Flagellum inheritance in *Trypanosoma brucei* requires a kinetoplastid-specific protein phosphatase

Revised for publication, April 16, 2018; Papers in Press, April 17, 2018; DOI: 10.1074/jbc.RA118.002106

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Edited by Ronald C. Wek

*Trypanosoma brucei* causes sleeping sickness in humans and nagana in cattle in sub-Saharan Africa and alternates between its mammalian hosts and its insect vector, the tsetse fly. *T. brucei* uses a flagellum for motility, cell division, and cell-cell communication. Proper positioning and attachment of the newly assembled flagellum rely on the faithful duplication and segregation of flagellum-associated cytoskeletal structures. These processes are regulated by the polo-like kinase homolog TbPLK, whose activity and abundance are under stringent control to ensure spatiotemporally regulated phosphorylation of its substrates. However, it remains unclear whether a protein phosphatase that counteracts TbPLK activity is also involved in this regulation. Here, we report that a putative kinetoplastid-specific protein phosphatase, named KPP1, has essential roles in regulating flagellum positioning and attachment in *T. brucei*. KPP1 localized to multiple flagellum-associated cytoskeletal structures and co-localized with TbPLK in several cytoskeletal structures at different cell-cycle stages. KPP1 depletion abolished basal body segregation, inhibited the duplication of the centrin arm and the hook complex of the bilobe structure, and disrupted the elongation of the flagellum attachment zone, leading to flagellum misplacement and detachment and cytokinesis arrest. Importantly, KPP1-depleted cells lacked dephosphorylation of TbCentrin2, a TbPLK substrate, at late cell-cycle stages. Together, these results suggest that KPP1-mediated protein dephosphorylation regulates the duplication and segregation of flagellum-associated cytoskeletal structures, thereby promoting flagellum positioning and attachment. These findings highlight the requirement of reversible protein phosphorylation, mediated by TbPLK and KPP1, in regulating flagellum inheritance in *T. brucei*.

This work was supported by National Institutes of Health R01 Grants AI101437 and AI118736 (to Z. Li). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains Figs. S1 and S2.

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taining flagellum attachment to the cell body (8). TbCentrin2, a TbPLK substrate in the centrin arm (17), promotes bilobe duplication (3). Phosphorylation of TbCentrin2 at Ser-54 by TbPLK is important for FAZ elongation and flagellum attachment, but dephosphorylation of TbCentrin2 at Ser-54 is necessary for FAZ elongation and flagellum attachment (17), indicating that an unidentified protein phosphatase is involved in dephosphorylating TbCentrin2. TbPLK itself is under tight control, as excess TbPLK also abolishes basal body segregation and FAZ elongation (18, 19), in part through constitutively phosphorylating TbCentrin2 in the centrin arm (19).

Reversible protein phosphorylation plays crucial roles in regulating many cellular processes in eukaryotes, and requires a protein kinase and an antagonizing protein phosphatase (20). In humans, the function of the polo-like kinase homolog Plk1 is antagonized by myosin phosphatase-targeting subunit 1 (MYPT1)-regulated protein phosphatase 1 catalytic subunit isoform β (PP1Cβ) at the centrosomes (21) and by B56-regulated protein phosphatase 2A (PP2A) at the kinetochores (22). MYPT1-PP1Cβ dephosphorylates Thr-210 in the T-loop of Plk1 to inactivate Plk1 at the centrosomes (21), whereas B56-PP2A dephosphorylates Plk1 substrates to counteract Plk1 function at the kinetochores (22). TbPLK is also activated by phosphorylation of Thr-198, which is analogous to Thr-210 in Plk1, in its T-loop (16), but neither the protein kinase responsible for Thr-198 phosphorylation nor the protein phosphatase responsible for Thr-198 dephosphorylation have been identified. It is also unclear whether any protein phosphatase in T. brucei antagonizes TbPLK function by dephosphorylating TbPLK substrates, but the requirement for TbCentrin2 dephosphorylation in the mitotic phase (17) suggests the involvement of an unidentified protein phosphatase.

Here we report that a putative kinetoplastid-specific protein phosphatase co-localizes with TbPLK in different flagellum-associated cytoskeletal structures and regulates the duplication and segregation of these cytoskeletal structures, thereby promoting flagellum positioning and adhesion. These findings highlight the involvement of reversible protein phosphorylation in flagellum inheritance in T. brucei.

