Maintenance of hook complex integrity and centrin arm assembly facilitates flagellum inheritance in *Trypanosoma brucei*

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Inheritance of the newly assembled flagellum in the human parasite *Trypanosoma brucei* depends on the faithful duplication and segregation of multiple flagellum-associated cytoskeletal structures, including the hook complex and its associated centrin arm. The biological functions of this unique hook complex–centrin arm assembly remain poorly understood. Here, we report a hook complex–associated protein named BOH2 that plays an essential role in promoting flagellum inheritance. BOH2 localizes to the hooked part of the hook complex by bridging the hook complex, the centrin arm, and the flagellum attachment zone filament. Depletion of BOH2 caused the loss of the shank part of the hook complex and its associated protein TbSmee1, disrupted the assembly of the centrin arm and the recruitment of centrin arm–associated protein CAAP1, inhibited the assembly of the flagellum attachment zone, and caused flagellum mispositioning and detachment. These results demonstrate crucial roles of BOH2 in maintaining hook complex integrity and promoting centrin arm formation and suggest that proper assembly of the hook complex–centrin arm structure facilitates flagellum inheritance.

*Trypanosoma brucei* is a flagellated microbial eukaryote and the causative agent of sleeping sickness in humans and nagana in cattle in sub-Saharan Africa. This early divergent parasite alternates between the insect vector tsetse fly and mammals and proliferates through binary fission inside the insect and mammalian hosts. During its cell division cycle, *T. brucei* duplicates and segregates its flagellum and multiple flagellum–associated cytoskeletal structures and, upon cell division, produces two identical daughter cells, each of which inherits a single copy of flagellum and flagellum–associated cytoskeletal structures. The flagellum is nucleated from the basal body, a centriole-like microtubule-organizing center located at the posterior portion of the cell (1, 2), exits the cell through the flagellar pocket, and extends toward the cell anterior. The extracellular portion of the flagellum attaches, along the majority of its length, to the cell body via a specialized structure termed the flagellum attachment zone (FAZ) consisting of multiple subdomains located at the junction between the flagellum and the cell body (3). The flagellum is required for cell motility, cell morphogenesis, cell division, and cell-cell communication (4–6); hence, faithful inheritance of the flagellum during the cell cycle is essential for trypanosome viability.

Proper positioning and attachment of the newly assembled flagellum in *T. brucei* require the faithful duplication and segregation of various flagellum–associated cytoskeletal structures, such as the basal body, the FAZ, the flagellar pocket collar, and the bilobe structure (7–14) (Fig. S1A). The bilobe structure was first reported as a centrin protein–marked bilobed structure connecting the old and the new Golgi apparatus and mediating Golgi duplication (15). Earlier work identified a set of proteins that localize to the bilobe, including two centrin proteins, TbCentrin2 and TbCentrin4 (15, 16), the Polo-like kinase homolog TbPLK (17), the MORN (membrane occupation and recognition nexus)-containing protein TbMORN1 (18), and the leucine-rich repeats–containing protein TbLRRP1 (8). Subsequent work using EM showed that TbMORN1 and TbCentrin4 define a hairpin-like structure consisting of a TbMORN1–marked fishhook-like structure termed the hook complex and a TbCentrin4–marked bar-shaped structure termed the centrin arm (19, 20). The hooked part of the hook complex sits atop the TbdilBO1-labeled flagellar pocket collar and runs alongside the specialized quartet microtubules, and the shank part of the hook complex and the centrin arm flank the FAZ filament (19). Although the precise functions of the hook complex and the centrin arm remain largely unknown, functional characterizations of some hook complex–localizing proteins indicated that proteins localized to different subdomains of the hook complex might play distinct functions (8, 21, 22). Knockdown of TbdilRP1 suggests that the hook complex is involved in FAZ assembly and flagellum attachment (8). Knockdown of TbSmee1, which localizes to the shank part of the hook complex and the new FAZ tip, disrupts hook complex morphology (21). Knockdown of BOH1, which localizes to the fishhook part of the hook complex resembling a fishing bait hung on a fishhook, impairs flagellum inheritance and cytokinesis initiation (22). Through proximity-dependent biotinylation (BioID) using TbMORN1 as bait (23) and proteome-wide protein localization (24), more than 50 proteins have been localized to the hook complex. It is therefore of paramount interest to localize these proteins to specific subdomains of the hook complex and determine their functions for a better understanding of the function of this unusual flagellum–associated cytoskeletal structure.
In this paper, we report a kinetoplastid-specific protein named BOH2, which partly co-localizes with BOH1 at the hooked part of the hook complex, and characterize its function by RNAi-mediated gene ablation in the procyclic form of *T. brucei*. Our results suggest that BOH2 maintains the integrity of the hook complex and promotes the biogenesis of the centrin arm, thereby facilitating flagellum inheritance. These findings highlight an unusual role of BOH2 in controlling the assembly of the flagellum-associated hook complex–centrin arm structure and demonstrate the essential function of the hook complex in FAZ nucleation and elongation and flagellum inheritance.

