The CIF1 protein is a master orchestrator of trypanosome cytokinesis that recruits several cytokinesis regulators to the cytokinesis initiation site

Qing Zhou1, Tai An1, Kieu T. M. Pham, Huiqing Hu, and Ziyin Li2

From the Department of Microbiology and Molecular Genetics, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, Texas 77030

Edited by Xiao-Fan Wang

To proliferate, the parasitic protozoan Trypanosoma brucei undergoes binary fission in a unidirectional manner along the cell’s longitudinal axis from the cell anterior toward the cell posterior. This unusual mode of cell division is controlled by a regulatory pathway composed of two evolutionarily conserved protein kinases, Polo-like kinase and Aurora B kinase, and three trypanosome-specific proteins, CIF1, CIF2, and CIF3, which act in concert at the cytokinesis initiation site located at the distal tip of the newly assembled flagellum attachment zone (FAZ). However, additional regulators that function in this cytokinesis signaling cascade remain to be identified and characterized. Using proximity biotinylation, co-immunofluorescence microscopy, and co-immunoprecipitation, we identified 52 CIF1-associated proteins, and validated six CIF1-interacting proteins, including the putative protein phosphatase KPP1, the katanin p80 subunit KAT80, the cleavage furrow–localized proteins KLIF and FRW1, and the FAZ tip–localized proteins FAZ20 and FPRC. Further analyses of the functional interplay between CIF1 and its associated proteins revealed a requirement of CIF1 for localization of a set of CIF1-associated proteins, an interdependence between KPP1 and CIF1, and an essential role of katanin in the completion of cleavage furrow ingress. Together, these results suggest that CIF1 acts as a master regulator of cytokinesis in Trypanosoma brucei by recruiting a cohort of cytokinesis regulatory proteins to the cytokinesis initiation site.

Trypanosoma brucei, a devastating eukaryotic pathogen causing sleeping sickness in humans and nagana in cattle, poses a significant public health burden in sub-Saharan Africa. This unicellular parasite has a complex life cycle, alternating between the tsetse fly vector and the mammalian hosts, in which the parasite proliferates as an insect (procyclic) form and a bloodstream form, respectively. T. brucei possesses a motile flagellum that is nucleated from the flagellar basal body located at the posterior portion of the cell, exits the cell body through the flagellar pocket, and extends along the cell body toward the cell anterior. The flagellum is attached, along most of its length, to the cell body through a specialized cytoskeletal structure termed the flagellum attachment zone (FAZ),3 which is composed of multiple subcellular structures located within the cell body and the flagellum (1). The length of the flagellum and the FAZ defines the cell division plane, and the anterior tip of the FAZ constitutes the site of cytokinesis furrow ingression, which proceeds unidirectionally toward the cell posterior, as visualized by live-cell video microscopy (2). Cleavage furrow ingression in yeast, amebae, and animals is mediated by the actomyosin contractile ring assembled along the short axis of the cell (3). However, the lack of a type II myosin (4) and the unlikely involvement of actin in cytokinesis in T. brucei (5) suggest an actomyosin-independent mechanism for cleavage furrow ingression in T. brucei.

Understanding how cell division is controlled in T. brucei is of paramount interest, as its unusual mechanism of cytokinesis suggests the existence of trypanosome-specific cytokinesis pathways/regulators that may be exploited as potential drug targets. Great efforts have been devoted to delineating the cytokinesis signaling pathway, starting with the characterization of two evolutionarily conserved protein kinases, the Polo-like kinase homolog TbPLK (6, 7) and the Aurora B kinase homolog TbAUK1 (8, 9). TbPLK localizes to the cytokinesis initiation site from S phase to early anaphase (10), and TbAUK1 localizes to the cytokinesis initiation site from late anaphase to telophase and then to the cleavage furrow during cytokinesis (2, 11). Subsequent identification of CIF1 as an essential cytokinesis regulator bridging TbPLK and TbAUK1 unveiled the mechanistic role of TbPLK in cytokinesis initiation and delineated a novel cytokinesis signaling pathway from TbPLK through CIF1 to TbAUK1 (12). CIF1 forms two separate protein complexes with two trypanosome-specific proteins, CIF2 (13) and CIF3 (14). The CIF1–CIF2 complex appears to function during S phase (13), and CIF1 is required for recruiting CIF2 to the new FAZ tip (15). The CIF1–CIF3 complex likely acts from G2 phase to cytokinesis, and the two protein subunits exert distinct effects on each other, with CIF1 maintaining CIF3 stability and CIF3

3 The abbreviations used are: FAZ, flagellum attachment zone; BioID, proximity-dependent biotin identification; HA, hemagglutinin; GST, glutathione S-transferase; aa, amino acids; KLIF, kinesin localized in ingressing furrow; FPRC, FAZ tip protein required for cytokinesis; pAb, polyclonal antibody; PTP, protein A/tobacco etch virus protease site/protein C.

This work was supported by National Institutes of Health Grants AI101437 and AI118736 (to Z. L.). 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–S7 and Tables S1–S3.

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Tel.: 713-500-5139; Fax: 713-500-5499; E-mail: Ziyin.Li@uth.tmc.edu.
CIF1 is a master regulator of cytokinesis

maintaining CIF1 localization (14). These studies built the framework for delineating the cytokinesis regulatory pathway and in-depth understanding of the mechanism of cytokinesis in T. brucei.

Our previous proximity biotin identification (BioID) using CIF1 fused with a C-terminal BirA* as the bait identified 160 proteins as potential CIF1-associated partners (13), but the majority of them have not been validated and functionally characterized. We recently identified CIF3 as a binding partner of CIF1 (14), but CIF3 was not detected by CIF1 BioID, suggesting that certain CIF1-associated proteins may have been missed in our previous BioID experiments, potentially because of interference of the C-terminal BirA* tag. Therefore, we carried out new BioID experiments using CIF1 fused with an N-terminal BirA* as the bait and identified a total of 305 potential CIF1-associated proteins. We further validated the CIF1-associated proteins by co-immunofluorescence microscopy and co-immunoprecipitation and investigated the functional interplay of CIF1 with its associated proteins. Our results suggest that CIF1 functions as a master regulator of cytokinesis by targeting a cohort of cytokinesis regulators to the cytokinesis initiation site.

Results

Generation and validation of an anti-CIF1 polyclonal antibody

To facilitate study of the CIF1-mediated cytokinesis pathway, we generated an anti-CIF1 polyclonal antibody by immunizing rabbits with a purified CIF1 C-terminal domain containing the two zinc-finger motifs. Western blotting showed that this antibody detected a major band of ~120 kDa that was gradually depleted upon CIF1 RNAi induction (Fig. S1A). Immunofluorescence microscopy with anti-CIF1 antibody, but not the pre-immune serum and the anti-CIF1 antibody that was preincubated with excess antigen, detected a specific fluorescence signal at the new FAZ tip that disappeared after CIF1 knockdown (Fig. S1B), confirming the specificity of this antibody. Further, co-immunofluorescence microscopy of cells expressing 3HA-tagged CIF1 with anti-CIF1 antibody and anti-HA antibody showed localization of CIF1 to the new FAZ tip and the cleavage furrow (Fig. S1C), thus validating the results obtained with epitope-tagged CIF1 (12).

Identification of CIF1-interacting proteins

Among the 160 potential CIF1-associated proteins identified by BioID with CIF1-BirA*-HA as the bait (13), only 24 proteins have been confirmed to localize to CIF1-associated structures, which include the hook complex, the new FAZ tip, and the cleavage furrow. They include 18 proteins that localize to the FAZ or the FAZ tip and six proteins that localize to the hook complex (13, 16). To identify additional CIF1-associated proteins that may have been missed in previous CIF1-BirA* BioID experiments, we performed BioID using CIF1 tagged with an N-terminal BirA* (BirA*-CIF1) as the bait (Fig. S2) and analyzed the purified proteins by LC-MS/MS. From the more than 500 proteins thus identified, we excluded tubulins, ribosomal proteins, histones, and metabolic enzymes, which were commonly detected in our BioID experiments with different bait proteins and are thus regarded as nonspecific hits (Table S1). As a result, a total of 305 potential CIF1-associated proteins were identified (Table S2).

