochemical approaches, we demonstrate the *in vitro* and *in vivo* interactions between SPBB1 and TbPLK and verify SPBB1 as an *in vitro* TbPLK substrate. We also demonstrate that SPBB1 is a novel basal body protein and co-localizes with TbPLK during early cell cycle stages. Importantly, RNAi against SPBB1 recapitulates the defects caused by TbPLK depletion, *i.e.* inhibition of basal body segregation, detachment...
of the flagellum, and defective cytokinesis. Finally, we show that RNAi of SPBB1 restricts TbPLK at the basal body and the bilobe structure, where the latter constitutively phosphorylates TbCentrin2 at the bilobe.

**Experimental Procedures**

Yeast Two-hybrid Screening and Directional Yeast Two-hybrid Assay—To construct the Gal4 activation domain (AD) fusion library for two-hybrid screening, trypanosome total RNA was purified and used to generate a cDNA library cloned in the pGADT7 vector using the Matchmaker™ library construction and screening kit (Clontech). The full-length coding sequence of the kinase-dead mutant TbPLK-K70R and the sequence encoding the PBD of TbPLK (PBD<sub>TbPLK</sub>) were each cloned into pGBK7 vector for expression of Gal4 binding domain fusion proteins (bait). The Gal4 AD fusion library was transformed into strain AH109 (mating type α), whereas the bait plasmids (pGBK-TbPLK-K70R and pGBK-PBD<sub>TbPLK</sub>) were transformed into strain Y187 (mating type α). After mating the haploids, the diploids were plated on SD-Leu-Trp-His plates to screen for positive clones.

For directional yeast two-hybrid assay, the full-length coding sequence of SPBB1 was cloned into the pGADT7 vector for expression of Gal4 AD-fused SPBB1 (prey). Full-length TbPLK, TbPLK-K70R, and the PBD alone were each cloned into the pGBK7 vector to express Gal4 binding domain-fused proteins (bait). The prey plasmid was transformed into strain AH109, and the bait plasmids were transformed into strain Y187. The yeast strains carrying both the bait and the prey plasmids were obtained by mating the two haploids at 30 °C overnight, plating the diploid on SD-Leu-Trp-His plates, and incubating them at 30 °C for 2–3 days. Each combination strain was spotted in three 10-fold serial dilutions onto SD-Leu-Trp and SD-Leu-Trp-His plates, and the growth of yeast on SD-Leu-Trp-His plate indicates the interaction between the bait and the prey proteins.

Purification of GST Fusion Proteins, GST Pulldown, and in Vitro Kinase Assay—The full-length coding sequence of SPBB1 was cloned into the pGEX-4T-3 vector for expression of recombinant GST-SPBB1 in bacteria. However, the recombinant protein was insoluble. We therefore expressed GST-fused SPBB1 truncations, SPBB1-N (amino acids 1–500) and SPBB1-C (amino acids 501–980), in bacteria. Recombinant GST-SPBB1-N and GST-SPBB1-C were expressed in *Escherichia coli* BL21 cells and purified through a column of glutathione-Sepharose 4B beads (GE Healthcare). For GST pulldown, trypanosome cells overexpressing TbPLK-3HA or TbPLK-K70R-3HA were lysed in trypanosome lysis buffer (25 mM Tris-HCl, pH 7.6, 500 mM NaCl, 1 mM DTT, 1% Nonidet P-40, and protease inhibitor cocktail) on ice for 30 min and cleared by centrifugation at the highest speed in a microcentrifuge. The cleared lysate (500 μl) was then incubated with GST-fused SPBB1-N or SPBB1-C or GST bound to glutathione-Sepharose 4B beads at room temperature for 1 h. The beads were then washed six times with the lysis buffer, and bound proteins were eluted by boiling the beads in SDS-PAGE sampling buffer for 5 min and separated on SDS-PAGE. Western blotting was then carried out with anti-HA antibody to detect TbPLK-3HA and TbPLK-K70R-3HA.

The full-length coding sequence of TbPLK was cloned into pET41 (18), and recombinant GST-TbPLK was purified from the soluble fraction. Purified recombinant proteins (GST-TbPLK and GST-SPBB1-C) were dialyzed against 50 mM Tris-Cl, pH 7.6, and 50 mM NaCl. Purified GST fusion proteins were incubated in kinase buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, and 1 mM DTT) containing 1 μCi of [γ-32P]ATP at room temperature for 60 min. Reactions were stopped by adding 1x SDS-PAGE sampling buffer and boiling for 5 min. Proteins were separated on SDS-PAGE, and the gel was exposed to x-ray film. GST-SPBB1-C was detected by Coomassie Blue staining of the SDS-PAGE gel after exposure. GST-TbPLK was detected by Western blotting with anti-GST antibody due to its low abundance.

Trypanosome Cell Culture and RNAi—The procyclic trypanosome strain 29-13 (19) was cultured at 27 °C in SDM-79 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc.), 15 μg/ml G418, and 50 μg/ml hygromycin B. The procyclic trypanosome strain 427 was maintained in SDM-79 medium containing 10% fetal bovine serum. Cells were routinely diluted when the density reached 5 × 10⁶/ml.

