The trypanosome-specific proteins FPRC and CIF4 regulate cytokinesis initiation by recruiting CIF1 to the cytokinesis initiation site

Received for publication, August 6, 2019, and in revised form, September 18, 2019 Published, Papers in Press, September 20, 2019, DOI 10.1074/jbc.RA119.010538

Huiqing Hu, Tai An, Yasuhiro Kurasawa, Qing Zhou, and Ziyin Li
From the Department of Microbiology and Molecular Genetics, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, Texas 77030
Edited by Chris Whitfield

The evolutionarily early divergent human parasite Trypanosoma brucei proliferates through binary cell fission in both its tsetse fly vector and mammalian host. The parasite divides unidirectionally along the longitudinal cell axis from the anterior cell tip toward the posterior cell tip through a mechanism distinct from that in the cells of its host. Initiation of cytokinesis in T. brucei is regulated by two evolutionarily conserved protein kinases, the Polo-like kinase TbPLK and the Aurora B kinase TbAUK1, and a cohort of trypanosome-specific proteins, including the three cytokinesis initiation factors CIF1, CIF2, and CIF3. Here, using RNAi, in situ epitope tagging of proteins, GST pulldown, and communoprecipitation assays, and immunofluorescence and scanning electron microscopy analyses, we report the identification and functional characterization of two trypanosome-specific proteins, flagellum attachment zone tip–localizing protein required for cytokinesis (FPRC) and CIF4. We found that the two proteins colocalize to the distal tips of the new and the old flagellum attachment zones and are required for cytokinesis initiation. Knockdown of FPRC or CIF4 disrupted the localization of CIF1, suggesting that they function upstream of CIF1. Moreover, depletion of CIF4 abolished FPRC localization, indicating that CIF4 acts upstream of FPRC. Together, these results identify two new cytokinesis regulators in T. brucei and integrate them into the CIF1-mediated cytokinesis regulatory pathway. These findings highlight the existence of a cytokinesis pathway in T. brucei that is different from that of its mammalian host and therefore suggest that cytokinesis in T. brucei could potentially be exploited as a new drug target.

Trypanosoma brucei belongs to the Excavata supergroup of eukaryotes (1) and is a flagellated unicellular protozoan parasite causing sleeping sickness in humans and nagana in cattle in sub-Saharan Africa. T. brucei has a complex life cycle, alternating between the insect vector, tsetse fly, and mammalian hosts, and has devastating effects on agriculture and human health in Africa. In the host, the parasite proliferates through binary cell fission along the longitudinal axis of the cell without the involvement of an actomyosin contractile ring, an evolutionarily conserved cytokinetic apparatus in eukaryotes (2). During early stages of the trypanosome cell division cycle, the parasite assembles a new flagellum, which, like the old flagellum, is adhered to the cell body via a cytoskeletal structure called the flagellum attachment zone (FAZ) (3). The length of the newly assembled flagellum and its associated FAZ determines the cell division plane (4, 5). This is strikingly different from other eukaryotic organisms, in which the cell division plane is usually defined by the position of the central spindle (6), highlighting the unusual mechanism of cytokinesis in T. brucei, which diverged from the eukaryotic ancestor earlier than yeast. Prior to cleavage furrow ingression in T. brucei, membrane invagination occurs between the new and the old flagella, forming a so-called division fold (7). Along the preformed division fold, the cleavage furrow ingresses unidirectionally from the anterior tip of the new-flagellum daughter cell toward the nascent posterior tip of the old-flagellum daughter cell, bisecting the biflagellum mother cell into two daughter cells, each inheriting one flagellum and one copy of all other organelles (7, 8).

The molecular mechanism underlying the unusual mode of cytokinesis in T. brucei remains largely elusive. Nothing is known about how membrane invagination is regulated to form the division fold and how cleavage furrow ingression is controlled in the absence of the actomyosin contractile ring. The signaling pathway that controls cytokinesis initiation is, however, gradually being elucidated. Two evolutionarily conserved protein kinases, the Aurora B kinase homolog TbAUK1 and the Polo-like kinase homolog TbPLK, were first characterized as essential cytokinesis regulators (9–12), and the two protein kinases appear to function sequentially at the distal tip of the new FAZ (13). Subsequent works identified a cohort of trypanosome-specific cytokinesis regulators, including three cytokinesis initiation factor (CIF) proteins CIF1, CIF2, and CIF3 (14–16); two cleavage furrow–localizing proteins, KLIF and FRW1 (17–19); a kinetoplastid-specific protein phosph...

This work was supported by National Institutes of Health Grants AI101437 and AI18736. 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 Table S1.

1 To whom correspondence should be addressed. E-mail: Ziyin.Li@uth.tmc.edu.

This is an open access article under the CC BY license.

© 2019 Hu et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.
tase called KPP1 (20); and a hook complex–localizing protein, BOH1, that recruits TbPLK to the new FAZ tip (21). Through genetic analyses, the order of action of these cytokinesis regulators has been determined, with BOH1 functioning at the most upstream of the cytokinesis pathway, followed by TbPLK, the three CIF proteins, TbAUK1, and finally FRW1 and KLIF, acting sequentially at the new FAZ tip and the cleavage furrow (14–16, 18, 21). Despite these advances, however, much remains to be learned about the functional interplay among these cytokinesis regulators and their mechanistic roles in controlling cytokinesis initiation and/or completion.

We aim to delineate the cytokinesis signaling pathway and dissect the mechanisms underlying the unusual mode of cytokinesis in *T. brucei*. To achieve these goals, it is necessary to first identify all of the cytokinesis regulatory proteins and study their functions. Here we report two new cytokinesis regulators, FPRC and CIF4, and further demonstrate that they function in the CIF1-mediated cytokinesis pathway. These findings add new components to the existing cytokinesis regulatory pathway and lay the foundation for completely delineating the pathway and understanding the mechanism of cytokinesis, one of the most fundamental aspects of trypanosome biology that might be exploited as new drug targets for treatment of human sleeping sickness.

**Results**

**FPRC localizes to the distal tips of the flagellar attachment zone filaments and the cleavage furrow**

We recently carried out proximity-dependent biotin identification (BioID) (22) using CIF1 as the bait and identified an evolutionarily conserved katanin complex, the KAT60a–KAT80 complex, and a cohort of trypanosome-specific proteins that regulate cytokinesis in the procyclic form of *T. brucei* (15, 18). Among these trypanosome-specific proteins is a CIF1-interacting protein called FAZ tip–localizing protein required for cytokinesis (FPRC) (18) whose subcellular localization and physiological function have not been characterized in the previous work. FPRC contains two coiled-coil motifs (Fig. 1A), suggesting that it might function in a large CIF1-containing protein complex. In G1 cells, FPRC localizes to the distal tip of the FAZ (Fig. 1B), and in S phase cells, when a new FAZ is assembled, FPRC additionally emerges at the distal tip of the new FAZ (Fig. 1B). From G2 phase to telophase, FPRC is constantly localized at the distal tips of both the new and the old FAZ filaments, but during cytokinesis, it additionally emerges at the cleavage furrow (Fig. 1B). Because FPRC interacts with CIF1 in vivo in trypanosomes (18), we examined their colocalization during the cell cycle by immunofluorescence microscopy. During G1 phase, CIF1 was not detectable, and FPRC was localized at the FAZ tip (Fig. 1C). From S phase onward, FPRC and CIF1 colocalized at the distal tip of the newly assembled FAZ (Fig. 1C). Unlike FPRC, however, CIF1 was not detectable at the distal tip of the old FAZ throughout the cell cycle (Fig. 1C), consistent with its absence at the FAZ tip in G1 cells, in which the FAZ turned into an old FAZ upon the assembly of a new FAZ from S phase. This result suggests that only a portion of the FPRC protein forms a complex with CIF1 at the new FAZ tip and the cleavage furrow.