**Results**

**BOH2 partly co-localizes with BOH1 to the hooked part of the hook complex**

To identify the binding partners and near neighbors of BOH1, we carried out BioID using BOH1 as bait. BOH1 fused with a C-terminal BirA*-HA was expressed in the procyclic form of *T. brucei* (Fig. S1B) and was localized to the hook complex (Fig. S1C). Biotinylated proteins from both the non-induced control and tetracycline-induced cells were purified (Fig. S1D) and analyzed by MS (Tables S1 and S2). Among the highly enriched (Mascot score > 100) proteins identified by BOH1 BioID, eight proteins localize to the vicinity of BOH1 (Fig. S1E and Table S2), of which two proteins (Tb927.5.570 and Tb927.10.3010) localize to the hook complex. Tb927.10.3010 localizes to the entire hook complex, displaying a fishhook-like morphology similar to TbMORN1 and TbLRRP1 (23), whereas Tb927.5.570 displays a bar-shaped structure similar to BOH1 (www.tryptag.org).

Immunofluorescence microscopy confirmed that Tb927.5.570 partly co-localized with BOH1 (Fig. 1A); therefore, we named it BOH2 for bait on hook 2. BOH2 is a large protein (2127 aa) containing four coiled-coil motifs (aa 358–385, 602–622, 853–873, and 1483–1503), and its homologs are only found in kinetoplastid parasites, including *Trypanosoma cruzi* (TcCLB.507867) and *Leishmania major* (LmjF.05.1190), suggesting that BOH2 is a kinetoplastid-specific protein. Using three-dimensional structured illumination microscopy (3D-SIM) superresolution microscopy, BOH2 was localized to the posterior portion of BOH1 with a slight shift toward the ventral side of BOH1 (Fig. 1B and Movie S1). Both BOH2 and BOH1 proteins remained to associate with the cytoskeleton when cells were treated with detergent (Fig. 1C).

To test whether BOH2 interacts with BOH1 in *vivo*, we carried out co-immunoprecipitation using cells co-expressing PTP-BOH1 and BOH2-3HA. We lysed the cells by sonication, which partially solubilized both proteins (Fig. 1C). Immunoprecipitation of PTP-BOH1 was not able to pull down BOH2-3HA (Fig. 1D). These results suggest that BOH2 and BOH1 are two neighboring proteins in the hook complex without forming a complex or interacting with each other.

We further investigated the localization of BOH2 relative to those flagellum-associated cytoskeletal structures, such as the hook complex, the centrin arm, and the FAZ filament, by immunofluorescence microscopy and 3D-SIM superresolution microscopy. Figure 1B shows the localization of BOH2 and BOH1 detected by immunofluorescence microscopy. BOH2 and BOH1 were endogenously tagged with a triple HA tag and a PTP tag, respectively. Scale bar, 5 μm. B, 3D-SIM to examine the co-localization of BOH2 and BOH1. Scale bar, 1 μm. C, BOH2 and BOH1 proteins associate with the cytoskeleton. Shown are Western blotting of cells lysed by detergent treatment (PEME containing 1% Nonidet P-40) and by sonication. S, soluble fraction; P, pellet (cytoskeletal) fraction. D, co-immunoprecipitation to test the interaction between BOH2 and BOH1. PTP-BOH1 was pulled down with IgG beads, and immunoprecipitated (IP) proteins were immunoblotted (IB) with anti-HA antibody and anti-ProA antibody.