To validate CIF1-associated proteins, the 305 proteins identified by BirA*-CIF1 BioID and the remaining 126 proteins that were identified by the previous CIF1-BirA* BioID but were not validated were first searched against the TriTryp database (www.tritrypdb.org)4 and the TrypTag database (www.tryptag.org)4 (17) for known localization data. Proteins whose localization data are unavailable were endogenously tagged with a triple HA epitope, and their localization was determined by immunofluorescence microscopy. As CIF1 localizes to the new FAZ tip from S phase to early cytokinesis and to the cleavage furrow during cytokinesis (12), its associated proteins are anticipated to localize to the new FAZ tip during any cell cycle stage from S phase to telophase or to the cleavage furrow during cytokinesis. Additionally, as the hook complex partly overlaps with the newly assembled FAZ during early S phase, proteins that localize to the hook complex during early S phase are also considered CIF1-associated proteins. Based on this criterion, a total of 52 proteins were considered CIF1-associated proteins; 33 of these were detected by BioID with both CIF1-BirA* and BirA*-CIF1 as baits, three were only detected by BioID with CIF1-BirA* as the bait, and 16 were detected by BioID with BirA*-CIF1 as the bait (Table S3).

Among the 52 CIF1-associated proteins, five proteins localize to the new FAZ tip, including CIF2, FAZ18 (13), KPP1 (16), and two subunits of katanin, KAT60a and KAT80 (18). Three proteins localize to the new FAZ tip and the cleavage furrow, including CIF3 (14), an orphan kinesin (Tb927.8.4950) that was recently named KLIF (kinesin localized at ingressing furrow) (19), and a G2/M-enriched protein (Tb927.10.870) (20) we named FRW1 (furrow 1). Seven proteins localize to both the new and old FAZ tips, including four known FAZ tip proteins (TbSAS-4 (33), FAZ11, FAZ14, and FAZ20 (13)) and three new proteins named FAZ21 (Tb927.7.5240), FAZ24 (Tb927.10.720), and FPRC (FAZ tip protein required for cytokinesis, Tb927.10.6360). The remaining 37 proteins localize either to the entire FAZ (22 proteins) or to the hook complex (15 proteins) (Table S3). Only CIF2 and CIF3 have been confirmed to interact with CIF1 in vivo in trypanosomes (13, 14).

Because we aimed to delineate the CIF1-mediated cytokinesis regulatory pathway, we investigated the potential co-localization and in vivo interaction between CIF1 and its associated proteins that localize to the new FAZ tip or both the new and old FAZ tips. The first set of associated proteins includes KPP1, KAT60a, KAT80, KLIF, FRW1, and FAZ18, and the second set of associated proteins includes FAZ11, FAZ14, FAZ20, FAZ21, FAZ24, and FPRC. Co-immunostaining with anti-CIF1 antibody and FITC-conjugated anti-HA antibody demonstrated the co-localization of CIF1 and its associated proteins, which were endogenously tagged with a triple HA epitope, at the new FAZ tip (Fig. 1, A and B). To test whether any of these CIF1-associated proteins interact with CIF1, we performed immunoprecipitation with anti-CIF1 antibody. Among the CIF1-associated proteins that localize to the new FAZ tip, KPP1, KAT80, 4 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party–hosted site.
KLIF, and FRW1, but not KAT60a and FAZ18, were co-precipitated with CIF1 (Fig. 1C, left panel). Among the CIF1-associated proteins that localize to both the new and old FAZ tips, FAZ20 and FPRC, but not FAZ11, FAZ14, FAZ21, and FAZ24, were co-precipitated with CIF1 (Fig. 1C, right panel). These results suggest that CIF1 interacts with a subset of its associated proteins in vivo in trypanosomes.

**Bioinformatics analysis of CIF1-interacting proteins**

Bioinformatics analyses of the six new CIF1-interacting proteins with hidden Markov models (21), SWISS-MODEL (37), and COILS algorithm (38) identified various structural motifs (Fig. 2A), indicating diverse biochemical functions of these CIF1-interacting proteins. Two proteins are involved in signal transduction. The putative phosphatase KPP1 contains an N-terminal Plus3 domain (E-value, 1.68e−04) that binds to phospho-threonine-containing peptides (22) and single-stranded DNA (23) and a conserved protein phosphatase 1 catalytic (PP1C) domain (E-value, 1.58e−76) at the C terminus, and the putative protein kinase FAZ20 contains an N-terminal serine/threonine kinase domain (E-value, 4.15e−07) and multiple coiled-coil motifs at the C terminus (Fig. 2A). The katanin p80 subunit KAT80 contains four WD40 repeats at the N terminus and a katanin con80 domain (E-value, 3.2e−39) at the C terminus (Fig. 2A). The tropomyosin-like domains (TPM1 and TPM2) in KLIF share 30% sequence identity to the tropomyosin protein from vertebrate animals (24)(Fig. S3B) and possess a similar rod-shaped coiled-coil structure that is potentially capable of dimerization like the vertebrate tropomyosin (Fig. S3C). The tropomyosin-like domains appear to be a part of a repetitive sequence con-
CIF1 is a master regulator of cytokinesis

Consisting of 19 repeats of a 28-amino acid sequence (Fig. S3D). Tropomyosin, consisting of rod-shaped coiled coils, acts as a hetero- or homodimer to regulate muscle contraction in muscle cells and a variety of cellular functions in nonmuscle cells (25). In fission yeast, the tropomyosin homolog Cdc8 is essential for cytokinesis (26). FRW1 and FPRC contain only coiled-coil motifs (Fig. 2A), suggesting that they likely function as the structural components of protein complexes at the FAZ tip.

Requirement of the zinc-finger motifs in CIF1 for interaction with CIF1-interacting proteins

CIF1 contains a coiled-coil motif at the N terminus and two CCHC-type zinc-finger motifs at the C terminus (Fig. 2A) (where CCHC is Cys-Cys-His-Cys), and the zinc-finger motifs are required for interaction with CIF2 and CIF3 (14, 15). To test whether the zinc-finger motifs are required for interaction with the six new CIF1-interacting proteins, we carried out in vitro GST pulldown experiments. To this end, GST-fused WT zinc-finger motifs (GST-CIF1-ZnF1&2) and two mutant zinc-finger motifs bearing point mutations of the zinc-coordinating residues in each of the two zinc-finger motifs (CIF1-ZnF1mut and CIF1-ZnF2mut) (15) were used to pull down each of the six CIF1-interacting proteins tagged with a triple HA epitope. The results showed that all six CIF1-interacting proteins were pulled down by the WT zinc-finger domain of CIF1, whereas neither zinc-finger motif mutant was able to pull down these CIF1-interacting proteins (Fig. 2B). These results demonstrated that the two zinc-finger motifs mediate the interaction of CIF1 with its binding partners.