Results

KPP1 co-localizes with TbPLK at several flagellum-associated cytoskeletal structures

A putative serine/threonine protein phosphatase, encoded by Tb927.5.4380, was previously identified as a near neighbor of CIF1 (23), suggesting that it may localize to the new FAZ tip and plays roles in cytokinesis initiation. It was previously annotated as a kinetoplastid-specific phosphoprotein phosphatase (kPPP) (24). Bioinformatics analysis and homology modeling by SWISS-MODEL (25) revealed an N-terminal domain, which is structurally similar to the Plus3 domain in human RNA polymerase II-associated RTF1 (Fig. 1, A–C), and a C-terminal phosphatase catalytic domain, which exhibits ~28/60% sequence identity/similarity to the human protein phosphatase 1 catalytic subunit isoform γ (PP1Cγ) (Fig. 1, A, D, and E, and Fig. S1) and the eight PP1 homologs and two PP2A homologs from T. brucei (Fig. S1). Given its unusual N-terminal Plus3 domain, the C-terminal protein phosphatase catalytic domain, and the presence of close homologs in other kinetoplastid parasites (Figs. S1 and S2), we named this protein KPP1 for Kinetoplastid-specific Protein Phosphatase 1. The Plus3 domain in human RTF1 consists of six α helices intervened by six β sheets in a mixed α/β-fold (26), and is involved in binding to a phosphothreonine-containing repeat sequence of the transcription elongation factor Spt5 (27). Notably, four of the five residues in the RTF1 Plus3 domain that are involved in direct binding to the phosphothreonine residue of Spt5 are also found in the Plus3 domain of KPP1 (Fig. 1B, blue arrowheads). Therefore, the Plus3 domain in KPP1 may be similarly involved in binding to phosphothreonine-containing proteins in T. brucei.

The modeled catalytic domain of KPP1 adopts a structure composed of an α/β-fold, with a β sandwich situated between two α-helical domains (Fig. 1D), similar to that of the human PP1Cγ (Fig. 1E) and other PP1 catalytic subunit proteins (28). It possesses all six conserved residues (Asp-336, His-338, Asp-371, Asn-403, His-462, and His-557) in the catalytic site (Fig. S1, green boxes) to coordinate two manganese ions, which are located at the three-way joint of the two α-helical domains and the β sandwich (Fig. 1, E and F).

KPP1 was endogenously tagged with a triple HA epitope and its subcellular localization during the cell cycle was determined by immunofluorescence microscopy. At G1 phase, KPP1 localizes to the basal body and the centrin arm, which were labeled with the anti-LdCen1 antibody that detects centrins at the basal body and the centrin arm, and co-localizes with TbPLK at both structures (Fig. 2, A and B). From S phase to mitotic phases, KPP1 localizes to the duplicated basal bodies, the duplicated centrin arms, and the new FAZ tip (Fig. 2, A, C, and D) and co-localizes with TbPLK at the new FAZ tip (Fig. 2A). FAZ was labeled with the anti-CC2D antibody, and basal body was labeled with the anti-TbSAS-6 antibody. In post-mitotic cells during which TbPLK disappears from the new FAZ tip, KPP1 also disappears from the new FAZ tip, but it remains in the basal bodies and the centrin arms (Fig. 2A). Given that the KPP1 fluorescence signal at the new FAZ tip in mitotic cells is much weaker than in pre-mitotic cells (Fig. 2, A and D), it suggests that starting from mitotic phases KPP1 gradually disappears from the new FAZ tip. Additionally, KPP1 is also detected at the flagella connector region (Fig. 2, A and C), where it co-localizes with TbPLK (Fig. 2A). Moreover, KPP1 is also detected as some weaker punctate dots in the cytosol (Fig. 2), suggesting the distribution of KPP1 in some cytosolic compartments.

We also investigated whether mutation of the active sites in the catalytic domain of KPP1 affects KPP1 localization. Immunofluorescence microscopy showed that ectopically expressed WT KPP1, which was tagged with a C-terminal triple HA epitope, displayed normal localization patterns (Fig. 2E). However, ectopically expressed mutant KPP1 that bore point mutations at two of the six conserved active sites (D371N and H557K) localized to the cytosol (Fig. 2E), indicating that the activity of KPP1 is required for KPP1 localization. Altogether, the localization of KPP1 to various flagellum-associated cytoskeletal structures indicates that KPP1 may play roles in the duplication and/or segregation of these cytoskeletal structures.

