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Two distinct cytokinesis pathways drive trypanosome cell division initiation from opposite cell ends

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Cytokinesis in *Trypanosoma brucei*, an early branching protozoan, occurs along its longitudinal axis uni-directionally from the anterior tip of the new flagellum attachment zone filament toward the cell's posterior end. However, the underlying mechanisms remain elusive. Here we report that cytokinesis in *T. brucei* is regulated by a concerted action of Polo-like kinase, Aurora B kinase, and a trypanosome-specific protein CIF1. Phosphorylation of CIF1 by Polo-like kinase targets it to the anterior tip of the new flagellum attachment zone filament, where it subsequently recruits Aurora B kinase to initiate cytokinesis. Consistent with its role, CIF1 depletion inhibits cytokinesis initiation from the anterior end of the cell, but, surprisingly, triggers cytokinesis initiation from the posterior end of the cell, suggesting the activation of an alternative cytokinesis from the opposite cell end. Our results reveal the mechanistic roles of CIF1 and Polo-like kinase in cytokinesis initiation and elucidate the mechanism underlying the recruitment of Aurora B kinase to the cytokinesis initiation site at late anaphase. These findings also delineate a signaling cascade controlling cytokinesis initiation from the anterior end of the cell and uncover a backup cytokinesis that is initiated from the posterior end of the cell when the typical anterior-to-posterior cytokinesis is compromised.

cytokinesis | Polo-like kinase | Aurora B kinase | backup cytokinesis | *Trypanosoma brucei*

In eukaryotes, regulation of cytokinesis, the final step of cell division, involves a complex interplay of numerous proteins at the cytokinesis initiation site and the cleavage furrow. The mechanisms underlying cytokinesis in fungi and metazoa have been well understood, and the core regulatory pathways appear to be evolutionarily conserved (1). Along the cell division plane, which is defined by the position of the central spindle or the nucleus, animals and fungi assemble an actomyosin contractile ring, the cytokinesis apparatus that appeared about 1 billion years ago in the common ancestor of fungi, amoebas, and animals (2). In metazoa, the signaling pathway driving the transition from mitosis to cytokinesis involves two evolutionarily conserved protein kinases, the Polo-like kinase and the Aurora B kinase. Both kinases are concentrated on the central spindle and the midbody during late cell cycle stages and cooperate to recruit the centralspindlin complex to the central spindle and the midbody (3). Subsequently, the centralspindlin complex recruits Ect2, a guanine nucleotide exchange factor, to the midbody, which then recruits and activates the small GTPase RhoA at the midbody. Activation of RhoA further promotes the formation of the actomyosin contractile ring to drive cytokinesis (3).

Unlike many eukaryotic organisms that divide along the cell's short axis, the early branching protozoan *Trypanosoma brucei* undergoes cytokinesis along its longitudinal axis (4). The cell division plane in *T. brucei* is positioned by the newly assembled flagellum and its associated cytoskeletal structure termed the flagellum attachment zone (FAZ) filament (5, 6). Thus, cytokinesis is initiated from the anterior tip of the new FAZ filament, and cleavage furrow ingression occurs uni-directionally along the longitudinal axis toward the posterior end of the cell (4, 7) without forming an actomyosin contractile ring at the cleavage furrow (8).

As in fungi and metazoa, the Polo-like kinase (TbPLK in *T. brucei*) and the Aurora B kinase (TbAUK1 in *T. brucei*) are also required for cytokinesis in *T. brucei* (9, 10). TbPLK is concentrated in the flagellar basal body and the bilobe at late G1 phase, but from early S phase it is concentrated at the new FAZ tip and remains there until early anaphase (11). At the new FAZ tip, TbPLK is believed to promote cytokinesis initiation, but the underlying mechanism is unclear. TbAUK1 forms an unusual chromosomal passenger complex (CPC) with TbCPC1 and TbCPC2, and the complex displays a dynamic localization during the cell cycle. The complex is located in kinetochores from S phase to metaphase and on the central spindle during anaphase, but finally is degraded at the central spindle after late anaphase. However, starting from late anaphase, newly synthesized CPC proteins emerge at the new FAZ tip and then transfer to the cleavage furrow during cytokinesis (12). Localization of TbAUK1 to the new FAZ tip at late anaphase is crucial for cytokinesis initiation (13), but how it is recruited remains mysterious. The sequential recruitment of TbPLK and TbAUK1 to the new FAZ tip led us to hypothesize that an unknown factor is targeted by TbPLK to the new FAZ tip, which subsequently recruits TbAUK1 for cytokinesis initiation.

Here we report the identification of this factor, named CIF1, that links the TbPLK- and TbAUK1-signaling pathways. We also report the delineation of a cytokinesis regulatory pathway in

Significance

Cytokinesis occurs along a cell's short axis in many organisms, including bacteria, archaea, and eukaryotes. In many protozoa it occurs along the cell's longitudinal axis. The mechanism underlying this mode of cytokinesis is unknown. We delineate the signaling cascade that regulates cytokinesis along the longitudinal axis of *Trypanosoma brucei*, which is totally different from that in its human host. Additionally, we discover an alternative cytokinesis pathway that drives trypanosome cell division along the same division plane as the typical cytokinesis, but in an opposite direction. This alternative cytokinesis is activated only when the typical cytokinesis pathway is defective, suggesting that trypanosomes have evolved a backup cytokinesis mechanism to prevent the failure of cell division, thereby ensuring survival of this organism.