We previously proposed that the Plus3 domain of KPP1 is involved in interaction with TbPLK substrates (16). As CIF1 and KPP1 interact in vivo in trypanosomes (Fig. 1C), we tested whether the Plus3 domain of KPP1 mediates the interaction with CIF1. In vitro GST pulldown using the purified recombinant Plus3 domain of KPP1 (GST-Plus3KPP1) showed that it was able to pull down CIF1 (Fig. 2C). Mutation of either zinc-finger motif, but not deletion of the coiled-coil motif, abolished CIF1 binding to the Plus3 domain of KPP1 (Fig. 2C). These results demonstrated that interaction between CIF1 and KPP1 is mediated by the zinc-finger motifs of CIF1 and the Plus3 domain of KPP1. As the Plus3 domain is involved in binding to phospho-threonine-containing peptides (22) and the Plus3 domain in KPP1 contains four of the five residues involved in binding to phospho-peptides (16), we asked whether phosphorylation of CIF1 is required for CIF1 interaction with the Plus3 domain of KPP1. Treatment of trypanosome cell lysate with λ protein phosphatase disrupted the in vitro interaction of CIF1 with Plus3KPP1 (Fig. 2D). Together, these results suggest that inter-

Figure 2. Interaction between CIF1 and its interacting partners is mediated by the zinc-finger motifs. A, schematic of the structural motifs in CIF1-interacting proteins and CIF1. Plus3, Plus3 domain; PP1C, protein phosphatase 1 catalytic domain; WD40, WD40 repeats; con80, con80 domain of katanin; CC, coiled coil; ZnF, zinc finger. B, in vitro GST pulldown of CIF1-interacting proteins by the CIF1 zinc-finger motif. GST-CIF1-ZnF1&2, GST-CIF1-ZnF1mut, and GST-CIF1-ZnF2mut were used to pull down 3HA-tagged CIF1-interacting proteins, which were detected by Western blotting with anti-HA antibody. GST alone was used as the control. GST and GST fusion proteins were stained with Coomassie Blue. C, interaction between CIF1 and KPP1 is mediated by the zinc-finger motifs of CIF1 and the Plus3 domain of KPP1. GST-Plus3KPP1 was used to pull down 3HA-tagged WT and mutant CIF1 proteins, which were detected by Western blotting with anti-HA antibody. GST and the GST-fused Plus3 domain were stained with Coomassie Blue. D, interaction of CIF1 with KPP1 requires phosphorylation of CIF1. GST-Plus3KPP1 was used to pull down PTP-tagged CIF1 from cell lysate treated without or with λ protein phosphatase (λPPase).
action between CIF1 and KPP1 requires the Plus3 domain in KPP1 and the zinc-finger motifs in CIF1 and depends on the phosphorylation state of CIF1.

CIF1 is required for KPP1 localization, and KPP1 is required for maintaining CIF1 stability

The functional interplay between CIF1 and KPP1 was investigated. We first examined the effect of CIF1 depletion on KPP1 localization and protein stability. Knockdown of CIF1 disrupted KPP1 localization to the new FAZ tip, but localization of KPP1 to the basal body in 2N2K cells was not affected (Fig. 3, A and B). Western blotting showed that the level of KPP1 protein was not affected in CIF1 RNAi cells, in which the CIF1 protein level was reduced to ~20% of the control level at 48 h (Fig. 3C). These results demonstrated that CIF1 is required for KPP1 localization to the new FAZ tip.
Conversely, we investigated the effect of KPP1 depletion on CIF1 localization and protein stability. As KPP1 knockdown disrupted FAZ assembly from 72 h of RNAi induction (16), it is necessary to rule out the indirect effect of the FAZ assembly defect on the localization of CIF1 to the new FAZ tip. Therefore, we investigated CIF1 localization in KPP1 RNAi cells induced for 48 h when the new FAZ was still intact and the new flagellum was not detached in the majority of the cells (16). CIF1 localization to the new FAZ tip was not affected (Fig. 3D, b, and E) at 48 h of KPP1 RNAi when KPP1 protein was down-regulated to an undetectable level (Fig. 3F). At 72 h of KPP1 RNAi, however, CIF1 was either detected along most of the length of the new FAZ in 2N2K cells with a partially assembled new FAZ and a partially detached new flagellum (Fig. 3D, c), at the flagellar pocket region (Fig. 3D, d, and E), or undetectable in 2N2K cells with a short new FAZ, and a fully detached new flagellum (Fig. 3, D and E). Western blotting showed that, starting from 72 h and onward, the level of CIF1 protein gradually decreased, and treatment of cells with the proteasome inhibitor MG-132 stabilized CIF1 protein (Fig. 3F), demonstrating that CIF1 was destabilized when it was not localized to the new FAZ tip in KPP1 RNAi cells.

CIF1 is required for localizing the KAT60α–KAT80 complex to the new FAZ tip

Katanin is a microtubule-severing enzyme involved in regulating microtubule dynamics in eukaryotes. It is a heterodimer composed of the catalytic AAA-ATPase subunit p60 and the regulatory subunit p80 (27) (where AAA is ATPase associated with diverse cellular activities). Previous work showed that RNAi of the katanin p80 subunit (KAT80) in the procyclic form (18) slowed down cell proliferation and caused an accumulation of multinucleated cells, suggestive of cell division defects (18). Subsequent work in the bloodstream form of T. brucei showed down cell proliferation and caused an accumulation of multinucleated cells, suggestive of cell division defects (18). Subsequent work in the bloodstream form of T. brucei showed that KAT80 and all three katanin p60 subunit homologs (KAT60a, KAT60b, and KAT60c) are essential for cytokinesis in the bloodstream form (28). The subcellular localizations of KAT80, KAT60a, KAT60b, and KAT60c were investigated in the procyclic form by ectopic overexpression. With the exception of KAT60c, overexpressed KAT60a and KAT80 were localized to the cytosol, whereas overexpressed KAT60b was localized to the flagellar tip (18). However, we showed that endogenously triple HA-tagged KAT60a and KAT80 co-localized with CIF1 at the new FAZ tip (Fig. 1A) and interacted with CIF1 in vivo (Fig. 1C). We further examined the localization of KAT60a and KAT80, which were endogenously tagged with a triple HA epitope and PTP epitope, respectively, during the cell cycle. From G1 phase to G2 phase, the two proteins appeared to be spread in the cytosol but were enriched at the posterior cell tip (Fig. S4A, solid arrowhead). Starting from mitosis and onward, the two proteins were enriched at the new FAZ tip (Fig. S4, A and B, open arrowhead), and their fluorescence signal at the posterior cell tip was reduced (Fig. S4, A and B, solid arrowheads). Co-immunoprecipitation showed that KAT80-PTP was able to pull down KAT60α-3HA from trypanosome cell lysate (Fig. S4, C and D), demonstrating that KAT60a and KAT80 form a complex.

We investigated whether CIF1 is required for KAT60a and KAT80 localization to the new FAZ tip. Immunofluorescence microscopy showed that, in CIF1 RNAi cells induced for 24 h, both KAT60a and KAT80, which were each tagged with a triple HA epitope, were no longer detectable at the new FAZ tip in ~82% of the 2N2K cells examined (Fig. 4, A and B). Western blotting showed that the levels of KAT60a and KAT80 were not affected in CIF1 RNAi cells, in which CIF1 protein level was reduced to ~20% of the control level after 48 h (Fig. 4C). These results indicate that localization of both proteins to the new FAZ tip depends on CIF1. Conversely, the potential effect of KAT60a and KAT80 RNAi on CIF1 localization was also investigated. We generated RNAi cell lines for both KAT60a and KAT80, each of which showed defective cytokinesis (see below), and tagged CIF1 with a C-terminal triple HA epitope in the two RNAi cell lines. Immunofluorescence microscopy showed that CIF1 localization to the new FAZ tip was not affected by knockdown of KAT60a and KAT80 (Fig. 4D), indicating that CIF1 localization to the new FAZ tip is independent of the KAT60α–KAT80 complex.