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J. Biol. Chem. (2018) 293(22) 8508–8520 8509
KPP1 is essential for FAZ elongation and flagellum attachment

The physiological function of KPP1 was investigated by RNAi in the procyclic form of *T. brucei*. Western blotting confirmed the knockdown of KPP1 protein, which was endogenously tagged with a triple HA epitope, after 24 h of RNAi induction (Fig. 3A). KPP1-depleted cells had a growth rate similar to the noninduced control cells up to 48 h of RNAi induction, but stopped growing thereafter (Fig. 3B), suggesting that KPP1 is essential for cell proliferation. Quantitation of the cells containing different numbers of nuclei and kinetoplasts showed that starting from 72 h of RNAi induction, cells with one nucleus and one kinetoplast (1N1K) decreased to ~15% of the total population, and cells with more than two nuclei increased to ~67% of the total population after 96 h of RNAi induction (Fig. 3C), suggesting defective cytokinesis. Moreover, anucleated zoid cells (0N1K) and cells with two nuclei and one kinetoplast (2N1K) also emerged (Fig. 3C), indicating that aberrant cytokinesis occurred in some cells. The 2N1K cells could also be derived from 1N1K cells in the absence of kinetoplast segregation but normal mitosis.

The most notable phenotype caused by KPP1 depletion is flagellum detachment in ~60% of the total cell population after 72 h of RNAi induction (Fig. 3D). Flagellum detachment was observed in almost all cell types (Fig. 3E and F). To investigate whether flagellum detachment is due to the defects in FAZ assembly/elongation, cells were immunostained with anti-FAZ1 antibody, and the number of FAZ was tabulated (Fig. 3, E and F). The results showed that the 1N1K cells with one FAZ were significantly increased after KPP1 RNAi, which was accompanied by a significant decrease of the cells with two FAZs (Fig. 3, E and F). Similarly, the 1N2K and 2N2K cells with only one FAZ or with one short new FAZ (snFAZ) and one normal old FAZ emerged to more than 60% for each cell type, and consequently, the cells with two FAZs were significantly decreased after KPP1 RNAi (Fig. 3, E and F). Strikingly, ~94% of the 2N1K cells from the KPP1 RNAi cells contained either one normal old FAZ or one snFAZ and one normal old FAZ (Fig. 3, E and F). These results demonstrated that assembly/elongation of the new FAZ was disrupted by KPP1 depletion, thus leading to flagellum detachment.

KPP1 is required for proper positioning of the new flagellum

The flagellum was immunostained with L8C4 (anti-PFR2) antibody, which detects the paraflagellar rod 2 (PFR2) protein...
in the flagellum (Fig. 4A), and was tabulated in control and KPP1 RNAi cells. The results showed that formation of the new flagellum was not affected by KPP1 depletion, as the 1N1K cells with two flagella slightly increased and the majority of the 1N2K, 2N2K, and 2N1K cells had two flagella (Fig. 4B). However, the location of the newly assembled flagellum in the bi-flagellated 1N1K, 1N2K, 2N2K, and 2N1K cells collected from KPP1 RNAi-induced cell population was very close to that of the old flagellum (Fig. 4A, KPP1 RNAi), in striking contrast to the bi-flagellated control cells in which the new flagellum was separated from the old flagellum and moved toward the cell posterior (Fig. 4A, Control). These results suggest that positioning of the new flagellum was compromised by KPP1 depletion.

RNAi of KPP1 impairs basal body segregation

The emergence of 2N1K cells after KPP1 RNAi (Fig. 3C) suggests that kinetoplast segregation is likely impaired. Because kinetoplast segregation is mediated by basal body segregation (29), it suggests that KPP1 RNAi may inhibit basal body duplication and/or segregation. Therefore, we investigated the effect of KPP1 depletion on basal body duplication and/or segregation. Quantitation of the mBB, which was labeled by the YL 1/2 antibody, showed that after KPP1 RNAi, the 1N1K cells with two mBBs increased, and the 1N2K and 2N2K cells with two or more than two mBBs were slightly, but not significantly, decreased (Fig. 4B), suggesting that basal body duplication was not compromised. Co-immunostaining of the KPP1 RNAi cells with anti-TbSAS6 antibody, which labels the cartwheel structure of both the mBB and the pro-basal body (pBB), and the YL 1/2 antibody showed that all the 2mBB-containing cells also possess two pro-basal bodies, i.e. 2mBB-2pBB (Fig. 4C), further confirming that basal body duplication was not affected by KPP1 RNAi. Notably, in the 1N2K, 2N2K, and 2N1K cells, but not the 1N1K cells with an elongated kinetoplast, the distance between the two pairs of mBB–pBB in KPP1 RNAi cells was significantly shorter than that in the control cells (Fig. 4A, G1 phase, C, and D), suggesting defective basal body segregation.