CIF1 contains a coiled-coil (CC) motif in its N-terminal portion and two Cys-Cys-His-Cys (CCHC)-type zinc finger (ZnF) motifs at the C terminus (Fig. 1A), and the CC motif and both ZnF motifs (ZnF1 and ZnF2) are required for CIF1 function (23). The N-terminal domain (NTD) upstream of the CC motif and the intrinsically disordered region (IDR) between the CC motif and the ZnF motifs contain numerous phosphorylation sites (24), and the NTD and the IDR are also required for CIF1 function.3 Given the importance of these four domains for CIF1 function, we examined their potential requirement for interaction with FPRC. We carried out GST pulldown experiments using GST-fused CIF1 domains and found that the NTD and the ZnF motifs, but not the CC motif or the IDR, were capable of precipitating FPRC from the cell lysate (Fig. 1D). Conversely, we also investigated the requirement of the structural motifs in FPRC for interaction with CIF1. FPRC contains a CC motif at the NTD and a CC motif at the C-terminal domain (CTD) (Fig. 1), and we fused the NTD, CTD, and full-length protein with GST and carried out GST pulldown experiments. The results showed that the NTD and the full-length FPRC protein, but not the CTD, were able to pull down CIF1 (Fig. 1E). These results demonstrated that the interaction between CIF1 and FPRC is mediated by the NTD and the ZnF motifs of the CIF1 protein and the NTD of the FPRC protein.

**FPRC is required for cytokinesis initiation in the procyclic form of *T. brucei***

To study the biological function of FPRC, we carried out RNAi in the procyclic form of *T. brucei*. RNAi of FPRC resulted in gradual depletion of the level of FPRC protein, resulting in a reduction of the FPRC level to ~25% of the control level on day 4 (Fig. 2A). Ablation of FPRC by RNAi caused a moderate growth defect (Fig. 2B), indicating that FPRC is required for cell proliferation in the procyclic form. Quantification of cells with different numbers of nuclei (N) and kinetoplasts (K, the cell’s mitochondrial DNA network) showed that FPRC knockdown caused an increase in binucleated (2N2K) cells from ~10% to ~35% of the total cell population on day 2 of RNAi and a gradual accumulation of multinucleated (XNKK, X ≥ 2) cells to ~17% of the total cell population on day 3 of RNAi (Fig. 2C), suggesting defective cytokinesis. To further investigate which cytokinesis stage was defective, we examined the cleavage furrow in binucleated cells under both a light microscope and scanning electron microscope. The results showed that the percentage of binucleated cells that possessed a cleavage furrow at the anterior tip of the new-flagellum daughter cell (referred to here as the anterior furrow) was decreased from ~36% in control cells to ~12% after FPRC RNAi was induced for 48 h (Fig. 2, D and E), suggesting that the FPRC RNAi cells had failed to initiate cytokinesis from the anterior tip of the new-flagellum daughter cell. Similarly, ~8% of multinucleated cells contained an anterior furrow (Fig. 2E), further demonstrating that RNAi of FPRC inhibited cytokinesis initiation. Intriguingly, ~13% of FPRC RNAi–induced binucleated cells started to initiate a cleavage furrow (referred to here as the posterior furrow) from the nascent posterior tip of the old flagellum-daughter cell (Fig. 2E).

---

3 Y. Kurasawa and Z. Li, unpublished results.
Roles of CIF4 and FPRC in cytokinesis

2, D and E), similar to that caused by RNAi of CIF1 (14) and RNAi of CIF2 (15). Strikingly, ~35% of FPRC RNAi-induced multinucleated cells possessed one or multiple posterior furrows (Fig. 2, D and E). Scanning EM analysis showed the presence of an anterior furrow in control cells and a posterior furrow in FPRC RNAi cells at higher resolution (Fig. 2F). Together, these results demonstrate that FPRC is required for cytokinesis initiation from the anterior tip of the new-flagellum daughter cell in the procyclic form.

**FPRC functions upstream of CIF1 in the cytokinesis regulatory pathway**

The fact that FPRC and CIF1 interact in vivo in trypanosomes (18) suggests that the two proteins might function in the same
Roles of CIF4 and FPRC in cytokinesis

Figure 2. FPRC is required for cytokinesis initiation in the procyclic form of T. brucei. A, Western blotting to monitor the protein level of FPRC before and after RNAi induction. FPRC was endogenously tagged with a triple-HA epitope and detected by anti-HA antibody. TBPAS6 served as the loading control. B, depletion of FPRC caused a moderate growth defect. C, RNAi of FPRC caused cytokinesis defects. Shown is quantification of cells with different numbers of nuclei and kinetoplasts, which were stained with DAPI. A total of 200 cells were counted for each time point, and error bars indicate S.D. from three independent experiments (n = 3). d, day. D, RNAi of FPRC abolished cleavage furrow ingression from the anterior tip of the new-flagellum daughter cell and caused furrow ingression from the posterior end of the old-flagellum daughter cell. Cells were immunostained with anti-CC2D polyclonal antibody to label the FAZ filament and counterstained with DAPI for nuclei and kinetoplasts. A, anterior; P, posterior. Scale bar = 5 μm. E, quantification of cells without or with visible cleavage furrows in 2N2K cells from control and FPRC RNAi and in the XNXX cells from FPRC RNAi. 200 cells were counted for each time point and each cell type, and the results are presented as the mean percentage ± S.D. (n = 3). F, scanning EM analysis of control and FPRC RNAi cells. Scale bar = 5 μm.

cytokinesis regulatory pathway mediated by CIF1 and CIF1-associated proteins, including TbPLK, TbAUK1, CIF2, CIF3, KPP1, the KAT60a-KAT80 complex, KLIF, and FRW1 (14–20). To examine the potential functional interplay as well as to determine the order of action between FPRC and the other protein by immunofluorescence microscopy. Knockdown of FPRC appeared to disrupt CIF1 localization to the new FAZ tip in binucleated cells (Fig. 3A), resulting in a significant decrease in CIF1-positive (new FAZ tip+) binucleated cells (Fig. 3B). Western blotting showed that the protein level of CIF1 remained unchanged after FPRC RNAi (Fig. 3C), suggesting that the CIF1 protein was spread out in the cytosol in CIF1-negative (new FAZ tip−) binucleated cells (Fig. 3A). Conversely, knockdown of CIF1 did not affect the localization of FPRC to the distal tip of the new FAZ in all (100%) cells examined (>1000 cells) (Fig. 3D). Together, these results suggest that FPRC functions upstream of CIF1 in the cytokinesis regulatory pathway.