The KAT60a–KAT80 complex is required for cytokinesis furrow ingression

The finding that the KAT60a–KAT80 complex localizes to the new FAZ tip in a CIF1-dependent manner (Fig. 4) suggests that this complex may function downstream of CIF1 to promote cytokinesis. Previous work demonstrated that KAT80 is required for cell division in the procyclic form (18) but did not characterize the potential effect of KAT80 knockdown on cytokinesis and also failed to reveal any growth defect in KAT60a RNAi cells. We revisited the function of KAT60a and KAT80 in the procyclic form. RNAi was efficient to knock down KAT60a and KAT80 proteins to undetectable levels after 24 h (Fig. 5A), and a moderate growth defect was observed for both RNAi cell lines (Fig. 5B). Cells at different cell cycle stages following KAT60a and KAT80 RNAi were tabulated. We observed a slight increase of 2N2K cells from ~8% to 16% in KAT60a RNAi cells and from ~9% to ~21% in KAT80 RNAi cells and an emergence of multinucleated (>2N) cells to ~21% of KAT60a RNAi cells and ~30% of KAT80 RNAi cells (Fig. 5C), indicating that both proteins are required for cytokinesis. Notably, anucleate (zoïd) cells also emerged to ~15% of the total cell population (Fig. 5C), indicating that aberrant cytokinesis occurred in some cells. Further examination of the cytokinesis cleavage furrow in the 2N2K cells showed that the number of cells with a clearly visible cleavage furrow was significantly increased when KAT60a and KAT80 were knocked down (Fig. 5D). Scanning EM additionally detected many cells that possessed a visible cleavage furrow and had multiple (four or more) flagella (Fig. 5E). These results demonstrated that KAT60a and KAT80 are both required for the completion of cleavage furrow ingression in the procyclic form.

KAT60a and KAT80 are interdependent for maintaining their stability

We investigated the potential interplay between KAT60a and KAT80 by examining the effect of depletion of one subunit on the localization and protein stability of the other subunit. In
KAT60a RNAi cells induced for 72 h, the KAT80 fluorescence signal at the new FAZ tip was lost in 90% of the 2N2K cells examined, and the KAT80 signal at the posterior tip and the nascent posterior tip in some 2N2K cells was also lost (Fig. 6, A and B). The loss of KAT80 fluorescence signal in KAT60a RNAi cells was due to the decrease in KAT80 protein level to 25% of the control level after 72 h (Fig. 6C). Treatment of the KAT60a RNAi cells with MG-132 significantly increased the KAT80 protein level (Fig. 6C). These results suggest that KAT60a is required for maintaining KAT80 protein stability. Conversely, in KAT80 RNAi cells induced for 72 h, 96% of the 2N2K cells examined lost KAT60a fluorescence signal at the new FAZ tip as well as the KAT60a signal at the posterior tip (Fig. 6D and E). Western blotting showed that KAT60a protein was gradually decreased to 20% of the control level after 72 h upon KAT80 RNAi induction, but it was stabilized in the presence of MG-132 (Fig. 6F). These results indicate that KAT60a is required for maintaining KAT60a stability. Thus, KAT60a and KAT80 are interdependent for maintaining their stability.

**Identification of two cleavage furrow proteins, KLIF and FRW1, that depend on CIF1 for localization**

Our previous BioID experiment using CIF1-BirA* as the bait (13) and our current BioID experiment using BirA*-CIF1 as the bait both identified KLIF as a CIF1-associated protein (Table S3). We confirmed its co-localization and interaction with CIF1 in trypanosomes (Fig. 1, A and C). The subcellular localization of KLIF during the entire cell cycle was examined with endogenously triple HA-tagged KLIF in the procyclic form. From G1 to metaphase, KLIF was detected near the flagellar pocket region and occasionally at the posterior cell tip (Fig. S5). Starting from anaphase, however, KLIF was additionally detected at the distal tip of the new FAZ (Fig. 7A and Fig. S5). During early cytokinesis, KLIF was detected at the cleavage furrow (Fig. 7A).
CIF1 is a master regulator of cytokinesis

Figure 5. Depletion of KAT60a and KAT80 impaired cytokinesis completion. A, Western blotting to monitor the level of KAT60a-3HA in KAT60a RNAi cells and the level of KAT80-3HA in KAT80 RNAi cells. B, knockdown of KAT60a and KAT80-3HA were detected by anti-HA mAb, and TbPSA6 served as the loading control. B, effect of KAT60a and KAT80 RNAi on cell proliferation. C, depletion of KAT60a and KAT80 caused accumulation of binucleate cells (2N2K and 2N1K) and multinucleate cells (XN, X>2). A total of 300 cells for each time point were counted, and the results are presented as mean percentage ± S.D. (n = 3). N, nucleus; K, kinetoplast. D, quantification of cells without or with a visible cleavage furrow in the binucleate cells from control, KAT60a RNAi, and KAT80 RNAi. Total 2N2K cells counted for KAT60a RNAi cell line: 310 (control) and 312 (KAT60a RNAi). Total 2N2K cells counted for KAT80 RNAi cell line: 310 (control) and 408 (KAT80 RNAi). The results are presented as mean percentage ± S.D. (n = 3). **, p < 0.01. E, scanning electron microscopic images of control, KAT60a RNAi, and KAT80 RNAi cells undergoing cytokinesis. The red arrows indicate the two elongating new flagella in KAT60a RNAi and KAT80 RNAi cells that were undergoing cytokinesis. Scale bars, 5 µm.

Because KLIF is a CIF1-interacting protein and localizes to the new FAZ tip at the cell cycle stages later than CIF1, we asked whether KLIF localization to the new FAZ tip depends on CIF1. Immunofluorescence microscopy showed that the KLIF fluorescence signal at the new FAZ tip and the cleavage furrow was lost in ~90% of the CIF1-deficient 2N2K cells (Fig. 7, B and C). However, the KLIF fluorescence signal at the flagellar pocket region was not affected (Fig. 7, B and C). Western blotting showed that the level of KLIF protein was not affected by CIF1 RNAi, which reduced CIF1 protein to ~20% of the control level (Fig. 7D). These results suggest that CIF1 is required for targeting KLIF to the new FAZ tip and the cleavage furrow. Conversely, the effect of KLIF depletion on CIF1 localization was also investigated. Depletion of KLIF by RNAi did not affect CIF1 localization to the new FAZ tip and CIF1 protein stability (Fig. 7, E and F). These results suggest that CIF1 functions upstream of KLIF.

BioID using BirA*-CIF1 as the bait identified another cleavage furrow–localized protein, FRW1 (Tables S2 and S3). FRW1 was recently reported as a G2/M-enriched protein localizing to the new FAZ tip (20), but its subcellular localization throughout the cell cycle was not explored. We determined its subcellular localization during the cell cycle in procyclic trypanosomes. At G1 phase, FRW1 was detected near the flagellar pocket region and occasionally at the posterior cell tip (Fig. S6), but starting from S phase to anaphase, FRW1 was localized to the new FAZ tip in addition to the flagellar pocket region (Fig. 8A and Fig. S6). When cytokinesis started, the FRW1 fluorescence signal in the flagellar pocket region was somewhat diminished, but it was detected at the cleavage furrow (Fig. 8A and Fig. S6). At later stages of cytokinesis, FRW1 was detected at the leading edge of the ingressing cleavage furrow, and during final cell abscission, FRW1 appeared to be concentrated at the nascent posterior tip or the cytoplasmic bridge that connects the two daughter cells (Fig. 8A and Fig. S6).