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T. brucei, which involves a concerted action of TbPLK, CIF1, and TbAUK1 at the anterior tip of the new FAZ filament. CIF1 is targeted, by TbPLK-mediated phosphorylation, to the anterior tip of the new FAZ filament, where it subsequently recruits TbAUK1 to drive cytokinesis initiation. We also discover a backup cytokinesis pathway that promotes cell division initiation from the posterior end of the cell. *T. brucei* thus has evolved two distinct cytokinesis pathways that drive cell division along the same division plane but in opposite directions.

Results

CIF1 Colocalizes with TbPLK at Early Cell Cycle Stages and with TbAUK1 at Late Cell Cycle Stages. To identify the factor(s) that interacts with both TbPLK and TbAUK1, we carried out yeast two-hybrid library screening using the kinase-dead TbPLK (TbPLK-K70R) and TbAUK1 as baits. A hypothetical protein (Tb927.11.15800), which was previously identified as a partner of TbPLK-K70R by us (14), was also identified as a partner of TbAUK1 (Fig. S1 *A* and *B*). We named it CIF1 for Cytokinesis Initiation Factor 1 due to its essential role in cytokinesis initiation (see below). CIF1 contains two coiled-coil motifs between residues 125–205 and 218–245 and two putative zinc-finger motifs between residues 680–709 and 758–779 and is conserved in kinetoplastid parasites.

We investigated the spatiotemporal distribution of CIF1 and the colocalization of CIF1 with TbPLK and TbAUK1 during the cell cycle. During G1 phase, CIF1 was not detectable, but at early S phase, a small amount of CIF1 was detected at the newly assembled FAZ filament, and its level appeared to increase significantly at the tip of the elongating new FAZ subsequently (Fig. S1*C*). CIF1 remained at the new FAZ tip until telophase and finally was concentrated at the cleavage furrow during cytokinesis (Fig. S1*C*). At G1 phase, TbPLK was concentrated at the basal body and the bilobe structure (Fig. 1*A*), but at early S phase, TbPLK was detected at the newly assembled FAZ filament, where it colocalized with CIF1 (Fig. 1*A*). TbPLK disappeared from the new FAZ tip at late anaphase, whereas CIF1 remained (Fig. 1*A*). Intriguingly, when TbPLK disappeared at late anaphase, a small amount of TbAUK1 was detected at the new FAZ tip, where it colocalized with CIF1 (Fig. 1*B*). Both TbAUK1 and CIF1 finally localized to the cleavage furrow during cytokinesis (Fig. 1*B*). This temporal relationship of localization patterns suggests that TbPLK may target CIF1 to the new FAZ tip, where CIF1 subsequently recruits TbAUK1 to the new FAZ tip.

TbPLK Phosphorylation of CIF1 Is Required for Localizing CIF1 to the New FAZ Tip. CIF1 is a highly phosphorylated protein, containing 29 in vivo phospho-serine and phospho-threonine sites (15). To test whether CIF1 is an in vivo substrate of TbPLK, we treated the cells with GW843682X, a small-molecule Polo-like kinase inhibitor (16, 17). CIF1 was detected as a doublet on the Western blot, and treatment with GW843682X reduced the level of the upper band of CIF1, which was confirmed to be the phosphorylated form of CIF1, suggesting de-phosphorylation of CIF1 by TbPLK inhibition (Fig. 2*A*). Similarly, depletion of TbPLK by RNAi (Fig. S2 *A*–*C*) also caused CIF1 de-phosphorylation (Fig. 2*B*). This inhibition of TbPLK activity and depletion of TbPLK protein both disrupted CIF1 localization to the new FAZ tip (Fig. 2 *C*–*F*), suggesting that phosphorylation of CIF1 by TbPLK is required to target CIF1 to the new FAZ tip.

CIF1 Maintains TbPLK at the New FAZ Tip. We next investigated the effect of CIF1 depletion on TbPLK localization to the new FAZ tip. Depletion of CIF1 by RNAi (Fig. S3) impaired the localization of TbPLK to the new FAZ tip, leading to the accumulation of TbPLK in the basal body and the bilobe structure in ~40% of the binucleate cells (Fig. S4 *A* and *B*). At the bilobe, TbPLK phosphorylates TbCentrin2 at Ser-54 during late G1 and early S phase, but after TbPLK localizes to the new FAZ tip,