The 2N1K cells could also be generated if aberrant cytokinesis occurs in 2N2K cells, which also produces zoid (0N1K) cells that contain a flagellum and a pair of mBB–pBB. KPP1 RNAi did produce ~6% zoid cells (Fig. 3C), but it should be noted that ~70% of the 2N1K cells from KPP1 RNAi contained two flagella and two pairs of mBB–pBB (Fig. 4, B and C), suggesting
that they were not generated through aberrant cytokinesis of 2N2K cells. Rather, these 2N1K cells were produced due to failed basal body segregation, which is known to inhibit kinetoplast segregation (29). Collectively, these results suggest that KPP1 depletion impaired the segregation, but not duplication, of basal bodies.

**KPP1 is necessary for duplication and segregation of the flagellar pocket collar**

The defective positioning of the new flagellum in KPP1 RNAi cells (Fig. 4A) suggests that biogenesis and/or segregation of the flagellar pocket collar (FPC) may also be compromised. To test this possibility, we immunostained the cells with anti-BILBO1 antibody to label the FPC (Fig. 5A) and counted the numbers of FPC in control and KPP1 RNAi cells (Fig. 5B). The results showed that in the three cell types (1N1K, 1N2K, and 2N2K) examined, the cells with two FPCs were significantly decreased, accompanied by a corresponding increase of 1N1K cells with one FPC and emergence of 1N2K and 2N2K cells with one FPC (Fig. 5B). In the 2N1K cells from the KPP1 RNAi population, 65% contained only one FPC (Fig. 5, A and B). These results suggest that FPC duplication was impaired in KPP1 RNAi cells. We further measured the inter-FPC distance in cells containing two FPCs, and the results showed that the average inter-FPC distance in 2N2K and 2N1K cells was significantly decreased upon KPP1 RNAi (Fig. 5C), indicating that in these cells the duplicated FPCs failed to segregate. Scanning electron microscopy confirmed that the bi-flagellated KPP1 RNAi cells contained either only one flagellar pocket from which both flagella exited the cell body (Fig. 5D, panel b) or two closely associated flagellar pockets (Fig. 5D, panels c and d). Together, these results suggest that KPP1 depletion disrupted the duplication and segregation of the flagellar pocket.

**KPP1 is essential for the duplication of the centrin arm and the hook complex of the bilobe structure**

The previously described bilobe structure (3) contains a centrin arm, which is marked by TbCentrin2 and TbCentrin4 (3, 30), and a hook complex, which is marked by TbMORN1 (31). The localization of KPP1 to the centrin arm of the bilobe (Fig. 2B) suggests its potential role in bilobe duplication and/or segregation. To test this possibility, cells were co-immunostained with the pan-centrin antibody 20H5 to label the centrin arm and the anti-TbMORN1 antibody to label the hook complex, and the numbers of centrin arm and hook complex were counted and compared between the control and KPP1 RNAi cells. In the 1N1K, 1N2K, and 2N2K cells examined, those cells with one centrin arm and one hook complex were significantly increased after KPP1 RNAi, which was accompanied by a corresponding decrease of the cells with two centrin arms and two
hook complexes (Fig. 6, A and B). Similarly, the majority (~80%) of the 2N1K cells in the KPP1 RNAi cell population contained one centrin arm and one hook complex (Fig. 6, A and B). These results indicate that KPP1 depletion impaired duplication of both the centrin arm and the hook complex in the bilobe structure.