As FRW1 interacts with CIF1 (Fig. 1C), we asked whether FRW1 localization to the new FAZ tip and the cleavage furrow depends on CIF1. Knockdown of CIF1 disrupted FRW1 localization to the new FAZ tip and the cleavage furrow in ~98% of the 2N2K cells examined, but the FRW1 fluorescence signal at the flagellar pocket region was not affected (Fig. 8, B and C). Western blotting showed that the level of FRW1 protein was not affected in CIF1 RNAi cells, in which the CIF1 protein level was gradually reduced to ~20% of the control level after 72 h (Fig. 8D). These results demonstrated that FRW1 localization to the new FAZ tip and the cleavage furrow depends on CIF1.
Conversely, the potential effect of FRW1 depletion on CIF1 localization and protein stability was investigated. In FRW1 RNAi cells, CIF1 localization to the new FAZ tip and CIF1 protein stability were not affected (Fig. 8, E and F). Collectively, these results suggest that FRW1 functions downstream of CIF1.

CIF1 is required for localizing FAZ18 and FAZ20 to the new FAZ tip

The dependence of CIF1 for the localization of three FAZ tip–localizing proteins (KPP1, KAT60a, and KAT80) and two FAZ tip– and cleavage furrow–localizing proteins (KLIF and FRW1) suggests that CIF1 may be required for targeting a cohort of CIF1-associated proteins to the new FAZ tip. To test this possibility, we examined the localization of the remaining FAZ tip–localizing proteins in CIF1 RNAi cells. These proteins included FAZ11, FAZ14, FAZ18, FAZ20, FAZ21, FAZ24, and FPRC, among which FAZ18 localized to the new FAZ tip only and the others localized to both the new and the old FAZ tips (Table S3). Knockdown of CIF1 disrupted the localization of FAZ18 and FAZ20, but not the other FAZ tip–localizing proteins, to the new FAZ tip (Fig. 9, A and B, and Fig. S7). Western blotting showed that the levels of FAZ18 and FAZ20 proteins were not affected by CIF1 depletion (Fig. 9C). These results
demonstrated that CIF1 is required for targeting FAZ18 and FAZ20 to the new FAZ tip.

Discussion

The identification of CIF1 as the protein factor that bridges TbPLK and TbAUK1 in the cytokinesis regulatory pathway suggests a pivotal role of CIF1 in promoting cytokinesis initiation in T. brucei. It is thus necessary to identify all CIF1-interacting proteins and characterize their roles in cytokinesis and their functional interplay with CIF1 for a comprehensive understanding of the CIF1-mediated cytokinesis pathway. BioID is a powerful tool for identifying associated partners of

Figure 7. CIF1 is required for localization of the cleavage furrow protein KLIF to the new FAZ tip and the cleavage furrow. A, subcellular localization of KLIF during anaphase and cytokinesis. KLIF-3HA was expressed from the endogenous locus and detected by FITC-conjugated anti-HA mAb. FAZ was labeled with anti-CC2D pAb. The arrowheads indicate the KLIF signal at the flagellar pocket region. Scale bar, 5 μm. DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast. B, depletion of CIF1 disrupted KLIF localization. KLIF-3HA was expressed in CIF1 RNAi cells. Immunofluorescence microscopy was performed as above. The arrowheads indicate the KLIF signal at the flagellar pocket region. Scale bar, 5 μm. C, quantification of 2N2K cells with different KLIF localization patterns in control and CIF1 RNAi cells. Total 2N2K cells counted: 331 (control) and 316 (CIF1 RNAi, 24 h). The results are presented as mean percentage ± S.D. (n = 3). *** p < 0.001. D, effect of CIF1 RNAi on KLIF protein level. CIF1 RNAi was induced for 72 h. KLIF-3HA was detected by anti-HA mAb, CIF1-PTP was detected by anti-protein A pAb, and TbPSA6 was detected by anti-TbPSA6 pAb as the loading control. E, CIF1 localization to the new FAZ tip did not require KLIF. Cells were immunostained with anti-CIF1 pAb and FITC-conjugated anti-HA mAb to detect CIF1 and KLIF-3HA, respectively. The arrowheads indicate the KLIF signal at the flagellar pocket region. Scale bar, 5 μm. F, KLIF depletion did not affect CIF1 protein stability. CIF1 was detected by anti-CIF1 pAb, KLIF-3HA by anti-HA mAb, and TbPSA6 by anti-TbPSA6 pAb, which served as the loading control.
any protein of interest, but it has an inherent caveat in that numerous nonassociated proteins are often co-purified. Therefore, further validation by co-localization and co-immunoprecipitation is necessary to confirm their identity as associated partners or interacting partners. Without further validation by co-immunoprecipitation, it is premature to claim any associated proteins as interacting partners (19). Although a total of 465 potential CIF1-associated proteins were identified by CIF1 BioID (Table S2 and Ref. 13), only 52 proteins (Table S3) can be regarded as CIF1-associated proteins because of their co-localization or partial co-localization with CIF1. Of the 12 CIF1-associated proteins examined by co-immunoprecipitation, six proteins were validated as CIF1-interacting proteins (Fig. 1C). Therefore, at least nine proteins, including TbAUK1 (12), CIF2 (13), and CIF3 (14), interact with CIF1 at the new FAZ tip and/or the cleavage furrow. These proteins possess diverse biochemical functions, indicating that they may play distinct roles in regulating cytokinesis in T. brucei.

We recently demonstrated that the putative protein phosphatase KPP1 is necessary for maintaining flagellum–cell body

Figure 8. CIF1 is required for localization of the cleavage furrow protein FRW1 to the new FAZ tip and the cleavage furrow. A, subcellular localization of FRW1 during anaphase and cytokinesis. FRW1–3HA expressed from the endogenous locus was detected by FITC-conjugated anti-HA mAb, and the FAZ was labeled with anti-CC2D pAb. Arrowheads indicate the FRW1 signal at the flagellar pocket region. Scale bar, 5 μm. DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast. B, depletion of CIF1 disrupted FRW1 localization. FRW1–3HA was expressed from the endogenous locus in CIF1 RNAi cells and detected by FITC-conjugated anti-HA mAb. FAZ was labeled with anti-CC2D pAb. The arrowheads indicate the FRW1 signal at the flagellar pocket region. Scale bar, 5 μm. C, quantification of the 2N2K cells with different FRW1 localization patterns in control and CIF1 RNAi cells. Total 2N2K cells counted: 343 (control) and 439 (CIF1 RNAi, 24 h). The results are presented as mean percentage ± S.D. (n = 3). FP, flagellar pocket. *** p < 0.001. D, effect of CIF1 RNAi on the FRW1 protein level. CIF1 RNAi was induced for 72 h. FRW1–3HA was detected by anti-HA mAb, CIF1-PTP was detected by anti-protein A pAb, and TbPSA6 was detected by anti-TbPSA6 pAb, which served as the loading control. E, FRW1 is not required for CIF1 localization to the new FAZ tip. Cells were immunostained with anti-CIF1 pAb and FITC-conjugated anti-HA mAb to detect CIF1 and FRW1–3HA, respectively. The arrowheads indicate the FRW1 signal at the flagellar pocket region. Scale bar, 5 μm. F, CIF1 protein stability was not affected by FRW1 depletion. CIF1 was detected by anti-CIF1 pAb, FRW1–3HA by anti-HA mAb, and TbPSA6 by anti-TbPSA6 pAb, which served as the loading control.
attachment and for promoting cell division (16), but its mechanistic role in cytokinesis was not explored previously. The biochemical and genetic data presented in this report (Figs. 1–3) demonstrated the physical and functional interaction between KPP1 and CIF1. Although the two proteins appeared to be interdependent (Fig. 3), they exerted distinct effects on each other. CIF1 depletion impaired KPP1 localization at as early as 24 h of RNAi induction, whereas KPP1 depletion destabilized CIF1 protein after 72 h of RNAi induction (Fig. 3). Such differential effects suggest that CIF1 may recruit KPP1 to the new FAZ tip first and that KPP1, in turn, maintains CIF1 stability. However, precisely how CIF1 stability is maintained by KPP1 remains unknown. Previous studies demonstrated that formation of the CIF1–CIF2 protein complex is necessary for maintaining CIF1 stability (13, 15). It is thus possible that KPP1 may play a role in promoting CIF1–CIF2 complex assembly. Nonetheless, the requirement of KPP1 for maintaining CIF1 stability suggests an essential role of KPP1 in regulating cytokinesis initiation.