To silence SPBB1 by RNAi, a 530-bp DNA fragment corresponding to the N-terminal coding region of SPBB1 was PCR-amplified and cloned into the pZM4 vector (20). The resulting plasmid was linearized with NotI digestion and transfected into the 29-13 cell line. Transfectants were selected under 2.5 μg/ml phelemycin and cloned by limiting dilution on a 96-well plate. To induce RNAi, the transfectants were incubated with 1.0 μg/ml tetracycline, and cell growth was monitored daily by counting the cell number with a hemocytometer.

Epitope Tagging of Endogenous Proteins—A 500-bp DNA fragment corresponding to the N-terminal coding region of SPBB1 was cloned into pN-3HA-PAC and pN-PTP-PAC for N-terminal tagging of SPBB1 at the endogenous locus. The resulting constructs were linearized with appropriate restriction enzymes and transfected into the 427 cell line. Transfectants were selected under 1.0 μg/ml puromycin and cloned by limiting dilution in a 96-well plate. Subsequently, the pC-TbPLK-3HA-Neo vector (18) was linearized and transfected into the cells harboring the pN-PTP-SPBB1-PAC construct, and the transfectants were selected under 40 μg/ml G418 in addition to 1 μg/ml puromycin and cloned by limiting dilution.

Generation of Anti-TbPLK Antibody—To generate anti-TbPLK polyclonal antibody, DNA sequence encoding the Polo box domain of TbPLK was cloned into pET26 for expression of recombinant His-tagged PBD<sub>TbPLK</sub> in bacteria. The recombinant protein was purified under denaturing conditions and used to immunize rabbits to generate polyclonal antibody at Cocalico Biologicals, Inc. (Reamstown, PA). The anti-TbPLK serum was used directly for Western blotting and immunofluorescence.

Co-Immunoprecipitation and Western Blotting—Cells (10⁷) expressing N-terminal PTP-tagged SPBB1 were harvested by centrifugation, lysed in 1 ml of lysis buffer (25 mM Tris-HCl, pH 7.6, 500 mM NaCl, 1 mM DTT, 1% Nonidet P-40, and protease inhibitor cocktail), and incubated with 10 μl of settled IgG-Sepharose 6 Fast Flow beads (GE Healthcare) at 4 °C for 1 h. The beads were then washed six times with the immunopre-
A Novel TbPLK Substrate

Putative TbPLK-associated proteins and substrates identified by yeast two-hybrid screening

| Accession No. | MM | Protein description | K70R | PBD | Total |
|--------------|----|---------------------|------|-----|-------|
| Tb927.10.4620 | 13.6 | FKBP-type peptidyl-prolyl cis-trans isomerase | 2 | 12 | 14 |
| Tb927.8.6510 | 22.9 | Ubiquitin-conjugating enzyme | 2 | 2 | 4 |
| Tb927.6.2790 | 36 | L-Threonine 3-dehydrogenase | 1 | 1 | 2 |
| Tb927.8.7730 | 65.1 | Hypothetical protein | 2 | 2 | 4 |
| Tb927.10.14030 | 50.4 | Hypothetical protein | 8 | 8 | 16 |
| Tb927.11.6590 | 55.3 | Aminopeptidase, putative | 1 | 1 | 2 |
| Tb927.5.2150 | 60 | Hypothetical protein | 1 | 1 | 2 |
| Tb927.7.3100 | 69.7 | Hypothetical protein | 4 | 4 | 8 |
| Tb927.11.15800 | 89.6 | Hypothetical protein | 1 | 1 | 2 |
| Tb927.6.900 | 112 | SPBB1 | 1 | 1 | 2 |
| Tb927.10.15080 | 180.2 | Hypothetical protein | 1 | 1 | 2 |
| Tb927.6.620 | 301.8 | Hypothetical protein | 4 | 3 | 7 |

**Results**

**Identification of TbPLK-associated Proteins and Substrates by Yeast Two-hybrid Screening**—To identify potential substrates of TbPLK, we employed yeast two-hybrid screening using a kinase-dead mutant TbPLK (TbPLK-K70R) and the PBD as baits. There are advantages of using kinase-dead mutant and the PBD as baits in yeast two-hybrid screening for identification of TbPLK substrates. First, because kinase-substrate interaction is transient and the substrates often dissociate from the kinase immediately after being phosphorylated, using kinase-dead mutant kinase as the bait in yeast two-hybrid screening may trap the substrate on the kinase, thus allowing detection of kinase-substrate interaction. Second, the PBD is known to mediate interaction between PLK and its substrates (3–6). Therefore, using PBD as the bait may identity potential TbPLK substrates. However, it should be noted that some proteins identified in this screen might be TbPLK-interacting proteins instead of substrates.