In this paper, we report a kinetoplastid-specific protein named BOH2, which partly co-localizes with BOH1 at the hooked part of the hook complex, and characterize its function by RNAi-mediated gene ablation in the procyclic form of *T. brucei*. Our results suggest that BOH2 maintains the integrity of the hook complex and promotes the biogenesis of the centrin arm, thereby facilitating flagellum inheritance. These findings highlight an unusual role of BOH2 in controlling the assembly of the flagellum-associated hook complex–centrin arm structure and demonstrate the essential function of the hook complex in FAZ nucleation and elongation and flagellum inheritance.
microscopy. Co-immunofluorescence microscopic analysis of BOH2 and TbMORN1 showed that BOH2 localized to the hook part of the hook complex, resembling a fishing bait hung on a fishhook, similar to the localization of BOH1 (Fig. 2 (A and B) and Movie S2). The anterior portion of BOH2 overlapped with the centrin arm marked by TbCentrin4 (Fig. 2 (A and C) and Movie S3) and with the proximal ends of both the FAZ filament marked by CC2D (Fig. 2 (A and D) and Movie S4) and the shank part of the hook complex marked by TbSmee1 (Fig. 2 (A and E) and Movie S5). The localization of BOH2 to the hook part of the hook complex and its partial overlapping with other hook complex–associated structures suggest that BOH2 might play a role in maintaining hook complex morphology or promoting hook complex assembly.

**BOH2 is required for FAZ assembly and flagellum attachment**

To understand the biological function of BOH2, we carried out a tetracycline-inducible RNAi in the procyclic (insect) form of *T. brucei*. Induction of BOH2 RNAi by tetracycline caused a gradual depletion of BOH2 protein, which was endogenously tagged with a PTP epitope and detected by anti-ProtA antibody (Fig. 3A). Depletion of BOH2 caused severe growth defects after only 1 day of RNAi induction (Fig. 3B), indicating that BOH1 is essential for cell proliferation of the procyclic form. We next examined whether BOH2 RNAi affected cell-cycle progression by counting the cells with different numbers of kinetoplasts (K) and nuclei (N) before and after BOH2 RNAi induction. Trypanosomes at different cell-cycle stages contain different numbers of kinetoplast and nucleus. Cells in G1 to S phase contain one nucleus and one kinetoplast (1N1K), cells in G2 to metaphase contain one nucleus and two kinetoplasts (1N2K), and cells in subsequent cell-cycle stages contain two nuclei and two kinetoplasts (2N2K). Induction of BOH2 RNAi for 24 h caused a decrease of 1N1K and 1N2K cells and an increase of 2N2K cells (Fig. 3C). Some abnormal cell types, such as the cells containing only the kinetoplast but no nucleus (0N1K), cells containing two nuclei and one kinetoplast (2N1K), and cells containing multiple (>2) nuclei and various numbers (≥1) of the kinetoplast (xNyK, where x > 2 and y ≥ 1), also increased (Fig. 3C). After RNAi induction for 48 h, the 2N2K cells and the abnormal 0N1K and 2N1K cells all decreased, whereas the abnormal xNyK cells increased to ~80% of the total cell population (Fig. 3C). These results suggest that knockdown of BOH2 inhibited cell division.