Although the requirement of KAT60a and KAT80 for T. brucei cytokinesis has been demonstrated, their subcellular localization was not unambiguously determined previously (18, 28), making understanding their mechanistic roles in cytokinesis difficult. We showed that KAT60a and KAT80 form a complex and function downstream of CIF1 at the distal tip of the new FAZ (Fig. 4 and S4). The finding that KAT80, but not KAT60a, was pulled down by CIF1 in co-immunoprecipitation experiments (Fig. 1C) is surprising, as KAT60a forms a complex with KAT80 (Fig. S4) and, thus, is expected to be co-precipitated with KAT80. We speculate that this distinction may reflect the different function of the two subunits of katanin. The p80 subunit of katanin is known to play a regulatory role by targeting and activating the p60 subunit of katanin (27, 29). Therefore, in T. brucei CIF1 may bind directly to KAT80 and target it to the new FAZ tip, and subsequently KAT80 may target KAT60a to the new FAZ tip. In this regard, the observed effect of CIF1 depletion on KAT60a localization (Fig. 4) is attributed to the disruption of KAT80 localization by CIF1 depletion. Intriguingly, KAT60a and KAT80 are interdependent for maintaining their stability (Fig. 6). This unusual functional interplay between the regulatory subunit and the catalytic subunit of CIF1 is required for localization of FAZ18 and FAZ20 to the new FAZ tip. A, depletion of CIF1 disrupted the localization of FAZ18 and FAZ20 to the new FAZ tip. FAZ18–3HA and FAZ20–3HA were each expressed from their respective endogenous locus in CIF1 RNAi cells and were detected by FITC-conjugated anti-HA mAb. FAZ was labeled with anti-CC2D pAb. Scale bar, 5 μm. DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast. B, quantification of the 2N2K cells with different localization patterns of FAZ18 and FAZ20 in control and CIF1 RNAi cells. Total 2N2K cells counted for FAZ18 localization: 329 (control), 312 (CIF1 RNAi, 24 h), and 319 (CIF1 RNAi, 48 h). Total 2N2K cells counted for FAZ20 localization: 311 (control), 315 (CIF1 RNAi, 24 h), and 324 (CIF1 RNAi, 48 h). The results are presented as mean percentage ± S.D. (n = 3). **, p < 0.001. C, effect of CIF1 RNAi on the protein levels of FAZ18 and FAZ20. CIF1 RNAi was induced for 72 h. FAZ18–3HA and FAZ20–3HA were detected by anti-HA mAb, CIF1-PTP was detected by anti-protein A pAb, and TbPSA6 was detected by anti-TbPSA6 pAb, which served as the loading control. The asterisk in the FAZ20–3HA Western blot indicates a nonspecific band detected by anti-HA antibody.
katanin suggests that the regulation of the katanin complex in *T. brucei* is distinct from that in other organisms. Nonetheless, these results incorporated the microtubule-severing katanin protein into the CIF1-mediated cytokinesis pathway and demonstrated the essential role of the KAT60α–KAT80 complex in promoting the completion of cleavage furrow ingress in the procyclic form of *T. brucei*.

We identified two new FAZ tip– and cleavage furrow–localized proteins, KLIF and FRW1, that interact with CIF1 and depend on CIF1 for localization to the new FAZ tip and the cleavage furrow (Figs. 7 and 8), demonstrating that they are downstream of CIF1 in the cytokinesis pathway. KLIF has been recently demonstrated to be required for cytokinesis furrow ingress (19). Given that KLIF contains a kinesin motor domain, which is microtubule plus end–directed, and two tropomyosin-like domains, which may regulate myosin activities as the yeast tropomyosin homolog does (30), we propose a mode of action of KLIF in driving the unidirectional cleavage furrow ingress in *T. brucei*. Using its plus end–directed kinesin motor domain, KLIF may move along the cortical microtubules from the distal tip of the new FAZ toward the nascent posterior tip of the cell, and, in the meantime, KLIF may use its tropomyosin-like domains to regulate stalled unknown myosin to constrict the membrane at the cleavage furrow, leading to unidirectional ingress of the cleavage furrow, as observed by live-cell video microscopy (2).

The finding that CIF1 is required for the localization of five of the six CIF1-interacting proteins, KPP1, KAT80, KLIF, FRW1, and FAZ20 (Figs. 3, 4, and 7-9), and two of the six CIF1-associated proteins, KAT60α and FAZ18 (Figs. 4 and 9), but not vice versa, suggests that CIF1 functions as a central player in orchestrating its interacting partners and associated proteins at the new FAZ tip and/or the cleavage furrow. Although the five CIF1-interacting proteins may be recruited to the new FAZ tip via interaction with CIF1, the two CIF1-associated proteins, KAT60α and FAZ18, may be recruited through an indirect means, such as interaction with KAT80 (Fig. S4C) or interaction with any of the known or unknown CIF1-interacting proteins, respectively. It is noteworthy that two previously unidentified CIF1-interacting proteins, TbAUK1 and CIF2, are also recruited by CIF1 to the new FAZ tip at late anaphase and S phase, respectively (12, 15). However, localization of the CIF1-interacting protein CIF3 to the new FAZ tip is independent of CIF1, but the stability of CIF3 depends on the formation of the CIF1–CIF3 complex (14). The lack of effect of CIF1 depletion on the localization of the CIF1-interacting protein FPRC (Fig. S7) indicates that FPRC localization is independent of CIF1 and that the two proteins may interact after they are both loaded onto the new FAZ tip. Therefore, except FPRC and CIF3, localization of all other seven CIF1-interacting proteins to the new FAZ tip depends on CIF1. Moreover, six of these seven CIF1-interacting proteins, except FAZ20, which has not been functionally characterized, play essential roles in cytokinesis. Thus, CIF1 appears to act as a master regulator of cytokinesis by recruiting a cohort of cytokinesis regulators to the new FAZ tip and maintaining the stability of certain cytokinesis regulator(s), such as CIF3, at the new FAZ tip.

### Experimental procedures

**Purification of recombinant CIF1 protein and antibody production**

A 1161-bp fragment corresponding to the CIF1 C-terminal coding region (aa 418–804) containing the two zinc-finger motifs was PCR amplified from the genomic DNA and cloned into the pET26 vector for expressing a His<sub>j</sub>-fused CIF1 truncation protein in *Escherichia coli*. The construct was transformed into *E. coli* BL21 cells, and recombinant His-tagged CIF1 truncation protein was induced with isopropyl β-D-thiogalactopyranoside, purified through a nickel column, and used for immunizing rabbits to produce anti-CIF1 antibody at Cocalico Biologicals, Inc. (Reamstown, PA).

**Trypanosome cell culture**

*T. brucei* strain 427 was grown in SDM-79 medium containing 10% heat-inactivated fetal bovine serum at 27 °C. *T. brucei* strain 29–13 (31) was cultured in SDM-79 medium supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Inc.), 15 μg/ml G418, and 50 μg/ml hygromycin at 27 °C.

**Identification of CIF1-associated proteins by BioID and LC-MS/MS**

To overexpress Myc-BirA*-CIF1 for identification of CIF1-associated proteins, the full-length coding sequence of CIF1 was cloned into the pLew100-Myc-BirA* vector (32), and the resulting plasmid was transfected into the 29–13 strain. Transfectants were selected under 2.5 μg/ml phleomycin and cloned by limiting dilution in a 96-well plate. Expression of Myc-BirA*-CIF1 was induced with 0.1 μg/ml tetracycline and confirmed by Western blotting and immunofluorescence microscopy with anti-Myc mAb and anti-CIF1 polyclonal antibody.