**Depletion of KPP1 abolishes dephosphorylation of TbCentrin2 in mitotic cells**

We asked whether KPP1 is likely to counteract TbPLK function by dephosphorylating TbPLK substrate(s). So far only two TbPLK substrates, TbCentrin2 (14, 17) and SPBB1 (8), have been experimentally validated, and TbPLK phosphorylation of TbCentrin2 can be detected by the PS54 antibody, which was raised against the TbPLK-phosphorylated Ser-54 of TbCentrin2 (17). Using the PS54 antibody, we assessed the effect of KPP1 depletion on TbCentrin2 phosphorylation. In the noninduced control cells, PS54 detects phospho-TbCentrin2 at the centrin arm in ~45% of the 1N1K and 1N1eK cells (Fig. 7A). In ~56% of 2N2K cells, phospho-TbCentrin2 is still detectable in the two segregated centrin arms, but the signal becomes much weaker (Fig. 7A). In all of the 2N2K cells, the PS54 signal is undetectable (Fig. 7A), suggesting that TbCentrin2 is dephosphorylated in mitotic cells. Knockdown of TbPLK completely eliminated PS54 staining (Fig. 7B), confirming that Ser-54 is a site phosphorylated by TbPLK. In KPP1 RNAi cells, PS54 detects phospho-TbCentrin2 in ~41% of the 1N1K and 1N1eK cells and ~53% of the 1N2K cells (Fig. 7C and D), similar to that in the control cells. However, PS54 signal is still detectable in the centrin arms in ~63% of the bi-nucleated cells (2N2K and 2N1K) (Fig. 7C and D), suggesting that TbCentrin2 was not dephosphorylated during the mitotic phase in KPP1 RNAi cells. The phospho-TbCentrin2 detected in these mitotic cells could be attributed to failure in dephosphorylation. These results suggest that KPP1 may antagonize TbPLK function by controlling, either directly or indirectly, TbCentrin2 dephosphorylation after cells enter mitosis.

**Knockdown of KPP1 impairs TbPLK localization to the new FAZ tip at late cell-cycle stages**

We investigated the effect of KPP1 deficiency on the subcellular localization of TbPLK by immunofluorescence microscopy. In noninduced control cells, TbPLK localized to the basal body and the centrin arm in some 1N1K cells, and to the new FAZ tip in 1N1eK cells (S-phase), 1N2K cells (G2 to metaphase), and some 2N2K cells that are in the early anaphase stage (Fig. 8, A and B). It was not detectable in the 2N2K cells that are in late anaphase and telophase (Fig. 8, A and B). In KPP1 RNAi cells that were induced for 48 h, TbPLK localization was incomplete in the basal body and the centrin arm in 1N1K cells and to the new FAZ tip in
1N1eK cells was not affected (Fig. 8, A and B). However, TbPLK localization to the new FAZ tip in 1N2K and 2N2K cells was significantly impaired, resulting in the increase of cells either with undetectable TbPLK signal (no signal) or with TbPLK detected near the flagellar pocket region (FP region) (Fig. 8, A and B). These results suggest that KPP1 is required for maintaining TbPLK at the new FAZ tip during G2 to anaphase.

Discussion

Protein phosphorylation is one of the most important post-translational protein modifications and plays crucial roles in various cellular processes in eukaryotes. The cellular processes that are controlled by protein phosphorylation often are also regulated by an antagonizing protein phosphatase, which dephosphorylates the substrate(s) of the protein kinase or dephosphorylates the protein kinase to inactivate the latter (20). These counteracting actions by protein phosphatases provide a means to fine-tune the protein kinase-mediated signaling cascades required to carry out complex physiological and developmental processes. In humans, Plk1 is antagonized by two different protein phosphatases at different subcellular locations through distinct mechanisms (21, 22). It is counteracted by PP1Cβ through dephosphorylation of pThr-210 of Plk1 at centrosomes (21), and by PP2A through dephosphorylation of multiple Plk1 substrates at kinetochores (22). In T. brucei, a TbPLK-counteracting protein phosphatase is likely also required, as previous studies demonstrated that dephosphorylation of TbCentrin2, a TbPLK substrate in the centrin arm, is necessary for flagellum positioning and attachment (17) and that TbPLK activity is tightly controlled by phosphorylation/dephosphorylation of two threonine residues (Thr-198 and Thr-202) in its T-loop (16). Our findings that KPP1 co-localizes with TbPLK at multiple cytoskeletal structures (Fig. 2A and B) and that KPP1 dephosphorylation abolished the dephosphorylation of TbCentrin2 in mitotic cells (Fig. 7, B and C) suggest that KPP1 is likely able to antagonize TbPLK function. KPP1 may execute this function through dephosphorylation of phospho–Thr-198 and/or phospho–Thr-202 in the T-loop of TbPLK, as in the case of PP1Cβ-mediated counteraction of Plk1 in humans (21). In such a scenario, KPP1 depletion will cause constitutive activation of TbPLK due to lack of T-loop dephosphorylation, and the constitutively active TbPLK may phosphorylate TbCentrin2 in mitotic cells. Alternatively, KPP1 may dephosphorylate TbPLK...
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Figure 6. KPP1 is required for duplication of the centrin arm and the hook complex of the bilobe structure. A, co-immunostaining of control and KPP1 RNAi cells with the 20H5 mAb, which labels the centrins in the centrin arm (CA), and the anti-TbMORN1 pAb, which labels the hook complex (HC). NF, new flagellum; OF, old flagellum. Scale bar, 5 μm. B, quantitation of the centrin arm and the hook complex in control and KPP1 RNAi cells. A total of 100 cells for each cell type (1N1K/1N1eK, 1N2K, 2N2K, and 2N1K) were counted, and three repeats were conducted. Error bars indicate S.D. **, p < 0.01. ***, p < 0.001.