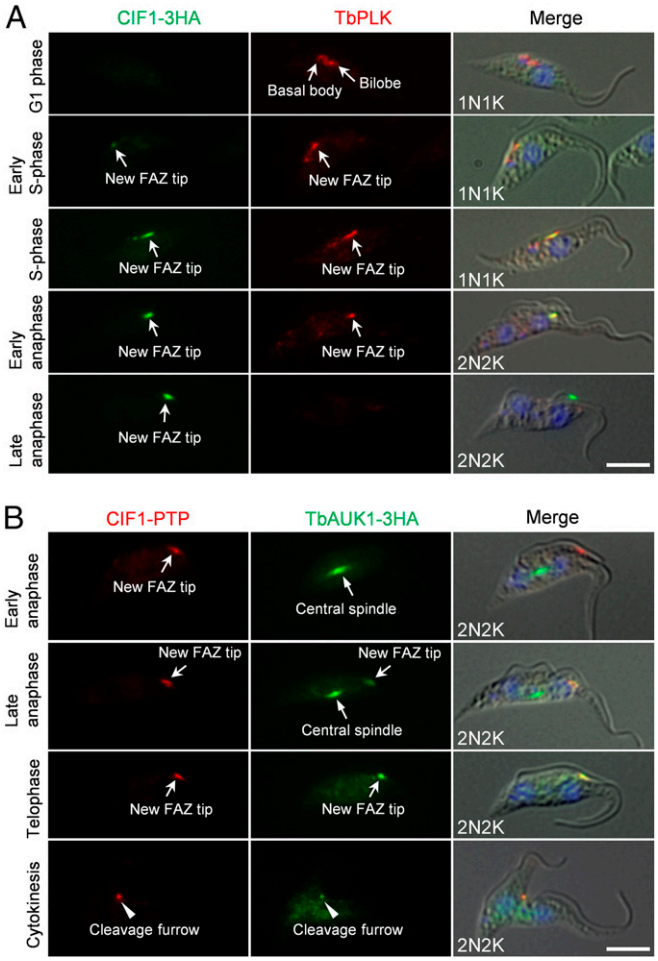

Fig. 1. CIF1 colocalizes with TbPLK during early cell cycle stages and with TbAUK1 during late cell cycle stages. (*A*) CIF1 colocalizes with TbPLK at the new FAZ tip from early S phase to early anaphase. CIF1-3HA was detected with FITC-conjugated anti-HA mAb and TbPLK was detected with anti-TbPLK pAb. N, nucleus; K, kinetoplast DNA. (*B*) CIF1 colocalizes with TbAUK1 at the new FAZ tip from late anaphase to telophase and at the cleavage furrow during cytokinesis. CIF1-PTP was labeled with anti-Protein A pAb, and TbAUK1-3HA was stained with FITC-conjugated anti-HA mAb. (Scale bars: 5 μm.)

TbCentrin2 is de-phosphorylated (18). To test whether TbCentrin2 was phosphorylated in CIF1-deficient binucleate cells, cells were immunostained with the PS54 antibody, which detects the phosphorylated Ser-54 in TbCentrin2 (18), and the results showed that TbCentrin2 was indeed phosphorylated (Fig. S4 *C* and *D*). This result provided another line of evidence to confirm that TbPLK was accumulated in the bilobe in a certain population of binucleate cells upon CIF1 depletion. Taken together, these results suggest that CIF1, once targeted to the new FAZ tip by TbPLK phosphorylation, in turn maintains TbPLK at the new FAZ tip. The two proteins thus are interdependent for their localization to the new FAZ tip.

CIF1 Is Required for Localizing TbAUK1 to the New FAZ Tip. Given that CIF1 and TbAUK1 colocalize at the new FAZ tip at late anaphase, we investigated whether CIF1 is required for localization of TbAUK1 to the new FAZ tip. In CIF1 RNAi cells, TbAUK1 localization to the new FAZ tip was disrupted (Fig. 3 *A* and *B*), despite the fact that the TbAUK1 protein level was not changed (Fig. 3*C*), indicating that TbAUK1 was dispersed in the cytosol. These results suggest that CIF1 recruits TbAUK1 to the