Affinity purification of biotinylated proteins was performed as described previously (33). Myc-BirA*-CIF1 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<sup>6</sup>) were washed three times with PBS and treated with PHEME buffer (100 mM PIPES (pH 6.9), 2 mM EGTA, 0.1 mM EDTA, and 1 mM MgSO<sub>4</sub>) 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 500 μl of prewashed streptavidin-coated Dynabeads (Invitrogen) at 4 °C for 4 h. The Dynabeads were washed five times with 50 mM ammonium bicarbonate and resuspended in 100 mM ammonium bicarbonate. 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 trypptic digest (in 2% acetonitrile/0.1% formic acid in water) was ana-
CIF1 is a master regulator of cytokinesis

lyzed by LC-MS/MS on an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific) interfaced with a Dionex UltiMate 3000 Binary RSLCnano system. Peptides were separated onto an Acclaim™ PepMap™ C_18 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, and 90% solvent B for 10 min (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile). The peptides were analyzed using the data-dependent acquisition method. Orbitrap Fusion was operated with measurements of FTMS1 at a resolution of 120,000, a scan range of 350–1500 m/z, automatic gain control (AGC) target 2E5, and a maximum injection time of 50 ms. During a maximum 3-s cycle time, the ITMS2 spectra were collected in rapid scan rate mode, with collision-induced dissociation (CID) normalized collision energy (NCE) 35, 1.6 m/z isolation window, AGC target 1E4, and a maximum injection time of 35 ms, and dynamic exclusion was employed for 30 s.

Raw data files were processed and searched using Thermo Proteome Discoverer software or the Mascot search engine. The protein search was against the T. brucei database. The search conditions used a peptide tolerance of 10 parts/million and an MS/MS tolerance of 0.8 Da with the enzyme trypsin and two missed cleavages.

RNAi

To generate RNAi cell lines, a 554-bp DNA fragment (nucleotides 17–570) from the coding region of KAT60a, a 558-bp DNA fragment (nucleotides 12–569) from the coding region of KAT80, a 548-bp DNA fragment (nucleotides 1143–1690) from the coding region of KLIF, and a 503-bp DNA fragment (nucleotides 117–569) from the coding region of KAT80, cells co-expressing 3HA-tagged KAT60a and PTP1. Transfectants were selected with 1 g/ml tetracycline. Cells were counted with a hemocytometer.

In situ epitope tagging of proteins

For epitope tagging of CIF1-associated proteins in the CIF1 RNAi cell lines, the PCR-based epitope tagging method was carried out. For epitope tagging of CIF1 in the RNAi cell lines targeting the genes encoding CIF1-interacting proteins, the plasmid pC-CIF1-PTP-PAC was used to tag CIF1 with a C-terminal PTP epitope from one of its endogenous loci. Transfectants were selected with 1 μg/ml puromycin and cloned by limiting dilution as described above.

In vitro GST pulldown

A 480-bp DNA sequence encoding the N-terminal domain (aa 1–160) containing the Plus3 domain of KPP1 gene and a 411-bp DNA sequence corresponding to the C-terminal zinc-finger motifs of CIF1 (aa 668–804) were each cloned into the pGEX-4T-3 vector (Clontech). Point mutations of the four zinc-coordinating residues in zinc-finger motifs 1 and 2 (CIF1-ZnF1 and CIF1-ZnF2) were generated by site-directed mutagenesis using the pGEX-CIF1-ZnF plasmid. The resulting plasmids were transformed into the E. coli BL21 strain. Expression of the GST-fused Plus3 domain of KPP1 and zinc-finger domain of CIF1 was induced with 0.1 mM isopropyl β-d-thiogalactopyranoside for 16 h at room temperature and purified through binding to GSH-Sepharose beads. Purified GST fusion proteins bound to the beads were incubated at 4 °C for 1 h with T. brucei lysate from 5 × 10^7 cells expressing either 3HA-tagged WT and mutant CIF1 proteins (for GST-Plus3 pulldown) or 3HA-tagged CIF1-interacting proteins (for GST-CIF1-ZnF, GST-CIF1-ZnF1mut, and GST-CIF1-ZnF2mut pulldown) in immunoprecipitation buffer (25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM DTT, 1% NP-40, and protease inhibitor mixture). The GSH-Sepharose beads were washed six times with the immunoprecipitation buffer, and bound proteins were eluted by boiling the beads in 1× SDS sampling buffer. Eluted proteins were separated on SDS-PAGE, transferred onto a polyvinylidene difluoride membrane and immunoblotted with anti-HA antibody to detect 3HA-tagged WT and mutant CIF1 proteins. GST alone was used as the negative control. GST and GST fusion proteins used for pulldown were stained with Coomassie Brilliant Blue dye.

Co-immunoprecipitation, Western blotting, and band intensity quantitation

For co-immunoprecipitation of CIF1 and its associated proteins, 5 × 10^7 cells expressing 3HA-tagged CIF1-associated proteins were lysed by sonication (1 s on and 5 s off for a total of 30 s) in 1 ml of immunoprecipitation buffer (25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM DTT, 1% NP-40, and protease inhibitor mixture). Lysed cells were centrifuged at 14,000 rpm (18,407 × g) for 5 min in a microcentrifuge. Lysed cell lysate was incubated with 1.0 μl of anti-CIF1 antibody for 1 h at 4 °C and then with 40 μl of protein A-Sepharose beads. For co-immunoprecipitation of KAT60a and KAT80, cells co-expressing 3HA-tagged KAT60a and PTP1-tagged KAT80 were lysed, and cleared cell lysate was prepared essentially as described above. Cell lysate was incubated with 20 μl of IgG-Sepharose 6 Fast Flow (Invitrogen) for 1 h at 4 °C. Beads were washed five times with the immunoprecipitation buffer. Immunoprecipitated proteins were then eluted by incubating the beads with 10% SDS at room temperature for 5 min. Eluted proteins were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and immunoblotted with anti-HA mAb to detect 3HA-tagged CIF1-associated proteins and with anti-CIF1 pAb to detect CIF1 or with anti-HA mAb to detect KAT60a-3HA and with anti-protein A to detect KAT80-PTP.

For Western blotting of the input proteins in co-immunoprecipitation experiments, a total of 1.0 μg of total protein was loaded onto each lane. For other Western blotting experiments, a total of 1 × 10^6 cell equivalents of lysate was loaded onto each lane. Quantitation of protein band intensity was performed with ImageJ software, and the relative protein levels after RNAi induction were normalized with the loading controls.
**Immunofluorescence microscopy**

Cells were washed once with PBS for 5 min and adhered to the glass coverslips at room temperature for 30 min. Cells on the glass coverslips were fixed with cold methanol (−20°C) for 30 min and then rehydrated with PBS for 10 min. Fixed cells were blocked with 3% BSA in PBS for 1 h and incubated with the primary antibody for 1 h at room temperature. The following primary antibodies were used: FITC-conjugated anti-HA mAb (1:400 dilution, Sigma-Aldrich, clone HA-7), anti-protein A (ProA) pAb (1:400 dilution, Sigma-Aldrich), anti-CC2D polyclonal antibody for the FAZ (1:1000 dilution) (36), and anti-CIF1 pAb (1:2000 dilution). Cells were washed three times with PBS for 5 min each and then incubated with FITC-conjugated anti-mouse IgG (1:400 dilution, Sigma-Aldrich) or Cy3-conjugated anti-rabbit IgG (1:400 dilution, Sigma-Aldrich) at room temperature for 1 h. Cells on the coverslips were washed three times with PBS for 5 min each and air dried at room temperature. Cells were then mounted with 4’,6-diamidino-2-phenylindole–containing VectaShield mounting medium (Vector Laboratories) and imaged using an inverted fluorescence microscope (Olympus IX71) equipped with a cooled charge-coupled device camera (model Orca-ER, Hamamatsu) and a PlanApo N ×60 1.42 numerical aperture lens. Images were acquired using the automatic exposure setting with Slidebook 5 software.