Identification of FPRC-proximal proteins by BioID uncovers known and new cytokinesis regulators

To identify additional cytokinesis regulators that function in the FPRC–CIF1–mediated pathway, we carried out proximity-dependent BioID using FPRC as the bait. To this end, FPRC was fused with BirA*-HA at its C terminus and ectopically expressed in the procyclic form. Expression of the FPRC-BirA*-HA fusion protein was confirmed by Western blotting with anti-HA antibody (Fig. 4A), and localization of the fusion protein to the FAZ tips was verified by immunofluorescence microscopy (Fig. 4B). Affinity purification of biotinylated proteins was then carried out (Fig. 4C), allowing identification of a total of 764 proteins as putative FPRC-interacting proteins and near neighbors (Table S1). We excluded 606 proteins that either are common contaminants in multiple BioID experiments using functionally irrelevant baits or localize to the subcellular structures far away from the FAZ tips (Table S1). Of the 158 potential FPRC-proximal proteins, 39 proteins were localized previously to the proximity of the FAZ tip, including 14 FAZ-localizing proteins, 15 FAZ tip–localizing proteins, and 10 hook complex–localizing proteins (Table S1). Six of the 14 FAZ tip–localizing proteins and one of the 10 hook complex–localizing protein have been functionally characterized as cytokinesis regulators (Fig. 4D), some of which additionally localize to other cytoskeletal structures, such as the basal body, the hook complex, or the cleavage furrow (14, 16–18, 21, 25, 26). Of the remaining 119 proteins, one protein (TbSpef1) localizes to the microtubule quartet (27), 15 proteins localize to the cortical cytoskeleton, 27 proteins localize to the cytosol, and 76 proteins have unknown localization (Table S1).

To identify new cytokinesis regulators, we focused on hypothetical proteins of unknown subcellular localization. One hypothetical protein, which is encoded by Tb927.10.8240, was located to the distal tips of both the new and the old FAZ filaments and was further characterized to be involved in cytokinesis initiation (Figs. 5 and 6 below). We named this protein CIF4, following our nomenclature for the other CIF proteins (CIF1, CIF2, and CIF3) (14–16). Like the cytokinesis regulators FPRC, CIF3, FRW1, and BOH1, CIF4 contains only coiled-coil motifs (Fig. 4D), indicating that it might be a component of a large protein complex.

CIF4 forms a complex with FPRC and CIF1

Because CIF4 was identified by FPRC BioID (Table S1), we investigated whether CIF4 forms a complex with FPRC by coimmunoprecipitation, and the results showed that immuno-
Roles of CIF4 and FPRC in cytokinesis

Figure 3. FPRC is required for localization of CIF1 to the new FAZ tip. A, localization of CIF1 in control and FPRC RNAi cells. CIF1 was detected with anti-CIF1 polyclonal antibody, and the FAZ was labeled with anti-FAZ1 (clone L3B2) mAb. The open arrowhead indicates CIF1 signal at the new FAZ tip. Scale bar = 5 μm. B, quantification of cells with CIF1 fluorescence signal at the new FAZ tip in binucleated cells from control and FPRC RNAi (48 h). A total of 200 cells were counted for each time point, and error bars indicate S.D. from three independent experiments (n = 3). New FAZ tip +, CIF1-positive at the new FAZ tip; New FAZ tip −, CIF1-negative at the new FAZ tip. C, Western blot to monitor the protein level of CIF1 in control cells and FPRC RNAi cells. CIF1 was detected by anti-CIF1 polyclonal antibody, and FPRC-PTP was detected by anti-protein A polyclonal antibody. TbPSA6 was detected by anti-TbPSA6 polyclonal antibody to serve as the loading control. D, effect of CIF1 RNAi on the localization of FPRC. Endogenously 3HA-tagged FPRC was detected by FITC-conjugated anti-HA mAb, and the FAZ was detected by anti-CC2D polyclonal antibody. Open arrowheads and solid arrowheads indicate FPRC signal at the new FAZ tip and old FAZ tip, respectively. Scale bar = 5 μm.

Figure 4. Identification of FPRC-proximal proteins by BioID. A, expression of FPRC–BirA*-HA in procyclic trypanosomes. FPRC was fused with a C-terminal BirA*-HA and ectopically expressed in a tetracycline-inducible manner. FPRC–BirA*-HA was detected by anti-HA antibody. TbPSA6 served as the loading control. B, localization of FPRC–BirA*-HA to the distal tips of FAZ filaments. FPRC-irA*-HA was detected by FITC-conjugated anti-HA mAb, and the FAZ was labeled by anti-CC2D polyclonal antibody. The open arrowhead and the solid arrowhead indicate FPRC–BirA*-HA signal at the new FAZ tip and old FAZ tip, respectively. Scale bar = 5 μm. C, affinity purification of biotinylated proteins from noninduced control cells and FPRC–BirA*-HA overexpression cells. Shown is the Western blot of the input samples and final elution samples detected by anti-HRP-streptavidin Western blotting. D, schematic of the structural motifs of seven known cytokinesis regulators and a new cytokinesis regulator, CIF4, identified by FPRC BioID. PB, Polo-box motif; Tpm, Tropomyosin-like domain. E, coimmunoprecipitation between PTP-tagged FPRC and 3HA-tagged CIF4. IP, immunoprecipitation. F, coimmunoprecipitation between CIF1 and 3HA-tagged CIF4 and FPRC. FAZ24–3HA served as a negative control.
precipitation of FPRC was able to pull down CIF4 from trypanosome cell lysate (Fig. 4E). Further, because FPRC forms a complex with CIF1 (18), we asked whether CIF4 also interacts with CIF1. Coimmunoprecipitation showed that CIF1 was able to pull down CIF4 from trypanosome cell lysate (Fig. 4F). It was noted that the amount of CIF4 protein coprecipitated by CIF1 was significantly less than the amount of FPRC protein coprecipitated by CIF1 (Fig. 4F), indicating that the interaction between CIF4 and CIF1 likely is weaker or less stable than that between FPRC and CIF1 under these experimental conditions. Nonetheless, these results suggest that CIF4 forms a complex with FPRC and CIF1 in vivo in trypanosomes.

We next examined the subcellular localization of CIF4 throughout the cell cycle by immunofluorescence microscopy using cells expressing CIF4–3HA from its endogenous locus. In G1 phase, CIF4 localizes to the distal tip of the FAZ, and from S phase onward, CIF4 additionally localizes to the distal tip of the new FAZ (Fig. 5A). During cytokinesis, when the cleavage furrow starts to ingress from the anterior tip of the new–flagellum daughter cell, CIF4 is concentrated at the cleavage furrow in addition to the distal tips of the new and the old FAZ filaments (Fig. 5A). CIF4 colocalizes with FPRC at the distal tip of the FAZ during G1 phase, at the distal tips of the new and the old FAZ filaments from S phase onward, and additionally at the cleavage furrow during cytokinesis (Fig. 5B). CIF4 colocalizes with CIF1 at the new FAZ tip from S phase onward and additionally at the cleavage furrow during cytokinesis (Fig. 5C), but CIF1 was not detectable at G1 phase, during which CIF4 localizes at the distal tip of the FAZ (Fig. 5C). Together with the results of the colocalization between FPRC and CIF1 (Fig. 1C), it appears that these three proteins (FPRC, CIF1, and CIF4) might form a complex at the new FAZ tip from S phase onward and at the cleavage furrow during cytokinesis. Another portion of the FPRC and CIF4 proteins might form a separate complex at the old FAZ tip throughout the cell cycle.