Figure 7. Depletion of KPP1 results in lack of TbCentrin2 dephosphorylation in the centrin arm in mitotic cells. A–C, immunostaining of control cells (A), TbPLK RNAi cells (B), and KPP1 RNAi cells (C) with the PS54 antibody, which detects the pSer-54 of TbCentrin2, and the 20H5 antibody, which labels the centrin arm (CA) and the basal body (BB). The enlarged image in the PS54 channel of the control 1N2K cell shows the longer exposure of the fluorescence signal. Scale bars, 5 μm. D, quantitation of PS54-positive and PS54-negative cells in control and KPP1 RNAi cells. A total of 100 cells for each cell group (1N1K/1N1eK, 1N2K, and 2N2K/2N1K) were counted, and three repeats were conducted. Error bars indicate S.D. ***, p < 0.001; ns, no statistical significance.
substrate(s) in the basal body, the centrin arm, or the new FAZ tip, as in the case of PP2A-mediated counteraction of Plk1 in humans (22). In this scenario, KPP1-deficient cells will fail to dephosphorylate TbCentrin2 in mitotic cells. Another possibility is that the mis-localized TbPLK at the flagellar pocket region of the KPP1 RNAi cells (Fig. 8) may continue to phosphorylate TbCentrin2, resulting in the observed phenotype of lack of TbCentrin2 dephosphorylation (Fig. 7). In this scenario, KPP1 dephosphorylates neither TbCentrin2 nor TbPLK, but regulates TbPLK localization through unknown mechanisms. Future efforts will be directed to test these possibilities, which will help ascertain the molecular function of KPP1.

The co-localization of KPP1 with TbPLK at the basal body, the centrin arm, and the new FAZ tip from G1 phase to early mitosis (Fig. 2A) raises an interesting question of how KPP1 counteracts TbPLK function at all these cell-cycle stages. It should be noted that the human Plk1-antagonizing protein phosphatase MYPT1-PP1C also co-localizes with Plk1 at multiple subcellular structures during the cell cycle (21). Previous results showed that TbPLK activity is under tight control during the cell cycle (16, 18), suggesting that TbPLK activity has to be maintained at a certain level at these cell-cycle stages to ensure proper regulation of its substrates. Moreover, previous results also showed that both hypophosphorylation and hyperphosphorylation of TbCentrin2 at Ser-54 caused a growth defect in T. brucei (17), suggesting that the phosphorylation level of TbCentrin2 is under tight control. We thus postulate that co-localization of KPP1 with TbPLK at these cell-cycle stages may balance the phosphorylation level of TbPLK and/or its substrates so as to maintain their phosphorylation level within a certain threshold. The level of phosphorylation that is higher or lower than this threshold will cause defects in the positioning and adhesion of the new flagellum.

The T. brucei genome encodes a total of 27 phosphoprotein phosphatase (PPP) homologs, including eight PP1 homologs, two PP2A homologs, two PP2B homologs, one PP4 homolog, one PP5 homolog, two PP7 homolog, and 11 kinetoplastid-specific PPP homologs (24). The catalytic domain of KPP1 exhibits ~30% sequence identity to all of these T. brucei PPP homologs, but the N-terminal Plus3 domain in KPP1 is lacking from the T. brucei PPP homologs and is not found in any PPPs from any eukaryotic organisms except the kinetoplastid parasites, including Trypanosoma cruzi and Leishmania spp. (Figs. S1 and S2). Furthermore, despite the high sequence similarity of the catalytic domain of KPP1 to the PPPs, phylogenetic analysis using the catalytic domain places the KPP1 homologs in a distinct clade (Fig. S2B), similar to the results obtained using the full coding sequence (Fig. S2A). These results suggest that KPP1 is a unique member of the PPP family. Importantly, our data demonstrated that KPP1 is essential for T. brucei proliferation.
Depletion of KPP1 caused accumulation of multinucleated (>2 nuclei) cells (Fig. 3C), indicative of defective cytokinesis, but a direct role for KPP1 in regulating cytokinesis remains to be determined. Multinucleated cells started to accumulate after 72 h of KPP1 RNAi induction, coincident with the accumulation of cells with detached flagella (Fig. 3D). Given that proper flagellum positioning and attachment are essential for cytokinesis in *T. brucei* (7, 12, 36, 37), it is difficult, if not impossible, to distinguish between the direct effect exerted by KPP1 RNAi and the indirect effect due to flagellum detachment. Nevertheless, KPP1 localizes to the distal tip of the new FAZ (Fig. 2), which constitutes the site from which cleavage furrow ingestion is initiated (38), and 2N2K cells increased from ∼11 to ∼21% after KPP1 RNAi for 48 h, prior to massive flagellum detachment occurred (Fig. 3C), indicating that KPP1 likely also plays a direct role in cytokinesis.