**Statistical analysis**

Statistical analysis was performed using Student’s t test and one-way analysis of variance in Microsoft Excel. The numbers of cells were counted, and the n values for each panel in the figures are stated in the corresponding legends. For immunofluorescence microscopy, images were taken randomly, and all cells in each image were counted.

**Author contributions**—Q. Z., T. A., and Z. L. formal analysis; Q. Z., T. A., K. T. M. P., and H. H. validation; Q. Z., T. A., K. T. M. P., and H. H. visualization; Q. Z., T. A., K. T. M. P., and H. H. methodology; Q. Z., T. A., K. T. M. P., H. H., and Z. L. writing-review and editing: Z. L. conceptualization; Z. L. supervision; Z. L. funding acquisition; Z. L. writing-original draft; Z. L. project administration.

**Acknowledgments**—We thank Dr. Cynthia Y. He (National University of Singapore) for providing the anti-CC2D antibody, Dr. Arthur Günzl (University of Connecticut Health Center) for providing the pC-PTP-PAC plasmid, and Dr. Christopher de Graffenried (Brown University) for providing the pLew100-Myc-BirA* vector. We also thank Li Li for MS at the Proteomics Core Facility of the University of Texas Health Science Center at Houston.

**References**

1. Sunter, J. D., and Gull, K. (2016) The flagellum attachment zone: “the cellular ruler” of trypanosome morphology. *Trends Parasitol.* 32, 309–324 CrossRef Medline
2. 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
3. Pollard, T. D., and Wu, J. Q. (2010) Understanding cytokinesis: lessons from fission yeast. *Nat. Rev. Mol. Cell Biol.* 11, 149–155 CrossRef Medline
4. Odronitz, F., and Kollmar, M. (2007) Drawing the tree of eukaryotic life based on the analysis of 2,269 manually annotated myosins from 328 species. *Genome Biol.* 8, R196 CrossRef Medline
5. García-Salcedo, J. A., Pérez-Morga, D., Gijón, P., Dilbeck, V., Pays, E., and Nolan, D. P. (2004) A differential role for actin during the life cycle of *Trypanosoma brucei*. *EMBO J.* 23, 780–789 CrossRef Medline
6. Kumar, P., and Wang, C. C. (2006) Dissociation of cytokinesis initiation from mitotic control in a eukaryote. *Eukaryot. Cell* 5, 92–102 CrossRef Medline
7. Hammarton, T. C., Kramer, S., Tetley, L., Boshart, M., and Mottram, J. C. (2007) *Trypanosoma brucei* Polo-like kinase is essential for basal body duplication, kDNA segregation and cytokinesis. *Mol. Microbiol.* 65, 1229–1248 CrossRef Medline
8. Li, Z., 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
9. 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 Medline
10. 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
11. Li, Z., Lee, J. H., Chu, F., Burlinge, 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
12. 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
13. 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
14. 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
15. 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
16. Zhou, Q., Dong, G., and Li, Z. (2018) Flagellum inheritance in *Trypanosoma brucei* requires a kinetoplastid-specific protein phosphatase. *J. Biol. Chem.* 293, 8508–8520 CrossRef Medline
17. Dean, S., Sunter, J. D., and Wheeler, R. J. (2017) *TrypTag* org: a trypanosome genome-wide protein localisation resource. *Trends Parasitol.* 33, 80–82 CrossRef Medline
18. Casanova, M., Crobu, L., Blaineau, C., Bourgeois, N., Bastien, P., and Pagès, M. (2009) Microtubule-severing proteins are involved in flagellar length control and mitosis in trypanosomatids. *Mol. Microbiol.* 71, 1353–1370 CrossRef Medline
19. Hilton, N. A., Sladewski, T. E., Perry, J. A., Pataki, 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
20. Crozier, T. W. M., Tinti, M., Wheeler, R. J., Ly, T., Ferguson, M. A. J., and Lamond, A. I. (2018) Proteomic analysis of the cell cycle of procyclic form *Trypanosoma brucei*. *Mol. Cell. Proteomics* 17, 1184–1195 CrossRef Medline
21. Finn, R. D., Clements, J., Arndt, W., Miller, B. L., Wheeler, T. J., Schreiber, F., Bateman, A., and Eddy, S. R. (2015) HMMER web server: 2015 update. *Nucleic Acids Res.* 43, W30–W38 CrossRef Medline
22. Wier, A. D., Mayekar, M. K., Héroux, A., Arndt, K. M., and VanDemark, A. P. (2013) Structural basis for Spf1-mediated recruitment of the Paf1 complex to chromatin. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17290–17295 CrossRef Medline
CIF1 is a master regulator of cytokinesis

23. de Jong, R. N., Truffault, V., Diercks, T., Ah, E., Daniels, M. A., Kaptein, R., and Folkers, G. E. (2008) Structure and DNA binding of the human Rtf1 Plus3 domain. *Structure* **16**, 149–159 CrossRef Medline

24. Whitby, F. G., and Phillips, G. N., Jr. (2000) Crystal structure of tropomyosin at 7 angstroms resolution. *Proteins* **38**, 49–59 CrossRef Medline

25. Gunning, P. W., Schevzov, G., Kee, A. J., and Hardeman, E. C. (2005) Tropomyosin isoforms: divining rods for actin cytoskeleton function. *Trends Cell Biol.* **15**, 333–341 CrossRef Medline

26. Balasubramanian, M. K., Helfman, D. M., and Hemmingsen, S. M. (1992) A new tropomyosin essential for cytokinesis in the fission yeast *S. pombe*. *Nature* **360**, 84–87 CrossRef Medline

27. Hartman, J. J., Mahr, J., McNally, K., Okawa, K., Iwamatsu, A., Thomas, S., Cheesman, S., Heuser, J., Vale, R. D., and McNally, F. J. (1998) Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. *Cell* **93**, 277–287 CrossRef Medline

28. Benz, C., Clucas, C., Mottram, J. C., and Hammarton, T. C. (2012) Cytokinesis in bloodstream stage *Trypanosoma brucei* requires a family of katanins and spastin. *PLoS ONE* **7**, e30367 CrossRef Medline

29. McNally, K. P., Bazirgan, O. A., and McNally, F. J. (2000) Two domains of p80 katanin regulate microtubule severing and spindle pole targeting by p60 katanin. *J. Cell Sci.* **113**, 1623–1633 Medline

30. Cranz-Mileva, S., MacTaggart, B., Russell, J., and Hitchcock-DeGregori, S. E. (2015) Evolutionarily conserved sites in yeast tropomyosin function in cell polarity, transport and contractile ring formation. *Biol. Open* **4**, 1040–1051 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. Morriswood, B., Havlicek, K., Demmel, L., Yavuz, S., Sealey-Cardona, M., Vidilaseris, K., Anrather, D., Kostan, J., Djinovic-Carugo, K., Roux, K. J., and Warren, G. (2013) Novel bilobe components in *Trypanosoma brucei* identified using proximity-dependent biotinylation. *Eukaryot. Cell* **12**, 356–367 CrossRef Medline

33. 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

34. 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

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

36. 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

37. Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195–201 CrossRef Medline

38. Lupas, A., Van Dyke, M., and Stock, J. (1991) Predicting coiled coils from protein sequences. *Science* **252**, 1162–1164 CrossRef Medline