**CIF4 is required for cytokinesis initiation in the procyclic form of T. brucei**

The localization of CIF4 to the new FAZ tip and the cleavage furrow (Fig. 5) and the interaction of CIF4 with FPRC and CIF1 (Fig. 4, E and F) suggest that CIF4 might be involved in cytokinesis. To examine the biological function of CIF4, we carried out the following experiments...

---

**Figure 5. Subcellular localization of CIF4 and colocalization of CIF4 with FPRC and CIF1.**

A, subcellular localization of CIF4 during the cell cycle of *T. brucei*. CIF4 was endogenously tagged with a triple-HA epitope and detected by FITC-conjugated anti-HA mAb. The FAZ was detected by anti-CC2D polyclonal antibody. Open arrowheads indicate CIF4 signal at the new FAZ tip, solid arrowheads indicate CIF4 signal at the old FAZ tip, and arrows indicate CIF4 signal at the cleavage furrow. Scale bar = 5 μm. B, CIF4 colocalizes with FPRC at the new FAZ tip (open arrowheads) and the old FAZ tip (solid arrowheads) and at the cleavage furrow (arrows). CIF4–3HA and FPRC-PTP were coexpressed from their respective endogenous loci and detected by FITC-conjugated anti-HA mAb and anti-protein A polyclonal antibody, respectively. Scale bar = 5 μm. C, CIF4 colocalizes with CIF1 at the new FAZ tip (open arrowheads) and the cleavage furrow (arrows). Endogenously 3HA-tagged CIF4 was detected by FITC-conjugated anti-HA mAb. CIF1 was detected by anti-CIF1 polyclonal antibody. Solid arrowheads indicate CIF4 signal at the old FAZ tip. Scale bar = 5 μm.
out RNAi in the procyclic form. Western blotting showed that the level of CIF4 protein was gradually decreased to ~13% of the control level after 3 days of RNAi (Fig. 6A). This depletion of CIF4 protein resulted in severe growth defects (Fig. 6B), demonstrating that CIF4 is required for cell proliferation in procyclic trypanosomes. To further examine the potential cell cycle defects caused by CIF4 RNAi, we quantitated the cells with different numbers of nuclei and kinetoplasts among control and CIF4 RNAi-induced cells. CIF4 RNAi caused a significant increase in 2N2K cells from ~10% to ~41% after only 1 day and subsequently an accumulation of XNXK (X > 2) cells to ~32% of the total cell population after 2 days and ~82% after 3 days (Fig. 6C). These results demonstrate that CIF4 is required for cytokinesis in the procyclic form.

To characterize in detail the cytokinesis defects caused by CIF4 RNAi, we examined the effect of CIF4 depletion on formation of the cleavage furrow by light microscopy and scanning EM. Similar to RNAi of CIF1 (14), RNAi of CIF2 (15), and RNAi of FPRC (Fig. 2), depletion of CIF4 also inhibited formation of the anterior furrow in 2N2K cells (Fig. 6, D and E), resulting in a decrease in anterior furrow–containing binucleated cells from ~37% to ~5% (Fig. 6E). Instead, after CIF4 RNAi induction for 48 h, ~29% of the binucleated cells possessed a posterior furrow (Fig. 6, D and E). For CIF4-deficient XNXK cells, ~51% possessed one or multiple posterior furrows (Fig. 6, D and E). Scanning EM showed that formation of the anterior furrow was blocked in CIF4 RNAi cells and confirmed formation of the posterior furrow in biflagellum cells and quadruple-flagellum cells after CIF4 RNAi for 48 h (Fig. 6F). Together, these results demonstrate that CIF4 is required for cytokinesis initiation from the anterior tip of the new-flagellum daughter cell in procyclic trypanosomes.

### CIF4 is required for localization of FPRC and CIF1

We investigated the potential effect of CIF4 depletion on the localization and stability of FPRC by immunofluorescence microscopy and Western blotting. To this end, FPRC was endogenously tagged with a triple-HA epitope in the CIF4 RNAi cell line. Induction of CIF4 RNAi exerted a strong effect on the localization of FPRC, abolishing the localization of FPRC to the new FAZ tip in ~97% of binucleated cells (Fig. 7A and B). In these binucleated cells, the FPRC fluorescence signal at the old FAZ tip was also significantly decreased (Fig. 7A) and in some cells was undetectable (data not shown). Western blotting showed that the protein level of FPRC was not altered by CIF4 depletion (Fig. 7C), indicating that CIF4 RNAi resulted in distribution of FPRC to the cytosol; thus, it became undetectable by immunofluorescence microscopy. Together, these results suggest that CIF4 is required for FPRC localization to the FAZ tips.

We also examined the potential effect of CIF4 depletion on the localization and stability of CIF1. Immunofluorescence microscopy using anti-CIF1 antibody showed that knockdown of CIF4 impaired CIF1 localization in ~96% of binucleated cells (Fig. 7, D and E). Western blotting showed that the level of CIF1 protein was not affected by CIF4 RNAi (Fig. 7F). These results demonstrate that CIF4 is required for CIF1 localization to the new FAZ tip.

### Discussion

We have identified two new cytokinesis regulatory proteins, FPRC and CIF4, and integrated them into the CIF1-mediated cytokinesis regulatory pathway that operates at the anterior tip of the new-flagellum daughter cell in the procyclic form of *T. brucei*. This trypanosome-specific cytokinesis regulatory...
pathway now comprises two evolutionarily conserved protein kinases, TbPLK (9, 12, 25) and TbAUK1 (10, 11); a conserved microtubule-severing enzyme complex, KAT60a–KAT80 (18); a kinetoplastid-specific protein phosphatase, KPP1 (17, 18, 20); an orphan kinesin with two C-terminal tropomyosin-like domains (17, 18); four CIF proteins, CIF1–CIF4 (14–16); and four trypanosome-specific proteins, TbSmee1 (26), FRW1 (18, 19), BOH1 (21), and FPRC (Fig. 2). Our previous efforts determined an order of action among several of the cytokinesis regulators (14–16, 18, 23). In this report, analysis of the functional interplay among FPRC, CIF4, and CIF1 placed FPRC and CIF4 upstream of CIF1 (Figs. 3 and 7). However, it is unlikely that there is a linear order of action from CIF4 to FPRC and then to CIF1, as FPRC depletion only exerted a moderate effect on CIF1 (Fig. 3), but CIF4 depletion severely impaired CIF1 localization (Fig. 7, D–F). It suggests that there is a triangle relationship among the three proteins, with FPRC directly regulating CIF1, CIF4 directly regulating FPRC, and CIF4 directly regulating CIF1 as well as indirectly regulating CIF1 via FPRC. An alternative model is that FPRC is only one of the multiple CIF4 downstream factors that regulate CIF1 and, hence, that depletion of each of these CIF4 downstream factors only partially affects CIF1 localization. On the other hand, the functional interplay between the CIF4–FPRC pair and the CIF4–CIF1 pair might be interdependent, as is the case for the TbPLK–CIF1 pair (14), the CIF1–CIF2 pair (15), and the CIF1–CIF3 pair (16). Whether there is any feedback effect on CIF4 by depletion of FPRC and CIF1 is currently under investigation. Finally, it is unclear whether FPRC and CIF4 are regulated by TbPLK, which is known to act upstream of CIF1, or whether they regulate TbPLK. On the Western blot, CIF4 appears to be detected as multiple bands on SDS-PAGE (Figs. 6A and 7, C and F), and, in light of the identification of six in vivo phosphosites on CIF4 by MS (24), the slower-migrating bands of CIF4 might be phosphorylated forms. It would also be interesting to test whether CIF4 is a substrate of TbPLK and/or TbAUK1. All in all, the identification of FPRC and CIF4 will enable us to further elucidate the cytokinesis signaling pathway and investigate the functional interplay among these cytokinesis regulators.