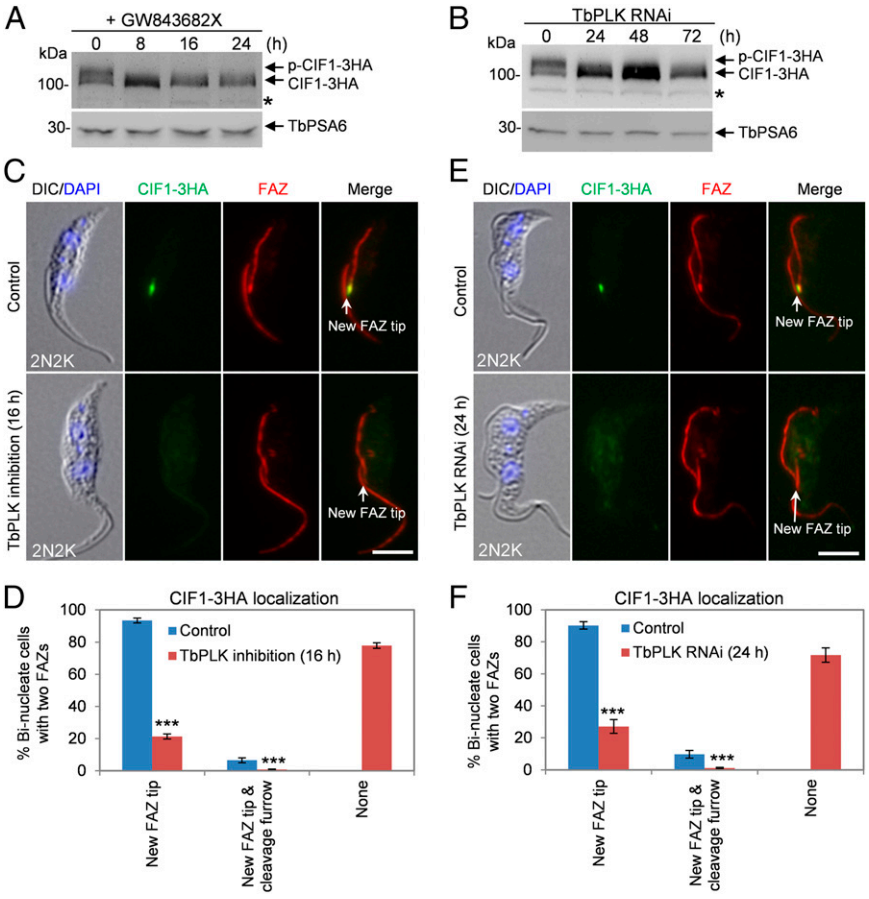

Fig. 2. Phosphorylation of CIF1 by TbPLK targets CIF1 to the new FAZ tip. (*A* and *B*). Inhibition of TbPLK activity by GW843682X (*A*) and depletion of TbPLK (*B*) caused de-phosphorylation of CIF1. CIF1-3HA was detected with anti-HA antibody. TbPSA6, a subunit of the 26S proteasome, served as the loading control. p-CIF1-3HA, phosphorylated CIF1-3HA. The asterisk indicates a nonspecific band. (*C*) Inhibition of TbPLK activity with GW843682X abolished CIF1 localization to the new FAZ tip. (*D*). Quantification of CIF1 localization in control and TbPLK-inhibited binucleate cells. (*E*) TbPLK depletion disrupted CIF1 localization. (*F*) Quantification of CIF1 localization in control and TbPLK-depleted binucleate cells. In *C* and *E*, CIF1-3HA was detected with anti-HA, and the FAZ filament was labeled with anti-CC2D. N, nucleus; K, kinetoplast DNA. (Scale bars: 5 μm.) In *D* and *F*, 300 binucleate cells were counted for each cell line, and results are presented as mean percentage ± SD (n = 3). ***$P < 0.001$.

new FAZ tip at late anaphase. It should be noted that the lack of TbAUK1 in the central spindle in CIF1-deficient binucleate cells was not a direct effect of CIF1 depletion. The fact that CIF1 RNAi arrested cells before cytokinesis initiation (see below) suggests that the CIF1-deficient binucleate cells have already exited mitosis and hence have disassembled the mitotic spindle. In terms of the cell cycle stage, these binucleate cells were equivalent to the telophase cells in wild-type cells, in which TbAUK1 is not detected in the central spindle (Fig. 1*B*).

We next investigated whether CIF1 is a substrate of TbAUK1 Both inhibition of TbAUK1 activity with Hesperadin, a small-molecule Aurora B kinase inhibitor (19, 20), and depletion of TbAUK1 by RNAi (Fig. S2 *D–F*) caused CIF1 de-phosphorylation (Fig. 3*D*), indicating that CIF1 is an in vivo substrate of TbAUK1. However, inhibition of TbAUK1 activity with Hesperadin or depletion of TbAUK1 by RNAi did not affect CIF1 localization to the new FAZ tip (Fig. 3*E*), suggesting that TbAUK1-mediated phosphorylation of CIF1 is not required to maintain CIF1 at the new FAZ tip from late anaphase to telophase.

TbPLK Is Required for Targeting TbAUK1 to the New FAZ Tip. If a signaling cascade from TbPLK through CIF1 to TbAUK1 does exist, then inhibition of TbPLK activity or depletion of TbPLK should also impair TbAUK1 localization to the new FAZ tip. To test this possibility, cells were treated with GW843682X, and the localization of TbAUK1 in TbPLK-inhibited cells was examined. This inhibition of TbPLK activity disrupted TbAUK1 localization to the new FAZ tip (Fig. S5 *A* and *B*), but did not affect the TbAUK1 protein level (Fig. S5*C*), suggesting that TbAUK1 was dispersed in the cytosol.

To further confirm the requirement of TbPLK for TbAUK1 localization to the new FAZ tip, we examined the localization of TbAUK1 in TbPLK RNAi cells, and the results showed that depletion of TbPLK by RNAi also impaired TbAUK1 localization to the new FAZ tip (Fig. S5 *D* and *E*). Similar to TbPLK inhibition (Fig. S5*C*), depletion of TbPLK did not alter the TbAUK1 protein level (Fig. S5*F*). These results indicate that TbPLK is required for localizing TbAUK1 to the new FAZ tip and also suggest that cytokinesis initiation in *T. brucei* requires a concerted action of TbPLK, CIF1, and TbAUK1 at the new FAZ tip.