A total of 43 positive clones were identified, of which 35 clones were identified by interaction with the PBD and 8 clones were identified by interaction with TbPLK-K70R. PCR amplification of the DNA insert in these clones identified 12 distinct genes, eight of which encode proteins of unknown function (Table 1). Because TbPLK plays unusual roles in trypanosomes, we focused on the eight novel proteins. Our preliminary RNAi experiments showed that Tb927.6.900 is essential in procyclic trypanosomes and, therefore, it was characterized in detail. Tb927.6.900 encodes an ~112-kDa protein of 980 residues, and was named SPBB1 for substrate of Polo-like kinase in the basal body 1. It contains mostly the coiled-coil motifs (from amino acids 140 to 740), and is only conserved in the kinetoplastida parasites (*T. brucei*, *Trypanosoma cruzi*, and *Leishmania*).

**SPBB1 Is an in Vitro Substrate of TbPLK**—In the yeast two-hybrid screen, SPBB1 was identified through interaction with TbPLK-K70R, but not the PBD (Table 1). To confirm the interaction between SPBB1 and TbPLK-K70R and to test whether SPBB1 interacts with wild-type TbPLK, we carried out directional yeast two-hybrid experiments. The results showed that...
SPBB1 only interacts with TbPLK-K70R, but not wild-type TbPLK and the PBD of TbPLK in yeast (Fig. 1A), which further validated our yeast two-hybrid screen result. We next carried out GST pulldown experiments to examine the in vitro interaction between SPBB1 and both TbPLK and TbPLK-K70R. Because the recombinant full-length SPBB1 was insoluble in E. coli, we purified truncations of SPBB1 and found that the C-terminal fragment (amino acids 501–980) of SPBB1 (designated SPBB1-C) was able to pull down both TbPLK and TbPLK-K70R, with more TbPLK-K70R precipitated by GST-fused SPBB1-C (Fig. 1B). This result suggests that the C-terminal fragment of SPBB1 mediates the interaction with TbPLK and confirms that SPBB1 interacts with wild-type TbPLK in vitro. To investigate whether SPBB1 and TbPLK interact in vivo in trypanosomes, we carried out co-immunoprecipitation. To this end, SPBB1 was endogenously tagged with a PTP (protein A-TEV-protein C) epitope (26) at its N terminus. Immunoprecipitation of PTP-SPBB1 was capable of pulling down TbPLK from the trypanosome cell lysate. In G2, TbPLK was detected as two closely associated bright fluorescence spots, which represent a mature basal body and its associated probasal body as marked by TbSAS-6 (Fig. 2A). From G2 phase and thereafter, TbPLK was only detected at the anterior tip of the new FAZ filament and did not co-localize with SPBB1, which remained in the basal body until the end of mitosis (Fig. 2B). The localization of SPBB1 to the basal body and the co-localization between SPBB1 and TbPLK in the basal body were further confirmed with PTP-tagged SPBB1 (Fig. 2C).

RNAi of SPBB1 Causes Defective Cytokinesis and Flagellum Detachment—To investigate the function of SPBB1, RNAi was carried out in the procyclic form of T. brucei. To monitor the efficiency of RNAi, SPBB1 was endogenously tagged with an N-terminal PTP epitope in SPBB1 RNAi cells, and Western blotting showed that after tetracycline induction for 3 days, the level of PTP-SPBB1 was decreased to about 30% of that in the
control cells (Fig. 3A, inset). This depletion of SPBB1 caused severe growth defect (Fig. 3A), suggesting that SPBB1 is essential for cell proliferation in the procyclic form. Non-induced control and RNAi-induced cells were tabulated for the numbers of cells with different numbers of nucleus (N) and kinetoplast (K), and the results showed that upon SPBB1 RNAi induction for up to 4 days, cells with two nuclei and one kinetoplast (2N1K) and cells with multiple nuclei (>2N) gradually emerged to ~14 and ~25%, respectively (Fig. 3B), suggesting an inhibition of kinetoplast duplication or segregation and defective cytokinesis. Additionally, flagellum detachment was clearly detectable in SPBB1 RNAi cells, and at day 6 of RNAi, ~78% of the cells possessed at least one detached flagellum (Fig. 3C). It appeared that the new flagellum was often detached in 2N1K and multi-nucleated (>2N) cells (Fig. 3D).

**SPBB1 Is Required for New FAZ Filament Assembly**—The flagellum in a trypanosome cell is attached to the cell body via the FAZ filament (27), and defects in the FAZ filament cause flagellum detachment (28–31). To examine whether flagellum detachment in SPBB1 RNAi cells was caused by defective assembly of the FAZ filament, cells were immunostained with the FITC-conjugated anti-HA mAb and anti-TbSAS-6 pAb, and then counterstained with DAPI for DNA. DIC, differential interference contrast. Scale bar: 5 μm. B, localization of 3HA-SPBB1 and TbPLK during the cell cycle. Cells were co-immunostained with the FITC-conjugated anti-HA mAb and anti-TbPLK pAb, and then counterstained with DAPI for DNA. Scale bar: 5 μm. C, co-localization of PTP-SPBB1 and TbPLK-3HA in the basal body. SPBB1 was endogenously tagged with the PTP epitope at the N terminus in the same cell line expressing endogenously 3HA-tagged TbPLK. Cells were co-immunostained with the FITC-conjugated anti-HA mAb and anti-protein A pAb to detect TbPLK-3HA and PTP-SPBB1, respectively. Scale bar: 5 μm.