Figure 7. CIF4 is required for localization of FPRC and CIF1 to the FAZ tip. A, localization of FPRC in control and CIF4 RNAi cells. FPRC was endogenously tagged with a triple-HA epitope and detected with FITC-conjugated anti-HA mAb, and the FAZ was labeled with anti-CC2D polyclonal antibody. The open arrowhead and the solid arrowheads indicate FPRC signal at the new FAZ tip and the old FAZ tip, respectively. Scale bar = 5 μm. B, quantification of cells with FPRC fluorescence signal at the new FAZ tip in binucleated cells from control and CIF4 RNAi (48 h). A total of 200 cells were counted for each time point, and error bars indicate S.D. from three independent experiments (n = 3). C, Western blotting to monitor the protein level of FPRC in control and CIF4 RNAi cells. FPRC-3HA was detected by anti-HA mAb, and TbPSA6 served as the loading control. D, localization of CIF1 in control and CIF4 RNAi cells. CIF1 was detected with anti-CIF1 polyclonal antibody, and the FAZ was labeled with anti-FAZ1 (clone L3B2) mAb. The open arrowhead indicates CIF1 signal at the new FAZ tip. Scale bar = 5 μm. E, quantification of cells with CIF1 fluorescence signal at the new FAZ tip in binucleated cells from control and CIF4 RNAi (48 h). A total of 200 cells were counted for each time point, and error bars indicate S.D. from three independent experiments (n = 3). F, Western blot to monitor the protein level of CIF1 in control cells and CIF4 RNAi cells. CIF1 was detected by anti-CIF1 polyclonal antibody, and CIF4–3HA was detected by anti-HA mAb. TbPSA6 served as the loading control.
Roles of CIF4 and FPRC in cytokinesis

tip, but the amount of CIF3 at the old FAZ tip appears to be significantly less than the amount of FPRC and CIF4 at the old FAZ tip (16). It is unclear whether FPRC, CIF4, and CIF3 play any roles at the old FAZ tip, but if they do, then such roles are likely unrelated to cytokinesis because cleavage furrow ingress occurs at the new FAZ tip. One possibility is that the FPRC, CIF4, and CIF3 proteins located at the old FAZ tip are remnants of the new FAZ tip after cell division. In this scenario, this suggests that the three proteins have a half-life longer than those proteins that only localize to the new FAZ tip. It is intriguing to also note that both FPRC and CIF4 emerge at the new FAZ tip as early as the S phase of the cell cycle (Figs. 1 and 5), but cytokinesis initiation (cleavage furrow ingress) only occurs after completion of mitosis (8). Other cytokinesis regulators that also localize to the new FAZ tip from S phase include TbPLK, CIF1, CIF2, CIF3, KPP1, and FRW1 (14–16, 18, 20, 25). However, TbAUK1, KLIF, and the KAT60a–KAT80 complex appear at the new FAZ tip from mitosis (18, 28). These observations raise the interesting question of why the cytokinesis regulators identified so far are all recruited to the future cytokinesis initiation site (the new FAZ tip) before cytokinesis is initiated. It suggests that T. brucei prepares for cytokinesis from as early as S phase of the cell cycle. This may reflect the lack of coordination between cytokinesis and mitosis in the procyclic form of T. brucei (29). T. brucei undergoes closed mitosis (30), and, hence, direct contacts between the cell cortex and the ana-phase spindle are not possible. Therefore, the recruitment of cytokinesis regulators is not influenced by progression of mitosis in T. brucei.

Knockdown of FPRC and CIF4 both impairs cytokinesis initiation (Figs. 2 and 6), but the extent of effects differs substantially between the two RNAi cell lines. FPRC RNAi only had a moderate effect on cell proliferation and cytokinesis initiation (Fig. 2), whereas depletion of CIF4 had a very strong effect on cell proliferation and cytokinesis initiation (Fig. 6). The discrepancy might be attributed to the different efficiency of RNAi, as FPRC RNAi resulted in a 75% reduction in FPRC protein level (Fig. 2A), but CIF4 RNAi resulted in a 87% reduction in CIF4 protein level (Fig. 6A). It appears that other approaches, such as conditional gene knockout, are necessary to achieve knockdown at a higher efficiency, and such an approach is currently being undertaken to reassess the function of FPRC. Nevertheless, the results of RNAi-mediated ablation of FPRC on cleavage furrow ingress (Fig. 2) and on CIF1 localization (Fig. 3) provide convincing evidence to support its role in cytokinesis initiation. We previously reported that knockdown of CIF1 or CIF2 in the procyclic form of T. brucei causes formation of the posterior furrow, and we suggested that depletion of CIF1 or CIF2 triggers an alternative cytokinesis route that operates in the direction opposite to the canonical cytokinesis route (14, 15, 31). Scanning EM results obtained from CIF1 RNAi cells (14), CIF2 RNAi cells (15), as well as FPRC RNAi (Fig. 3F) and CIF4 RNAi cells (Fig. 6F) suggest that the posterior furrow ingresses along the same division fold as the anterior furrow. The underlying mechanism for formation of the posterior furrow in these RNAi cell lines remains unclear. It is possible that the posterior furrow results from remodeling of the subpellicular microtubules and membrane fusion at the nascent posterior of the old-flagellum daughter cell, which causes separation of the nascent posterior from the cell body of the new-flagellum daughter cell. Microtubule remodeling at the nascent posterior of the old-flagellum daughter cell has been well described in T. brucei (7). However, it is unclear why such remodeling does not generate a posterior furrow in WT cells. Future work is directed toward understanding how microtubule remodeling and membrane fusion at the nascent posterior are regulated in the WT and these cytokinesis-deficient RNAi mutants, which might provide clues regarding formation of the posterior cleavage furrow.