In summary, we have identified a putative kinetoplastid-specific protein phosphatase, which plays essential roles in regulating the duplication and segregation of multiple flagellum-associated cytoskeletal structures to ensure proper positioning and attachment of the newly assembled flagellum. Our findings highlight the requirement of protein dephosphorylation in controlling flagellum inheritance, adding another layer of regulation to this process in addition to the previously discovered TbPLK-mediated protein phosphorylation.

**Experimental procedures**

**Trypanosome cell culture**

The procyclic *T. brucei* strain Lister 427 was cultured in SDM-79 medium supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Inc.) at 27 °C. The Lister 427-derived 29-13 strain (39), which stably expresses the T7 RNA polymerase and the tetracycline repressor, was grown at 27 °C in SDM-79 medium containing 10% heat-inactivated fetal bovine serum, 15 μg/ml of G418, and 50 μg/ml of hygromycin B. Cells were routinely diluted with fresh medium when the cell density reached $5 \times 10^6$/ml.

**RNAi**

A 583-bp DNA fragment (nucleotide 993–1575) corresponding to the C-terminal portion of the *KPP1* coding sequence was PCR amplified (forward primer: 5′-ATCTTAG-CCCGCTCGAGACAGTGTTCGGTGACATCCA-3′; underlined are XhoI and HindIII sites, respectively) and cloned into the pZJM vector (40). The resulting plasmid was linearized with NotI and electroporated into the 29-13 cell line. Transfectants were selected with 2.5 μg/ml of phleomycin and further cloned by limiting dilution in a 96-well plate containing the SDM-79 medium supplemented with 20% fetal bovine serum, 2.5 μg/ml of phleomycin, 15 μg/ml of G418, and 50 μg/ml of hygromycin B.

**Epitope tagging of endogenous proteins**

A 1,248-bp DNA fragment corresponding to the C-terminal coding sequence of KPP1 was PCR amplified (forward primer: 5′-GGCGAATTCTGATTTTACGCGCTCGGTCAGAAAAGGAATG-
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ACG-3'; reverse primer: 5'-AGGATATTCCCTAAGAACG-51-GCCTAAGACGCCG-G3'; underlined are KpnI and AflII sites, respectively) and cloned into the pc-3HA-PAC vector, which was derived from the pc-PTP-NEO vector (41) by replacing the PTP epitope with a triple HA epitope and the neomycin resistance gene (NEO) with the puromycin resistance gene (PAC). The resulting plasmid, pc-KPP1–3HA-PAC, was linearized with NcoI and electroporated into the 29-13 cell line. Ectopic overexpression of WT and mutant KPP1 in T. brucei

Ectopic overexpression of WT and mutant KPP1

The full-length coding sequence of KPP1 was PCR amplified from genomic DNA (forward primer: 5'-CATTCTTG-ACTCGAGATGGATCACCCTAATAGTACC-3'; reverse primer: 5'-AGGATATTCCCTAAGAACG-51-GCCTAAGACGCCG-G3'; underlined are XhoI and AflII sites, respectively) and cloned into the pLew1000-3HA vector, which was modified from the pLew100 vector (39). Site-directed mutagenesis of Asp-371 to asparagine (D371N) and His-557 to lysine (H557K) in the active sites of KPP1 was carried out using the QuikChange II site-directed mutagenesis kit (Agilent, Inc.) using the following primers (D371N: forward primer, 5'-TTGTTCCTTGGCACAATGTAGAT CGT-3'; reverse primer, 5'-ACGATCTCAGATGGCAGAGAACA-3'; H557K: forward primer, 5'-TACATATTTGCGTTTC-TAAGAAAAGCGCGGAT-3'; reverse primer, 5'-ATCCGGCCTTTTCTTGAACGCACGAAATATGTA-3'). Mutation of the DNA sequences encoding the two residues was confirmed by sequencing. The pLew100-KPP1–3HA vector and the pLew100-KPP1-D371N/H557K-3HA vector were linearized with NotI and electroporated into the 29-13 cell line. Ectopic expression of WT and mutant KPP1 proteins was induced with 0.1 µg/ml of tetracycline for 16 h before being fixed for immunofluorescence microscopy.