CIF1 Depletion Inhibits Cytokinesis Initiation from the Cell Anterior, but Triggers Cytokinesis Initiation from the Cell Posterior. CIF1 depletion caused an accumulation of binucleate cells initially and multinucleate (four or more nuclei) cells subsequently (Fig. S3*C*), suggesting a cytokinesis defect. We therefore investigated the effect of CIF1 depletion on cleavage furrow ingression. In wild-type cells, a division fold is formed along the longitudinal axis of the cell between the two flagella/FAZs before cytokinesis initiation (Fig. 4*A*). Subsequently, cleavage furrow ingression occurs from the anterior tip of the new FAZ filament and proceeds along the division fold toward the posterior end of the cell (Fig. 4 *B–E*, Fig. S6*A*, and Movie S1). At late cytokinesis stage, the two daughter cells are connected at the posterior region by a cytoplasmic bridge (Fig. 4*E*), which is finally cleaved.

In CIF1 RNAi cells, a similar division fold was also formed along the longitudinal axis of the cell between the two flagella/FAZs (Fig. 4*F*), but furrow ingression from the anterior tip of the new FAZ filament was inhibited (Fig. 4 *G–I* and Fig. S7), suggesting that CIF1 is required for cytokinesis initiation from the anterior tip of the new FAZ filament. Strikingly, CIF1-deficient cells started to initiate cytokinesis from the posterior end of the cell toward the anterior end of the cell, albeit at a rate slower than the typical anterior-to-posterior cytokinesis (Fig. 4 *G* and *H*,

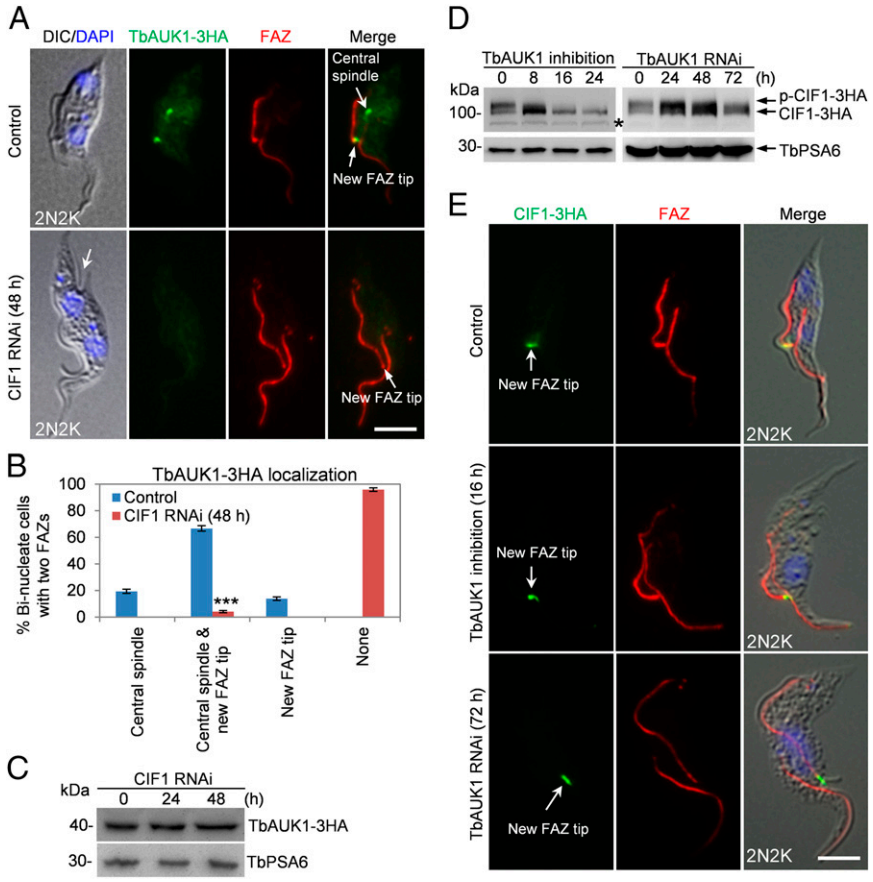

Fig. 3. CIF1 recruits TbAUK1 to the new FAZ tip. (*A*) CIF1 depletion abolished TbAUK1 localization to the new FAZ tip. TbAUK1-3HA was labeled with FITC-conjugated anti-HA mAb, and the FAZ filament was detected by anti-CC2D pAb. The white arrow in the DIC/DAPI panel indicates the cleavage furrow at the posterior end of the CIF1 RNAi cell. N, nucleus; K, kinetoplast DNA. (Scale bar: 5 μm.) (*B*) Quantification of TbAUK1 localization in control and CIF1-depleted cells. A total of 300 binucleate cells were counted for each cell line, and results were presented as mean percentage ±SD ($n = 3$). ***$P < 0.001$. (*C*) TbAUK1 protein level was not changed in CIF1 RNAi cells. TbAUK1-3HA was detected with anti-HA antibody. TbPSA6 served as the loading control. (*D*) Both inhibition of TbAUK1 activity with Hesperadin and depletion of TbAUK1 by RNAi caused de-phosphorylation of CIF1. p-CIF1-3HA, phosphorylated CIF1-3HA. The asterisk indicates a nonspecific band. (*E*) Inhibition of TbAUK1 activity or depletion of TbAUK1 did not abolish CIF1 localization to the new FAZ tip. CIF1-3HA was detected by anti-HA, and the FAZ filament was labeled by anti-CC2D. (Scale bar: 5 μm.)