Materials and methods

Trypanosome cell culture and RNAi

The procyclic form of the T. brucei 29-13 strain (32) was cultured in SDM-79 medium supplemented with 10% fetal bovine serum, 50 μg/ml hygromycin, and 15 μg/ml G418 at 27 °C. The procyclic form of the T. brucei Lister 427 strain was cultivated in SDM-79 medium containing 10% fetal bovine serum at 27 °C. Cells were routinely diluted with fresh medium whenever the cell density reached 5 × 10⁶/ml.

RNAi was carried out using the pZJM vector (33). To construct pZJM-FPRC, a 740-bp DNA fragment (nucleotides 30–769) of the FPRC gene was PCR-amplified using the following primers: forward primer 5′-ATCTAGCCCTCAGACTCCTTGAGGCCTTGGAGG-3′ and reverse primer 5′-TTCCGATATCAAGCTTCTAGGATGTTGCCGCTTGGAGG-3′. The PCR fragment was cloned into the Xhol/HindIII sites of pZJM. To construct pZJM-CIF4, a 1119-bp DNA fragment (nucleotides 4–1122) of the CIF4 gene was PCR-amplified using the following primers: forward primer 5′-ATCTAGCCCTCAGAGGACCAGGCTAAGGAATCGG-3′ and reverse primer 5′-TTCCGATATCAAGCTTCTAGGATGTTGCCGCTTGGAGG-3′. The RNAi plasmid was linearized by NotI digestion and electroporated into the 29-13 cell line according to our published procedures (34). Successful transfectants were selected with 2.5 μg/ml phleomycin and further cloned by limiting dilution in a 96-well plate. To induce RNAi, the clonal cell line was incubated with 1.0 μg/ml tetracycline. Cell growth was monitored by daily counting of cells with a hemacytometer. At least three clonal cell lines were analyzed. To minimize the off-target effect of RNAi, the DNA fragment used for RNAi was chosen after the sequence was searched against the T. brucei genome database to confirm the uniqueness for the specific gene. The CIF1 RNAi cell line has been described previously (14).

In situ epitope tagging of proteins

Epitope tagging of proteins at the endogenous locus was performed using a one-step PCR-based method described previously (35). For C-terminal epitope tagging, one PCR primer contained a 100-bp DNA sequence that overlapped with the 3′ coding sequence immediately upstream of the stop codon of the gene and a 25-bp DNA sequence that amplified the selection drug marker–containing plasmid vector. The other PCR primer contained a 100-bp DNA sequence that overlapped with the 3′ UTR immediately downstream of the stop codon of the gene and a 25-bp DNA sequence that amplified the plasmid
vector. For N-terminal epitope tagging, one PCR primer contained a 100-bp DNA sequence that overlapped with the 5' UTR immediately upstream of the initiation codon and a 25-bp DNA sequence that amplified the plasmid vector. The other PCR primer contained a 100-bp DNA sequence that overlapped with the 5' coding sequence immediately downstream of the initiation codon and a 25-bp sequence that amplified the plasmid vector. Detailed information regarding primer design can be found in the published literature (35).

**Identification of FPRC-associated proteins by BioID and LC-MS/MS**

To overexpress FPRC–BirA*-HA for identification of FPRC-associated proteins, the full-length coding sequence of FPRC was cloned into the pLew100–BirA*-HA vector (36), and the resulting plasmid was transfected into the 29-13 strain. Transfectants were selected under 2.5 µg/ml puromycin and cloned by limiting dilution. Expression of FPRC–BirA*-HA was induced with 0.1 µg/ml tetracycline and confirmed by Western blotting and immunofluorescence microscopy with the anti-HA mAb.

Affinity purification of biotinylated proteins was performed as described previously (36). FPRC–BirA*-HA was overexpressed by induction with 0.1 µg/ml tetracycline for 24 h, and cells were incubated with 50 µM biotin for an additional 24 h. Cells (~2.5 × 10^6) were washed with PBS and treated with PEME buffer (100 mM PIPES (pH 6.9), 2 mM EGTA, 0.1 mM EDTA, and 1 mM MgSO₄) containing 0.5% Nonidet P-40. Cytosolic (soluble) and cytoskeletal (pellet) fractions were separated, and the cytoskeletal fraction was further extracted with lysis buffer (0.4% SDS, 500 mM NaCl, 5 mM EDTA, 1 mM DTT, and 50 mM Tris- HCl (pH 7.4)). The cytosolic extract and the cytoskeletal extract were combined and incubated with 50% iodoacetamide was added for alkylation. 5% DTT was then added to the solution, and proteins were digested with trypsin overnight at 37 °C. Digestion was stopped by adding TFA to approximately pH 2.0. Trypsin-digested peptides were cleaned up with a C18 ZipTip (Millipore) and subsequently 50% iodoacetamide was added for alkylation, and 10% DTT was then added to reduce the disulfide bond, and subsequently 50% iodoacetamide was added for alkylation. 5% DTT was then added to the solution, and proteins were digested with trypsin overnight at 37 °C. Digestion was stopped by adding TFA to approximately pH 2.0. Trypsin-digested peptides were cleaned up with a C18 ZipTip (Millipore) and dried with a SpeedVac. An aliquot of the tryptic digest (in 2% acetonitrile/0.1% formic acid in water) was analyzed by LC-MS/MS on an Orbitrap Fusion™ Trubrid™ mass spectrometer (Thermo Scientific) interfaced with a Dionex UltiMate 3000 Binary RSLCnano system. Peptides were separated onto an Acclaim™ PepMap™ C18 column (75 µm inner diameter × 15 cm length, 2-µm particle size) at a flow rate of 300 nl/min. Gradient conditions were as follows: 3%–22% solvent B for 40 min; 22%–35% solvent B for 10 min; 35%–90% solvent B for 10 min; 90% solvent B for 10 min (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile). Peptides were analyzed using a data-dependent acquisition method. The Orbitrap Fusion was operated with measurement of FTMS1 at a resolution of 120,000, a scan range of 350–1500 m/z, automatic gain control (AGC) target of 2E5, and a maximum injection time of 50 ms. During a maximum of 3 s of cycle time, the ITMS2 spectra were collected in rapid scan rate mode, with collision-induced dissociation (CID) normalized collision energy (NCE) of 35, a 1.6 m/z isolation window, AGC target of 1E4, and a maximum injection time of 35 ms, and dynamic exclusion was employed for 30 s. Raw MS data were processed and searched using Thermo Proteome Discoverer software or the Mascot search engine. Proteins were searched against the Trypanosoma brucei database. The search conditions used a peptide tolerance of 10 ppm and MS/MS tolerance of 0.8 Da with the enzyme trypsin and two missed cleavages.

**GST pulldown and coimmunoprecipitation**

For GST pulldown experiments, the various domains of CIF1, the NTD (aa 1–120), the CC (aa 121–271), the IDR (aa 272–666), and the ZnF motifs (aa 667–804) as well as the two domains of FPRC, the NTD (aa 1–240), and the CTD (aa 241–487) were each fused with a N-terminal GST tag by cloning their corresponding DNA fragments into the pGEX-4T-3 vector (Clontech). The resulting plasmids were each transformed into the Escherichia coli BL21 strain, and recombinant GST fusion proteins were purified through a GSH-Sepharose 4B column (GE Healthcare). GST pulldown was carried out as described in our previous publication (21).