**RNAi of SPBB1 Inhibits Basal Body Separation**—Because the kinetoplast is physically attached to the basal body (32) and segregation of the duplicated kinetoplasts is mediated by basal body separation (33), we reasoned that the emergence of 2N1K cells upon SPBB1 RNAi was due to defective duplication or segregation of basal bodies. To test this hypothesis, we co-immunostained the cells with the YL 1/2 antibody, which labels the transition fibers near the mature basal body and serves as a marker for the mature basal body (34, 35), and anti-TbSAS-6, which detects the basal body cartwheel protein in both the mature basal body and the probasal body (25). We then quantified the numbers of mature basal bodies (mBB) and total basal bodies (BB) in 2N1K, 2N2K, and multi-nucleated (>2N) cells from SPBB1 RNAi and in 2N2K cells from the non-induced control. As expected, the 2N2K cells from the non-induced control all contained two mature basal bodies labeled by YL 1/2
and four basal bodies (both mature and probasal bodies) labeled by TbSAS-6 (Fig. 5, A and B). The two pairs of basal bodies in control 2N2K cells were normally separated, with an average distance of ~5.8 μm (Fig. 5, A, C, and D). In SPBB1-deficient 2N1K cells, ~40% of them contained one mature basal body and an associated probasal body, whereas the rest of them contained two mature basal bodies and two associated probasal bodies, but the two pairs of basal bodies were not well separated in these cells (Fig. 5, A and B). In SPBB1-deficient 2N2K cells, all of them contained two mature basal bodies and two associated probasal bodies, which were also not well separated (Fig. 5, A and B). For the 2N1K and 2N2K cells with two mature basal bodies (2mBB), the average distance between the mature basal bodies was calculated to be ~2.4 μm, which is less than half of the inter-basal body distance in the control 2N2K cells (Fig. 5, C and D). These results suggest that SPBB1 RNAi inhibited the separation of the duplicated basal bodies, thereby inhibiting kinetoplast segregation.

\textbf{TbPLK Is Not Required for SPBB1 Localization to the Basal Body—}Given that SPBB1 is phosphorylated by TbPLK, we asked whether phosphorylation of SPBB1 by TbPLK is required for SPBB1 localization. To test this possibility, we treated the cells stably expressing PTP-tagged SPBB1 with GW843286X, a small molecule inhibitor developed against human Polo-like kinase 1 (21). This molecule also showed potent inhibition of trypanosome TbPLK RNAi cells (Fig. 6A), suggesting that TbPLK activity is not required for targeting SPBB1 to the basal body. Furthermore, to test whether the interaction with TbPLK recruits SPBB1 to the basal body, PTP-SPBB1 was expressed endogenously in TbPLK RNAi cells, and immunostaining showed that PTP-SPBB1 was still localized to the basal body (Fig. 6B), suggesting that depletion of TbPLK did not abolish SPBB1 localization. Together, these results suggest that localization of SPBB1 to the basal body is independent of TbPLK activity and interaction with TbPLK.

\textbf{Depletion of SPBB1 Restricts TbPLK in the Basal Body and the Bilobe Structure—}To test whether SPBB1 RNAi might affect the localization of TbPLK, non-induced control and SPBB1 RNAi cells induced for 4 days were co-immunostained with anti-TbPLK antibody and the 20H5 antibody, which stains the basal body, PTP-SPBB1 was expressed endogenously in TbPLK RNAi cells, and immunostaining showed that PTP-SPBB1 was still localized to the basal body (Fig. 6B), suggesting that depletion of TbPLK did not abolish SPBB1 localization. Together, these results suggest that localization of SPBB1 to the basal body is independent of TbPLK activity and interaction with TbPLK.