Figs. S6*B* and S7, and Movie S2). A posterior cleavage furrow was found in ∼15 and ∼37% of the binucleate cells after CIF1 RNAi for 24 and 48 h, respectively, and in ∼61% of the multinucleate cells after CIF1 RNAi for 48 h (Fig. 4*I*). At late cytokinesis stage, the two daughter cells were also connected by a cytoplasmic bridge (Fig. 4*H* and Fig. S7 *J* and *K*), similar to that in the wild-type cell (Fig. 4*E*), but at the anterior end. These results suggest that inhibition of the typical anterior-to-posterior cytokinesis activated an alternative, backup cytokinesis from the opposite cell end. This backup cytokinesis appeared to use the same division plane as the typical cytokinesis (Fig. 4 *A* and *F*) and was initiated in binucleate cells, but due to its slower rate, these cells became multinucleated (four or more nuclei) before cell division was completed (Fig. 4*H* and Figs. S6*B* and S7). Consequently, this alternative cytokinesis appeared to generate two binucleate daughter cells with two flagella (Fig. 4*H* and Fig. S6*B*), instead of two uni-nucleate daughter cells as in the wild-type cells (Fig. 4*E* and Fig. S6*A*). Moreover, many of the multinucleate cells started to assemble additional cleavage furrows (Figs. S3*D* and S7 *K* and *L*) at the posterior end of the cell and, consequently, were likely to generate multiple (more than two) daughter cells (Figs. S3*D* and S7*K*). In some of the multinucleate cells, mitosis and the alternative cytokinesis appeared to be poorly coordinated, leading to the generation of anucleate daughter cells that contained only the kinetoplast DNA, the cell's unique mitochondrial DNA complex (Figs. S3*F* and S6*B*).

Discussion

Trypanosomes initiate cytokinesis from the anterior tip of the new FAZ filament, and it is believed that the regulators playing direct roles in cytokinesis initiation should localize to the new FAZ tip before cytokinesis initiation (21). CIF1 is the fifth protein, after TbAUK1 (12), TbCPC1 (12), TbCPC2 (12), and TbPLK (22), that concentrates at the new FAZ tip, and our RNAi studies demonstrated that CIF1 is essential for cytokinesis initiation from the anterior tip of the new FAZ filament (Fig. 4 and Fig. S3). CIF1 was also identified as a substrate of TbPLK in a recent study and was named TOEFAZ1 for Tip Of Extending FAZ 1 (23). However, given its additional localization to the cleavage furrow during cytokinesis (Fig. 1*B* and Fig. S1*C*), this nomenclature does not sufficiently reflect its localization and function. Although McAllaster et al. also showed that RNAi of this protein caused a cytokinesis defect by microscopic inspection, the underlying mechanism was not investigated (23). Our work, however, elucidated the mechanistic role of CIF1 in cytokinesis initiation. It recruits TbAUK1, a crucial regulator of cytokinesis initiation (13), to the new FAZ tip at late anaphase (Fig. 3 *A* and *B*) for the latter to initiate cytokinesis from the anterior tip of the new FAZ filament. Moreover, our results also uncovered the mechanism underlying the recruitment of TbAUK1 to the new FAZ tip at late anaphase, which has been a mystery since we first discovered that TbAUK1 emerges at the new FAZ tip at late anaphase (12). It should be noted that CIF1 also localizes to the cleavage furrow during cytokinesis (Fig. 1*B* and Fig. S1*C*). Therefore, CIF1 may play additional roles in furrow ingression and cell abscission, but these potential functions of CIF1 cannot be revealed in the RNAi studies because depletion of CIF1 arrested cells before cytokinesis initiation.

It was previously postulated that TbPLK regulates cytokinesis initiation based on the inhibited growth of the RNAi cells (9, 24), but the underlying mechanism has never been addressed. Given that TbPLK disappears from the new FAZ tip before cytokinesis initiation, this raises the question of whether TbPLK plays a direct role in cytokinesis initiation. Here we demonstrated that