Trypanosome cells coexpressing CIF4–3HA and FPRC-PTP from their respective endogenous loci were collected by centrifugation, washed with PBS, and resuspended in 500 µl of immunoprecipitation buffer (25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM DTT, 1% NP-40, and protease inhibitor mixture). Cells were lysed by sonication, and cell lysate was cleared by centrifugation. The supernatant was incubated with 30 µl of settled IgG–Sepharose beads (GE Healthcare) for 1 h at 4 °C with gentle rotation. The IgG beads were washed five times with immunoprecipitation buffer, and bound proteins were eluted with 30 µl of 10% SDS. Trypanosome cells expressing CIF4–3HA alone and FPRC-PTP alone were used as controls. Immunoprecipitated proteins were separated by SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted with anti-protein A polyclonal antibody (1:2000 dilution, Sigma-Aldrich, P3775) and anti-HA mAb (1:5000 dilution, Sigma-Aldrich, clone HA-7, H9658) to detect FPRC-PTP and CIF4–3HA, respectively.

Trypanosome cells expressing CIF4–3HA from its endogenous locus were lysed in immunoprecipitation buffer by sonication as described above, and cell lysate was cleared by centrifugation. The supernatant was incubated with 1.0 µl anti-CIF1 polyclonal antibody (18) for 1 h at 4 °C with gentle rotation and then incubated with 40 µl of protein A-Sepharose beads for 1 h at 4 °C with gentle rotation. The beads were washed five times with the immunoprecipitation buffer, and bound proteins were eluted with 30 µl of 10% SDS. Trypanosome cells expressing FA224–3HA (18) or FPRC-3HA were used as negative and positive controls, respectively. Immunoprecipitated proteins were separated by SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted with anti-HA mAb (1:5000 dilution, Sigma-Aldrich, clone HA-7, H9658) and anti-CIF1 polyclonal antibody (1:1000 dilution) to detect HA-tagged proteins and CIF1, respectively.

**Roles of CIF4 and FPRC in cytokinesis**
Roles of CIF4 and FPRC in cytokinesis

Western blotting

Western blotting was performed as described previously (37). An equal number of trypanosome cells was collected by centrifugation, lysed in SDS sampling buffer, and boiled for 5 min. The cell lysate was loaded onto a 10% SDS-PAGE gel, and proteins were separated by gel electrophoresis, transferred onto a PVDF membrane, and blotted in blocking buffer (3% nonfat milk in PBS). The membrane was then incubated with the primary antibody (anti-HA mAb, 1:1000 dilution; anti-CIF1 polyclonal antibody, 1:1000 dilution; anti-protein A polyclonal antibody, 1:1000 dilution; anti-TbPSA6 polyclonal antibody, 1:1000 dilution) for 1 h at room temperature, washed three times with PBS, and then incubated with HRP-conjugated secondary antibody (anti-mouse IgG, 1:1000 dilution; anti-rabbit IgG, 1:1000 dilution). The membrane was washed three times with PBS and then developed using the FluarChem HD2 system (ProteinSimple Inc.).

Immunofluorescence microscopy

*T. brucei* cells were collected by centrifugation, washed once with PBS, adhered to glass coverslips, and fixed in cold methanol (−20 °C) for 20 min. Cells on the coverslips were then incubated with blocking buffer (3% BSA in PBS) for 1 h at room temperature and then incubated with the primary antibody for 1 h at room temperature. The following antibodies were used: anti-CIF1 polyclonal antibody (1:1000 dilution) (18), anti-protein A antibody (1:400 dilution, Sigma-Aldrich), anti-CC2D polyclonal antibody (1: 1000 dilution) (5), anti-FaZ1 (clone L3B2) mAb (38), and FITC-conjugated anti-HA antibody (1:400 dilution, Sigma-Aldrich). Cells were then washed three times with PBS containing 0.1% Triton X-100. Except for the FITC-conjugated anti-HA antibody, cells were then incubated with Cy3-conjugated anti-rabbit IgG (Sigma-Aldrich) or Cy3-conjugated anti-mouse IgG (Sigma-Aldrich) at room temperature for 1 h. The slides were mounted in VectaShield mounting medium (Vector Laboratories) containing DAPI and examined with a Nikon fluorescence microscope (model IX71, Olympus) equipped with Cy3-conjugated anti-rabbit IgG (Sigma-Aldrich) or Cy3-conjugated anti-mouse IgG (Sigma-Aldrich).

Scanning EM

Scanning EM was performed as described previously (14). *T. brucei* cells were collected by centrifugation, washed three times with PBS, and settled onto glass coverslips. Cells were then fixed with 2.5% (v/v) glutaraldehyde in PBS for 30 min in the dark at room temperature. Cells were dehydrated with a series of alcohol (30%, 50%, 70%, 90%, and 100%) and dried by critical-point drying. Coverslips were then coated with a 8-nm metal film (Pt:Pd 80:20, Ted Pella Inc.) using a sputter coater (Cressington Sputter Coater 208 HR, Ted Pella Inc.). Cells on the coverslip were examined using Nova NanoSEM 230 (FEI). The parameters used were 5 mm for the scanning work distance and 8 kV for the accelerating high voltage.

Statistical analysis

For statistical analysis, we used the Student’s *t* test provided in the Microsoft Excel software. The *n* values for each panel in the figures are indicated in the corresponding figure legends. For counting of cells from immunofluorescence microscopy, images were taken randomly, and all cells in each image were counted. Data were collected from three independent experiments.

Acknowledgments—We thank Dr. George A. M. Cross (Rockefeller University) for providing the 29-13 cell line, Dr. Keith Gull (University of Oxford) for providing the anti-FaZ1 antibody, and Dr. Cynthia Y. He (National University of Singapore) for providing the CC2D antibody. We also thank Dr. James Gu (Houston Methodist Research Institute) for assistance with scanning EM and Dr. Li Li (Clinical and Translational Service Center, UT Health Science Center at Houston) for assistance with mass spectrometry.