\textbf{FIGURE 3. SPBB1 RNAi causes cytokinesis defects and flagellum detachment.} A, RNAi of SPBB1 caused growth defects. \textit{Inset} shows the Western blot to monitor the level of PTP-SPBB1, which was endogenously tagged in the SPBB1 RNAi cell line, before and after tetracycline (Tet) induction of RNAi. The level of the α-6 subunit of the 26S proteasome (TbPSA6) was included as the loading control. B, tabulation of cells with different numbers of kinetoplast (K) and nucleus (N) before and after SPBB1 RNAi for up to 4 days. About 200 cells were counted from each time point. \textit{Error bars} represent S.D. calculated from three independent experiments. C, the percentage of cells with detached flagellum upon SPBB1 RNAi. About 200 cells were counted from each time point, and \textit{error bars} represent S.D. calculated from three independent experiments. ***, \( p < 0.001 \). D, morphology of SPBB1 RNAi cells, showing the 2N1K and XN1K cells with a detached new flagellum (arrows). DIC, differential interference contrast. Scale bar: 5 μm.
In the bilobe, TbPLK is known to phosphorylate the bilobe-resident TbCentrin2 at serine 54 during late G1 and early S-phase of the cell cycle, but not at late S-phase and beyond (16). Importantly, expression of phospho-deficient and phospho-mimic mutants of TbCentrin2, TbCentrin2-S54A and TbCentrin2-S54E, caused defective FAZ filament assembly and flagellum detachment (16). The enrichment of TbPLK in the bilobe in 2N1K and 2N2K cells upon SPBB1 RNAi (Fig. 7A) prompted us to investigate whether TbCentrin2 was still phosphorylated in these cells. To this end, non-induced control and SPBB1 RNAi cells were immunostained with the PS54 antibody, which detects the TbPLK-phosphorylated Ser-54 residue in TbCentrin2 (16). We confirmed that in the non-induced control, TbCentrin2 was phosphorylated in the bilobe in G1/S (1N1K) cells but not in mitotic (2N2K) cells (Fig. 7, C and D). However, in the majority (~81%) of the SPBB1-deficient 2N1K and 2N2K cells, TbCentrin2 was phosphorylated at Ser-54 in the two segregated bilobe structures (Fig. 7, C and D), presumably because TbPLK remained at the bilobe structures (Fig. 7A) and phosphorylated TbCentrin2 at Ser-54 in these cells. Together, these results suggest that RNAi of SPBB1 confined TbPLK to the basal body and the bilobe structure, where TbPLK constitutively phosphorylated TbCentrin2 in mitotic and post-mitotic cells.

**Discussion**

In this study, we reported the identification and functional characterization of a new TbPLK substrate, SPBB1, in *T. brucei*. SPBB1 is the first basal body protein identified as a TbPLK substrate. TbCentrin2, a bilobe protein involved in bilobe biogenesis, Golgi duplication, and flagellum adhesion, is also a TbPLK substrate (8, 16, 17). TbPLK is known to differ significantly from its yeast and animal homologs in terms of subcellular localization and cellular functions (7–9, 15), and previous studies have demonstrated that TbPLK is required for basal body segregation, bilobe duplication, flagellum attachment, and cytokinesis initiation (7, 8, 14, 15, 36). Given its localization to multiple subcellular structures and its multiple roles in organelle biogenesis, TbPLK may phosphorylate proteins located in the basal body, the bilobe structure, and the anterior tip of the new FAZ filament. Although our yeast two-hybrid screening with TbPLK-K70R and PBD as baits has identified several TbPLK-interacting partners (Table 1), their candidacy as TbPLK substrates remains to be experimentally verified. Using proximity-dependent biotin identification (BioID) and stable isotope labeling in the cell culture (SILAC) coupled with quantitative mass spectrometry, a recent study identified a few basal body proteins as TbPLK close neighbors and identified several other proteins as TbPLK substrates (37). This study confirms that TbPLK regulates many downstream factors in the basal body, the bilobe structure, and the FAZ filament.

RNAi against SPBB1 recapitulated the defects caused by TbPLK RNAi in procyclic trypanosomes, although the defects appeared to be less severe than that caused by TbPLK RNAi, presumably because TbPLK has additional downstream targets, including TbCentrin2, which may play redundant roles in executing TbPLK functions. As a support of this assumption, mutation of the TbPLK-phosphorylated Ser-54 in TbCentrin2 disrupted the assembly of the new FAZ filament, leading to detachment of the new flagellum, and caused defects in bilobe duplication and cytokinesis in *T. brucei* (16). It is notable that...
expression of TbCentrin2-S54A or TbCentrin2-S54E also caused less severe defects than TbPLK RNAi (16). It is also interesting to note that despite the distinct subcellular localizations of SPBB1 and TbCentrin2, deficiency in SPBB1 and deficiency in TbPLK-mediated TbCentrin2 phosphorylation caused similar phenotypes, i.e. malformation of the new FAZ filament, flagellum detachment, inhibition of basal body segregation, and defective cytokinesis (Figs. 3–5). However, SPBB1 RNAi appeared to exert little effect on bilobe duplication, although bilobe segregation appeared to be inhibited in the 2N1K cells (Fig. 7, A and C). In contrast, expression of TbCentrin2-S54A and TbCentrin2-S54E inhibited bilobe duplication (16). This difference in bilobe duplication/segregation defects between SPBB1 RNAi and TbCentrin2-S54A/S54E mutants is...
likely to be attributed to the fact that TbCentrin2 is localized in the bilobe, in addition to the basal body and the flagellum (16, 36), whereas SPBB1 is localized only in the basal body (Fig. 2). Therefore, defective segregation of the duplicated bilobe structures in SPBB1 RNAi cells is likely an indirect effect, presumably due to the inhibited segregation of basal bodies.