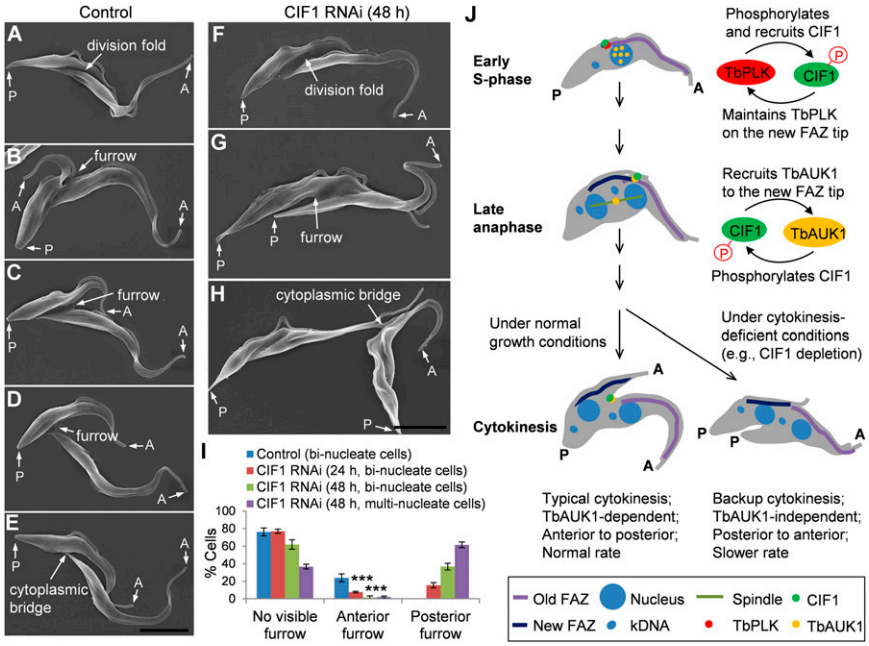

Fig. 4. CIF1 depletion inhibits cytokinesis initiation from the cell anterior, but leads to cytokinesis from the cell posterior. (*A*) Formation of a division fold along the longitudinal axis in a control cell. (*B–E*). Cleavage furrow ingression occurs from cell anterior to cell posterior in control cells. (*E*) Two daughter cells connected by a cytoplasmic bridge at the posterior region of the cells. (*F*) Formation of a division fold along the longitudinal axis in a CIF1 RNAi cell. (*G* and *H*) Cleavage furrow ingression occurs from cell posterior to cell anterior in CIF1 RNAi cells. (*H*) Two daughter cells connected by a cytoplasmic bridge at the anterior region of the cells. (Scale bars: 5 μm.) (*I*) Quantification of cells without and with visible cleavage furrow(s) in control and CIF1 RNAi cells. Binucleate and multinucleate cells (200 cells for each cell type and each time point) were counted. Data were presented as mean percentage ±SD ($n = 3$). ***$P < 0.001$. (*J*) Model for cytokinesis initiation regulated by TbPLK, CIF1, and TbAUK1 and the existence of a backup cytokinesis in *T. brucei*. A, anterior; P, posterior.

TbPLK-mediated phosphorylation of CIF1 is required for localizing CIF1 to the new FAZ tip (Fig. 2), which uncovered, for the first time, the mechanistic role of TbPLK in cytokinesis initiation. TbPLK targets CIF1 to the new FAZ tip at early cell cycle stages for the latter to recruit TbAUK1 at later cell cycle stages to initiate cytokinesis from the anterior tip of the new FAZ filament.

We have delineated a signaling cascade that regulates cytokinesis along the longitudinal axis of a trypanosome cell. During early S phase, TbPLK phosphorylates CIF1, thereby targeting CIF1 to the new FAZ tip. In turn, CIF1 maintains TbPLK at the new FAZ tip before it recruits TbAUK1 to the new FAZ tip, where TbAUK1, likely together with CIF1, drives cytokinesis from the anterior tip of the new FAZ filament toward the posterior end of the cell (Fig. 4*J*). This signaling pathway is totally different from that in metazoa, in which Polo-like kinase and Aurora B kinase cooperate to recruit the centralspindlin complex to the central spindle and the midbody for assembling the actomyosin contractile ring (3) at the cell division plane that is defined by the position of the spindle (25). This mechanism of cytokinesis through the actomyosin contractile ring appeared after *T. brucei* diverged from the last common eukaryotic ancestor (26). Therefore, *T. brucei* may have adopted the ancestral cytokinesis pathway or have evolved a trypanosome-specific cytokinesis pathway by adopting some crucial regulators, such as Polo-like kinase and Aurora B kinase, from the ancestral cytokinesis pathway and by incorporating trypanosome-specific factor(s), such as CIF1, in accordance with its unusual cell division plane that is defined by the flagellum and the FAZ filament.

Our findings indicate the existence of two distinct cytokinesis pathways that drive trypanosome cell division along the same division plane but in opposite directions, which is unprecedented. The typical, Polo-like kinase- and Aurora B kinase-dependent cytokinesis occurs from the anterior end of the cell toward the posterior end of the cell under normal growth conditions. The alternative cytokinesis, which is Polo-like kinase- and Aurora B kinase-independent, occurs from the posterior end of the cell toward the anterior end of the cell and is activated only when the typical anterior-to-posterior cytokinesis is inhibited (Fig. 4*J*). Initiation of cytokinesis from the posterior end of the cell in CIF1 RNAi cells is unlikely to be attributed to the misdirection of the typical cytokinesis machinery, as TbAUK1, the downstream factor of CIF1 and the key regulator of the typical anterior-to-posterior cytokinesis, is not targeted to the posterior end of the cell but is dispersed in the cytosol (Fig. 3 *A–C*). This alternative cytokinesis appears to be less efficient and slower than the typical cytokinesis (compare Movies S1 and S2) and sometimes generates anucleate daughter cells (Figs. S3*C* and S6*B*), but it also generates normal daughter cells (Figs. S6*B* and S7) and enables trypanosomes to proliferate, albeit at a rate slower than that of the wild-type cells (Fig. S3*B*). Cytokinesis is one of the most critical steps in the trypanosome cell cycle, and any defect in cytokinesis can lead to cell death. Trypanosomes undergo a complex life cycle by alternating between the insect vector and the mammalian host and must combat with diverse environmental challenges for survival. It is highly likely that under certain adverse conditions in certain life cycle stages, the typical anterior-to-posterior cytokinesis could be defective, and thus a backup plan would be necessary to maintain a critical cell population. Our findings suggest that trypanosomes have evolved a backup cytokinesis mechanism, which is invoked to ensure survival of this organism when the typical cytokinesis is defective.