References

1. Burki, F. (2014) The eukaryotic tree of life from a global phylogenomic perspective. Cold Spring Harb. Perspect. Biol. 6, a016417 CrossRef Medline

2. Li, Z. (2012) Regulation of the cell division cycle in *Trypanosoma brucei*. Eukaryot. Cell 11, 1180–1190 CrossRef Medline

3. Sunter, J. D., and Kohl, L. (2013) Novel roles for the flagellum in cell morphogenesis and cytokinesis of trypanosomes. EMBO J. 32, 5336–5346 CrossRef Medline

4. 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 CrossRef Medline

5. Oliferenko, S., Chew, T. G., and Balasubramanian, M. K. (2009) Positioning cytokinesis. Genes Dev. 23, 660–674 CrossRef Medline

6. Wheeler, R. J., Scheumann, N., Wickstead, B., Gull, K., and Vaughan, S. (2013) Cytokinesis in *Trypanosoma brucei* differs between bloodstream and tsetse trypanostage forms: implications for microtubule-based morphogenesis and mutant analysis. Mol. Microbiol. 90, 1393–1355 CrossRef Medline

7. Tu, X., Kumar, P., Li, Z., and Wang, C. C. (2006) Dissociation of cytokinesis initiation from mitotic control in a eukaryote. Eukaryot. Cell 5, 92–102 CrossRef Medline

8. 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 CrossRef Medline

9. Kumar, P., and Wang, C. C. (2006) Dissociation of cytokinesis initiation from mitotic control in a eukaryote. Eukaryot. Cell 5, 92–102 CrossRef Medline

10. Tu, X., Kumar, P., Li, Z., and Wang, C. C. (2006) An aurora kinase homologue is involved in regulating both mitosis and cytokinesis in *Trypanosoma brucei*. J. Biol. Chem. 281, 9677–9687 CrossRef Medline

11. Tu, X., and Wang, C. C. (2006) Changing roles of aurora-B kinase in two life cycle stages of *Trypanosoma brucei*. Eukaryot. Cell 5, 1026–1035 CrossRef Medline

12. Hammerton, 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 CrossRef Medline
Roles of CIF4 and FPRC in cytokinesis

13. Li, Z., Umeyama, T., Li, Z., and Wang, C. C. (2010) Polo-like kinase guides cytokinesis in Trypanosoma brucei through an indirect means. *Eukaryot. Cell* 9, 705–716 CrossRef Medline

14. Zhou, Q., Gu, J., Lun, Z. R., Ayala, F. J., and Li, Z. (2016) Two distinct cytokinesis pathways drive trypanosome cell division initiation from opposite cell ends. *Proc. Natl. Acad. Sci. U.S.A.* 113, 3287–3292 CrossRef Medline

15. Zhou, Q., Hu, H., and Li, Z. (2016) An EF-hand-containing protein in *Trypanosoma brucei* regulates cytokinesis initiation by maintaining the stability of the cytokinesis initiation factor CIF1. *J. Biol. Chem.* 291, 14395–14409 CrossRef Medline

16. Kurasawa, Y., Hu, H., Zhou, Q., and Li, Z. (2018) The trypanosome-specific protein CIF3 cooperates with the CIF1 protein to promote cytokinesis in *Trypanosoma brucei*. *J. Biol. Chem.* 293, 10275–10286 CrossRef Medline

17. Hilton, N. A., Sladewski, T. E., Perry, J. A., Patalki, Z., Sinclair-Davis, A. N., Muniz, R. S., Tran, H. L., Wurster, J. I., Seo, J., and de Graffenried, C. L. (2018) Identification of TOEFAZ1-interacting proteins reveals key regulators of *Trypanosoma brucei* cytokinesis. *Mol. Microbiol.* 109, 306–326 CrossRef Medline

18. Zhou, Q., An, T., Pham, K. T. M., Hu, H., and Li, Z. (2018) The CIF1 protein is a master orchestrator of trypanosome cytokinesis that recruits several cytokinesis regulators to the cytokinesis initiation site. *J. Biol. Chem.* 293, 16177–16192 CrossRef Medline

19. Zhang, X., An, T., Pham, K. T. M., Lun, Z. R., and Li, Z. (2019) Functional analyses of cytokinesis regulators in bloodstream stage *Trypanosoma brucei* parasites identify functions and regulations specific to the life cycle stage. *mSphere* 4, e00199-19 CrossRef Medline

20. Zhou, Q., Dong, G., and Li, Z. (2018) Flagellar inheritance in *Trypanosoma brucei* requires a kinetoplast-specific protein phosphatase. *J. Biol. Chem.* 293, 8508–8520 CrossRef Medline

21. Pham, K. T. M., Zhou, Q., Kurasawa, Y., and Li, Z. (2019) BOH1 cooperates with Polo-like kinase to regulate flagellar inheritance and cytokinesis initiation in *Trypanosoma brucei*. *J. Cell Sci.* 132, jcs230581 CrossRef Medline

22. Roux, K. J., Kim, D. I., Raida, M., and Burke, B. (2012) A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J. Cell Biol.* 196, 801–810 CrossRef Medline

23. Hu, H., Majneri, P., Li, D., Kurasawa, Y., An, T., Dong, G., and Li, Z. (2017) Functional analyses of the CIF1-CIF2 complex in trypanosomes identify the structural motifs required for cytokinesis. *J. Cell Sci.* 130, 4108–4119 CrossRef Medline

24. Urbaniak, M. D., Martin, D. M., and Ferguson, M. A. (2013) Global quantitative SILAC phosphoproteomics reveals differential phosphorylation is widespread between the procyclic and bloodstream form lifecycle stages of *Trypanosoma brucei*. *J. Proteome Res.* 12, 2233–2244 CrossRef Medline

25. 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 CrossRef Medline

26. Perry, J. A., Sinclair-Davis, A. N., McCaIster, M. R., and de Graffenried, C. L. (2018) TbSmeel regulates hook complex morphology and the rate of flagellar pocket uptake in *Trypanosoma brucei*. *Mol. Microbiol.* 107, 344–362 CrossRef Medline

27. Geieratmand, L., Brasseur, A., Zhou, Q., and He, C. Y. (2013) Biochemical characterization of the bi-lobe reveals a continuous structural network linking the bi-lobe to other single-copied organelles in *Trypanosoma brucei*. *J. Biol. Chem.* 288, 3489–3499 CrossRef Medline

28. 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 CrossRef Medline

29. Ploubidou, A., Robinson, D. R., Docherty, R. C., Ogbadouyi, E. O., and Gull, K. (2000) Architecture of the *Trypanosoma brucei* nucleus during interphase and mitosis. *Chromosoma* 108, 501–513 CrossRef Medline

30. Zhou, Q., and Li, Z. (2016) A backup cytokinesis pathway in *Trypanosoma brucei*. *Cell Cycle* 15, 2379–2380 CrossRef Medline

31. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 99, 89–101 CrossRef Medline

32. 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 CrossRef Medline

33. Wei, Y., Hu, H., Lun, Z. R., and Li, Z. (2013) The cooperative roles of two kinetoplastid-specific kinesins in cytokinesis and in maintaining cell morphology in bloodstream trypanosomes. *PLoS ONE* 8, e73869 CrossRef Medline

34. Shen, S., Arbin, G. K., Ullu, E., and Tschudi, C. (2001) *In vivo* epistasis tagging of *Trypanosoma brucei* genes using a one step PCR-based strategy. *Mol. Biochem. Parasitol.* 113, 171–173 CrossRef Medline

35. Hu, H., Zhou, Q., and Li, Z. (2015) SAS-4 protein in *Trypanosoma brucei* controls life cycle transitions by modulating the length of the flagellum attachment zone filament. *J. Biol. Chem.* 290, 30453–30463 CrossRef Medline

36. Kohl, L., Sherwin, T., and Gull, K. (1999) Assembly of the paraflagellar rod linking flagellar pocket uptake in *Trypanosoma brucei*. *J. Eukaryot. Microbiol.* 46, 105–109 CrossRef Medline