Duplication of the flagellar basal body represents one of the earliest cytoskeletal events in the trypanosome cell cycle. A number of proteins have been localized to the basal body in *T. brucei*, but only a few of them are localized to both the probasal body and the mature basal body, which includes TbCentrin2 (17, 36), TbCentrin4 (38), TbSAS-6 (25), and an unknown protein that is recognized by a monoclonal antibody BBA4 (39). Centrins and SAS-6 are evolutionarily conserved components of the centriole/basal body, and are present in all of the eukaryotic organisms investigated so far (40, 41). However, SPBB1 appears to be unique to the kinetoplastida parasites; therefore, its function might also be specific to these early diverged organisms. Unlike TbCentrin2, which is required for basal body biogenesis (17), SPBB1 is required for segregation of the duplicated basal bodies (Fig. 5), but its precise role in basal body segregation remains unclear. In *T. brucei*, segregation of basal bodies appears to be coupled with the elongation of the new FAZ filament during the cell cycle (42). SPBB1 RNAi also caused a defective assembly/elongation of the new FAZ filament (Fig. 4) in addition to the inhibition of basal body segregation (Fig. 5). These results further confirmed that basal body segregation and new FAZ elongation are well coordinated in *T. brucei*.

An unexpected but intriguing observation made in this study is the restriction of TbPLK in the basal body and the bilobe, leading to constitutive TbCentrin2 phosphorylation by TbPLK. A, effect of SPBB1 RNAi on TbPLK localization. Non-induced control and SPBB1 RNAi-induced (4-day) cells were immunostained with anti-TbPLK pAb and 20H5, which labels the basal body, the bilobe structure, and the flagellum in trypanosomes. Scale bar: 5 μm. B, quantification of 2N cells (2N2K cells from the control and 2N1K and 2N2K cells from SPBB1 RNAi) with different TbPLK localization patterns. About 200 cells were counted, and error bars represent S.D. calculated from three independent experiments. ***, p < 0.001. C, effect of SPBB1 RNAi on TbPLK-mediated TbCentrin2 phosphorylation. Non-induced control and SPBB1 RNAi-induced (4-day) cells were immunostained with PS54 for Ser-54-phosphorylated TbCentrin2 and with 20H5 for the bilobe structure. Scale bar: 5 μm. D, quantification of 2N cells (2N2K cells from the control and 2N1K and 2N2K cells from SPBB1 RNAi) with different PS54 (Ser-54-phosphorylated TbCentrin2) localization patterns. About 200 cells were counted, and error bars represent S.D. calculated from three independent experiments. ***, p < 0.001.
prevented TbPLK from being targeted from the basal body and the bilobe to the anterior tip of the new FAZ filament. It is likely that the failure for TbPLK to localize to the anterior tip of the new FAZ was due to the absence of new FAZ filament assembly, thus restricting TbPLK at the basal body and the bilobe structure. In this regard, this defect in TbPLK localization could be an indirect effect.

Although we have proven the candidacy of SPBB1 as an in vitro TbPLK substrate and have demonstrated the essential role of SPBB1 in basal body segregation and FAZ filament assembly, the mechanistic role(s) of SPBB1 in regulating these cellular processes and how TbPLK regulates SPBB1 to execute its function remain unclear. Through immunoprecipitation and mass spectrometry, we have identified nine in vivo phosphorylation sites in SPBB1 (Ser-265, Ser-276, Thr-647, Thr-659, Ser-742, Ser-818, Ser-892, Ser-900, and Ser-901). Notably, seven out of the nine phosphosites are located in the C terminus of SPBB1, which was used for the in vitro kinase assay (Fig. 1D). Future work will be directed to investigate whether phosphorylation of SPBB1 by TbPLK is required for TbPLK function. Given that SPBB1 only contains the coiled-coil motifs, which are known to be involved in protein-protein interactions (43), SPBB1 is unlikely to possess any catalytic activity. It could serve as a scaffold in a multi-protein complex, although this still needs further investigation. Moreover, because inhibition of TbPLK activity by a small molecule inhibitor or depletion of TbPLK by RNAi did not abolish SPBB1 targeting to the basal body (Fig. 6), this suggests that the physical interaction with TbPLK and the activity of TbPLK are not required for SPBB1 localization. SPBB1 might just serve as one of the downstream effectors of TbPLK to execute the function of TbPLK in controlling basal body segregation and assembling the new FAZ filament to maintain flagellar adhesion.