Immunofluorescence microscopy

Control cells and KPP1 RNAi cells induced for 48 or 72 h were harvested by centrifugation, washed once with PBS, and then settled onto coverslips for 20 min at room temperature. Cells were immediately fixed with cold methanol at −20 °C for 30 min, rehydrated with PBS at room temperature for 10 min. After blocking with 3% BSA in PBS at room temperature for 30 min, cells were incubated with appropriate primary antibodies for 1 h at room temperature. The following primary antibodies were used: FITC-conjugated anti-HA mAb (1:400 dilution, Sigma), anti-TbPLK polyclonal antibody (1:1,000 dilution) (8), anti-LdCent1 polyclonal antibody (1:1000 dilution) (42), anti-CC2D polyclonal antibody (1:2,000 dilution) (7), anti-TbSAS-6 polyclonal antibody (1:2,000 dilution) (43), L8C4 (anti-PTFR2) mAb (1:50 dilution) (44), Y1L1/2 mAb (1:2,000 dilution, Millipore), L3B2 (anti-FAZ1) mAb (1:25 dilution) (44), 20H5 (anti-centrin) mAb (1:1,000 dilution, Millipore) (3), anti-TbBILBO1 polyclonal antibody (1:4,000 dilution) (45), anti-TbMORN1 polyclonal antibody (1:5,000 dilution) (31), and PS54 (anti-pSer-54 of TbCentrin2) polyclonal antibody (1:30,000 dilution) (17). After washing with PBS, cells were incubated with secondary antibodies for another 1 h at room temperature. The following secondary antibodies were used: Cy3-conjugated anti-rabbit IgG (1:400 dilution, Sigma), Cy3-conjugated anti-mouse IgG (1:400 dilution, Sigma), FITC-conjugated anti-rat IgG (1:400 dilution, Sigma), and FITC-conjugated anti-mouse IgG (1:400 dilution, Sigma). Cells were mounted with Vectashield mounting medium containing DAPI (Vector Labs), and visualized under an inverted fluorescence microscope (Olympus IX71) equipped with a cold CCD camera and a PlanApo N60X/1.42 immersion lens. Images were acquired with the Slidebook version 5.0 software.

Scanning electron microscopy

Scanning electron microscopic analysis of T. brucei cells was performed as described previously (23). To maintain cell shape and morphology, control cells and KPP1 RNAi cells induced for 72 h were washed once with PBS for 5 min at room temperature, and then fixed with 2.5% (v/v) glutaraldehyde for 2 h. Cells were washed three times with PBS, and settled on glass coverslips. After a brief washing with water, cells were dehydrated in alcohol (30, 50, 70, 90, and 100%, v/v) for 10 min in each solution. Samples were processed by critical point drying, and then coated with a 5-nm metal film (Pt:Pd 80:20, Ted Pella Inc.) using a sputter-coater (Cressington Sputter Coater 208 HR, Ted Pella Inc.). Cells were imaged under Nova NanoSEM 230 (FEI) using the following parameters: 5 mm for the scanning work distance and 8 kV for the accelerating high voltage.

Statistical analysis

Statistical analysis was performed using the t test in the Microsoft Excel software. Detailed p values for each panel in the figures were stated in the corresponding legends. For immunofluorescence microscopy, images were randomly taken and all cells in each image were counted.

Author contributions—Q. Z. and Z. L. conceptualization; Q. Z. validation; Q. Z., G. D., and Z. L. investigation; Q. Z. visualization; Q. Z. methodology; Q. Z. and Z. L. writing-original draft; Q. Z., G. D., and Z. L. writing-review and editing; G. D. software; G. D. and Z. L. formal analysis; Z. L. supervision; Z. L. funding acquisition; Z. L. project administration.

Acknowledgments—We thank Dr. Arthur Günzl for providing the epitope-tagging vector pc-PTP-NEO, Dr. Keith Gull for L3B2 (anti-FAZ1) and L8C4 (anti-PTFR2) antibodies, Dr. Cynthia He for the anti-LdCen1 polyclonal antibody, Dr. Briggs, L. J., McKean, 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 CrossRef Medline

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