The existence of a typical cytokinesis and a backup cytokinesis in *T. brucei* is analogous to the two pathways regulating nonhomologous end joining (NHEJ), an important enzymatic process for repairing DNA double-strand breaks (DSBs) in eukaryotes. In the absence of the canonical NHEJ (c-NHEJ), cells can repair a large portion of DSBs using an alternative pathway that employs a distinct set of factors (27). This alternative NHEJ, termed alt-NHEJ or b-NHEJ (for backup NHEJ), operates with an order of magnitude slower kinetics, frequently joins incorrect ends, and is suppressed by the c-NHEJ (28, 29). Additional examples of the existence of a backup pathway were also found in other cellular processes, such as cell death (30), endoplasmic reticulum-associated degradation (31), and mRNA decay (32). It is believed that evolution ensures the existence of a backup mechanism to prevent the failure of cellular processes that are critically important to life (33). The alternative pathway is cryptic under normal growth conditions, but will be activated once the canonical pathway fails to operate.

Materials and Methods

Trypanosome Cell Lines and RNAi. The procyclic 427 cell line and 29–13 cell line (34) were cultured according to our published procedures (14). To knock down CIF1, a fragment of *CIF1* gene was cloned into the pZJM vector (35) and transfected into the 29–13 cell line. Selection of transfectants, cloning,

and RNAi induction were carried out as described previously (14). TbPLK RNAi cell line and TbAUK1 RNAi cell line have been reported previously (12, 17).

Inhibition of TbPLK and TbAUK1 Kinase Activity by Small-Molecule Inhibitors. The small-molecule PLK inhibitor GW843682X (16) was previously demonstrated to inhibit TbPLK activity in vitro, with an in vitro IC_{50} of ~1.3 μM, and in vivo, inhibiting the insect-form trypanosomes with an IC_{50} of ~2 μM (17). The small-molecule Aurora B kinase inhibitor Hesperadin (20) was previously demonstrated to inhibit TbAUK1 activity in vitro, with an in vitro IC_{50} of 40 nM, and in vivo, inhibiting the insect-form trypanosomes with an IC_{50} of 0.55 μM (19).

Trypanosome cells harboring the pC-CIF1-3HA-PAC construct were treated with 1 μM Hesperadin for 24 h, and cells were collected for Western blotting with anti-HA antibody to monitor the de-phosphorylation of 3HA-tagged CIF1. Cells harboring the pC-CIF1-3HA-PAC construct or both the pC-CIF1-PTP-PAC and the pC-TbAUK1-3HA-NEO constructs were treated with 5 μM GW843682X for 16 h and were then collected for immunostaining with FITC-conjugated anti-HA antibody and anti-Protein A antibody. Cells harboring the pC-CIF1-3HA-PAC construct were treated with 1 μm Hesperadin for 16 h and were then collected for immunofluorescence microscopy with FITC-conjugated anti-HA antibody.

Immunofluorescence Microscopy. Cells were fixed with cold methanol and incubated with the primary antibody for 1 h at room temperature. The following primary antibodies were used: FITC-conjugated anti-HA antibody, anti-Protein A antibody, anti-CC2D antibody (6), 20H5 (36), and anti-TbPLK antibody (14). Cells were then incubated with FITC-conjugated anti-mouse IgG or Cy3-conjugated anti-rabbit IgG for 1 h at room temperature, mounted with DAPI-containing VectaShield mounting medium (Vector Labs), and imaged using an inverted fluorescence microscope (Olympus IX71) equipped with a cooled CCD camera (model Orca-ER, Hamamatsu) and a PlanApo N 60 × 1.42-NA lens. Images were acquired using Slidebook 5 software.

Scanning Electron Microscopy. Cells were settled onto coverslips and fixed with 2.5% (vol/vol) glutaraldehyde in PBS for 30 min at room temperature. After washing the cells on the coverslips three times with PBS, cells were dehydrated in a graded series of alcohol [30, 50, 70, 90, and 100% (vol/vol)] for 10 min each. After critical point drying, samples were 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.) and imaged using Nova NanoSEM 230 (FEI). The scanning work distance was at 5 mm, and the accelerating high voltage was at 8 kV.

Time-Lapse Video Microscopy. Time-lapse video microscopy was carried out as described previously (13). Cells were plated on a 0.6% agarose gel in SDM-79 medium and visualized under an inverted automatic microscope (Olympus IX81) equipped with a cooled CCD camera. Images were taken every 2–3 min, and time-lapse movies were generated using ImageJ.

Statistical Analysis. Statistical analysis was performed using the *t* test provided in the Microsoft Excel software. For immunofluorescence microscopy experiments, images were randomly taken and all of the cells in each image were counted.

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