Author Contributions—Z. L. conceived and designed the experiments; H. H. and Q. Z. performed the experiments; H. H., Q. Z., and Z. L. analyzed the data; and Z. L. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We are grateful to Dr. George Cross of Rockefeller University for providing the 29-13 cell line, Dr. Paul Englund of Johns Hopkins Medical School for providing the pZJM vector, Dr. Arthur M. Noro, N., Furuta, M., Emmitte, K. A., Gilmer, T. M., Mook, R. A., Jr., and Cheung, M. (2007) Identification of a novel chromosomal passenger complex and its unique localization during cytokinesis in Trypanosoma brucei. PLoS Pathog. 5, e1000575

References
1. Song, S., Grenfell, T. Z., Garfield, S., Erikson, R. L., and Lee, K. S. (2000) Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. Mol. Cell. Biol. 20, 286–298
2. Reynolds, N., and Okhura, H. (2003) Polo boxes form a single functional domain that mediates interactions with multiple proteins in fission yeast polo kinase. J. Cell Sci. 116, 1377–1387
3. Kumar, P., and Wang, C. C. (2006) Dissociation of cytokinesis initiation from mitotic control in a eukaryote. Eukaryot. Cell 5, 92–102
4. de Graffenried, C. L., Ho, H. H., and Warren, G. (2008) Polo-like kinase is required for Golgi and bilobe biogenesis in Trypanosoma brucei. J. Cell Biol 181, 431–438
5. Li, Z., Umeyama, T., and Wang, C. C. (2008) Polo-like kinase is expressed in S/G2/M phase and associated with the flagellum attachment zone in both procyclic and bloodstream forms of Trypanosoma brucei. Eukaryot. Cell 7, 1582–1590
6. Vaughan, S., and Gull, K. (2003) The trypanosome flagellum. J. Cell Sci. 116, 757–759
7. Briggs, L. J., McKeen, P. G., Baines, A., Moreira-Leite, F., Davidge, J., Vaughan, S., and Gull, K. (2004) The flagella connector of Trypanosoma brucei: an unusual mobile transmembrane junction. J. Cell Sci. 117, 1641–1651
8. Li, Z., Lee, J. H., Chu, F., Burlingame, A. L., Günzl, A., and Wang, C. C. (2008) Identification of a novel chromosomal passenger complex and its unique localization during cytokinesis in Trypanosoma brucei. PLoS One 3, e2354
9. Li, Z., Umeyama, T., and Wang, C. C. (2009) The Aurora kinase in Trypanosoma brucei plays distinctive roles in metaphase-anaphase transition and cytokinetic initiation. PLoS Pathog. 5, e1000575
10. Yu, Z., Liu, Y., and Li, Z. (2012) Structure-function relationship of the Polo-like kinase in Trypanosoma brucei. J. Cell Sci. 125, 1519–1530
11. Hammarton, T. C., Kramer, S., Tetley, L., Boshart, M., and Mottram, J. C. (2007) Trypanosoma brucei Polo-like kinase is essential for basal body duplication, kDNA segregation and cytokinesis. Mol. Microbiol. 65, 1229–1248
12. de Graffenried, C. L., Anrather, D., Von Raußendorf, F., and Warren, G. (2013) Polo-like kinase phosphorylation of bilobe-resident TbCentrin2 facilitates flagellar inheritance in Trypanosoma brucei. Mol. Biol. Cell 24, 1947–1963
13. He, C. Y., Pypaert, M., and Warren, G. (2005) Cytoskeletal role of the C-terminal domain of mammalian polo-like kinase. Mol. Biochem. Parasitol. 99, 89–101
14. Kohl, L., Sherwin, T., and Gull, K. (1999) Assembly of the paraflagellar rod and the flagellar attachment zone complex during the Trypanosoma brucei cell cycle. J. Eukaryot. Microbiol. 46, 105–109
15. Lansing, T. J., McConnell, R. T., Duckett, D. R., Spehar, G. M., Knick, V. B., Hassler, D. F., Noro, N., Furuta, M., Emmitte, K. A., Gilmer, T. M., Mook, R. A., Jr., and Cheung, M. (2007) In vitro biochemical activity of a novel small-molecule inhibitor of polo-like kinase 1. Mol. Cancer Ther. 6, 450–459
16. Kohl, L., Shriver, T., and Gull, K. (1999) Assembly of the paraflagellar rod and the flagellar attachment zone complex during the Trypanosoma brucei cell cycle. J. Eukaryot. Microbiol. 46, 105–109
17. Kilmartin, J. V., Wright, B., and Milstein, C. (1982) Rat monoclonal anti-centrosomal centrin is confined to the distal lumen of centrioles. J. Cell Sci. 576–582
18. de Graffenried, C. L., Anrather, D., Von Raußendorf, F., and Warren, G. (2013) Polo-like kinase phosphorylation of bilobe-resident TbCentrin2 facilitates flagellar inheritance in Trypanosoma brucei. Mol. Biol. Cell 24, 1947–1963
19. He, C. Y., Pypaert, M., and Warren, G. (2005) Polo-like kinase in Trypanosoma brucei plays distinctive roles in metaphase-anaphase transition and cytokinetic initiation. PLoS Pathog. 5, e1000575
20. Wang, Z., Morris, J. C., Drew, M. E., and Englund, P. T. (2000) Inhibition of Trypanosoma brucei gene expression by RNA interference using an integratable vector with opposing T7 promoters. J. Biol. Chem. 275, 40174–40179
21. Kilmartin, J. V., Wright, B., and Milstein, C. (1982) Rat monoclonal anti-centrosomal centrin is confined to the distal lumen of centrioles. J. Cell Sci. 576–582
22. Kohl, L., Sherwin, T., and Gull, K. (1999) Assembly of the paraflagellar rod and the flagellar attachment zone complex during the Trypanosoma brucei cell cycle. J. Eukaryot. Microbiol. 46, 105–109
23. Kilmartin, J. V., Wright, B., and Milstein, C. (1982) Rat monoclonal anti-centrosomal centrin is confined to the distal lumen of centrioles. J. Cell Sci. 576–582
24. Kilmartin, J. V., Wright, B., and Milstein, C. (1982) Rat monoclonal anti-centrosomal centrin is confined to the distal lumen of centrioles. J. Cell Sci. 576–582
biogenesis and flagellum assembly. *Eukaryot. Cell* **14**, 898–907, 10.1128/EC.00083-15

26. Schimanski, B., Nguyen, T. N., and Günzl, A. (2005) Highly efficient tandem affinity purification of trypanosome protein complexes based on a novel epitope combination. *Eukaryot. Cell* **4**, 1942–1950

27. Gull, K. (1999) The cytoskeleton of trypanosomatid parasites. *Annu. Rev. Microbiol.* **53**, 629–655

28. Zhou, Q., Liu, B., Sun, Y., and He, C. Y. (2011) A coiled-coil- and C2-domain-containing protein is required for FAZ assembly and cell morphology in *Trypanosoma brucei*. *J. Cell Sci.* **124**, 3848–3858

29. Vaughan, S., Kohl, L., Ngai, I., Wheeler, R. J., and Gull, K. (2008) A repetitive protein essential for the flagellum attachment zone filament structure and function in *Trypanosoma brucei*. *Protist* **159**, 127–136

30. LaCount, D. J., Barrett, B., and Donelson, J. E. (2002) *Trypanosoma brucei* FLA1 is required for flagellum attachment and cytokinesis. *J. Biol. Chem.* **277**, 17580–17588

31. Zhou, Q., Hu, H., He, C. Y., and Li, Z. (2015) Assembly and maintenance of the flagellum attachment zone filament in *Trypanosoma brucei*. *J. Biol. Chem.* **280**, 501–510

32. Ogbadoyi, E. O., Robinson, D. R., and Gull, K. (2003) A high-order transmembrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar basal bodies in trypanosomes. *Mol. Biol. Cell* **14**, 1769–1779

33. Robinson, D. R., and Gull, K. (1991) Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle. *Nature* **352**, 731–733

34. Sherwin, T., Schneider, A., Sasse, R., Seebeck, T., and Gull, K. (1987) Distinct localization and cell cycle dependence of COOH terminally tyrosinated α-tubulin in the microtubules of *Trypanosoma brucei*. *J. Cell Biol.* **104**, 439–446

35. Stephan, A., Vaughan, S., Shaw, M. K., Gull, K., and McKean, P. G. (2007) An essential quality control mechanism at the eukaryotic basal body prior to intraflagellar transport. *Traffic* **8**, 1323–1330

36. Ikeda, K. N., and de Graffenried, C. L. (2012) Polo-like kinase is necessary for flagellum inheritance in *Trypanosoma brucei*. *J. Cell Sci.* **125**, 3173–3184

37. McAllaster, M. R., Ikeda, K. N., Lozano-Núñez, A., Anrather, D., Unterwurzacher, V., Gossenreiter, T., Perry, J. A., Crickley, R., Mercadante, C. J., Vaughan, S., and de Graffenried, C. L. (2015) Proteomic identification of novel cytoskeletal proteins associated with TbPLK, an essential regulator of cell morphogenesis in *T. brucei*. *Mol. Biol. Cell* **26**, 3013–3029, 10.1091/mbc.E15-04-0219

38. Shi, J., Franklin, J. B., Yelinek, J. T., Ebersberger, I., Warren, G., and He, C. Y. (2008) Centrin4 coordinates cell and nuclear division in *T. brucei*. *J. Cell Sci.* **121**, 3062–3070

39. Woods, A., Sherwin, T., Sasse, R., MacRae, T. H., Baines, A. J., and Gull, K. (1989) Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.* **93**, 491–500

40. Carvalho-Santos, Z., Machado, P., Branco, P., Tavares-Cadete, F., Rodrigues-Martins, A., Pereira-Leal, J. B., and Bettencourt-Dias, M. (2010) Stepwise evolution of the centriole-assembly pathway. *J. Cell Sci.* **123**, 1414–1426

41. Hodges, M. E., Scheumann, N., Wickstead, B., Langdale, J. A., and Gull, K. (2010) Reconstructing the evolutionary history of the centriole from protein components. *J. Cell Sci.* **123**, 1407–1413

42. Absalon, S., Kohl, L., Branche, C., Blisnick, T., Toutirais, G., Rusconi, F., Cosson, J., Bonhivers, M., Robinson, D., and Bastin, P. (2007) Basal body positioning is controlled by flagellum formation in *Trypanosoma brucei*. *PLoS One* **2**, e437

43. Burkhard, P., Stetefeld, J., and Strelkov, S. V. (2001) Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol.* **11**, 82–88