Knockdown of BOH2 caused the detachment of the new flagellum, which occurred from as early as 16 h of RNAi and was detected in ~77% of the total cell population after 48 h (Fig. 3D and E). Moreover, the detached new flagellum also appeared to be not well-separated from the old flagellum (Fig. 3E). These results suggest that knockdown of BOH2 impaired the attachment and positioning of the newly formed flagellum. We examined the effect of BOH2 on FAZ assembly by immunofluorescence microscopy using anti-CC2D antibody to label Figure 2. BOH2 localizes to the hooked part of the hook complex. A, BOH2 localization relative to the hook complex labeled by TbMORN1 (anti-TbMORN1 antibody), the centrin arm labeled by TbCentrin4 (anti-LdCen1/TbCentrin4 antibody), the FAZ filament labeled by CC2D (anti-CC2D antibody), and the shank part of the hook complex labeled by TbSmee1, which was endogenously tagged with a PTP epitope and detected by anti-ProtA antibody. Scale bar, 5 μm. B–E, 3D-SIM superresolution microscopic analysis of BOH2 localization relative to the hook complex (B), the centrin arm (C), the FAZ filament (D), and the shank part of the hook complex (E). Cells were co-immunostained with anti-HA antibody to detect BOH2-3HA and anti-TbMORN1, anti-LdCen1/TbCentrin4, anti-CC2D, or anti-ProtA to label PTP-TbSmee1. The lowercase letters indicate the angle of viewing of the fluorescence-labeled structures. Scale bar, 1 μm.
the intracellular FAZ filament (9). Trypanosome cells start to assemble a new FAZ in the S-phase of the cell cycle, and the newly formed FAZ gradually elongates from a short, new FAZ (snFAZ) or a FAZ root to a full-length, new FAZ during cell-cycle progression from S-phase until the completion of mitosis. Therefore, among the noninduced control cells, the 1N1K cells (G1 and S phases) contain either one FAZ, one FAZ and one snFAZ, or two FAZs, whereas the 1N2K cells (G2 to metaphase) and the 2N2K cells (anaphase to telophase) all contain two FAZs (Fig. 3, E and F). Among the cells depleted of BOH2, the 1N1K cells with one FAZ increased by \( \frac{13}{210} \)%, whereas the 1N2K and 2N2K cells with two FAZs decreased by \( \frac{77}{263} \) and \( \frac{80}{47} \)% respectively (Fig. 3 F). Consequently, the 1N1K cells with two FAZs decreased by \( \frac{8}{174} \)% whereas the 1N2K and 2N2K cells with one FAZ increased to \( \frac{34}{203} \) and \( \frac{80}{217} \)% respectively (Fig. 3 F). Strikingly, among the abnormal 2N1K cells, \( \frac{74}{256} \)% of them contained one FAZ, and the rest contained one FAZ and one snFAZ (Fig. 3 F). The generation of cells without a new FAZ suggests that FAZ nucleation was inhibited, and the generation of cells with a snFAZ (or a FAZ root) suggests defective FAZ elongation. Thus, BOH2 is required for both the nucleation and the elongation steps of the FAZ assembly process.

**BOH2 maintains the shank part of the hook complex and its associated protein TbSmee1**

The localization of BOH2 to the hook complex (Figs. 1 and 2) implies that BOH2 might play roles in promoting hook complex formation or maintaining hook complex morphology. We investigated these possibilities by immunofluorescence microscopy using TbMORN1 and TbLRRP1-3HA to label the hook complex and by 3D-SIM superresolution microscopy using TbMORN1 to label the hook complex. In the noninduced control cells, both the new and the old hook complexes displayed a typical fishhook-like morphology (Fig. 4 (A and B) and Movie S6). In BOH2 RNAi cells, the old hook complex retained the fishhook-like morphology, but the new hook complex appeared to lose the shank part, and the hooked part assumed a bar-shaped morphology instead of a hook-shaped morphology.
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observed in the control cells (Fig. 4, A and B) and Movie S7).

To quantitatively analyze the defect of BOH2 knockdown on the hook complex, we measured the length of the hook complex, which was defined as the distance from the end of the hooked part of the hook complex to the distal tip of the shank part of the hook complex. We found that the old hook complex was not affected by BOH2 RNAi, but the new hook complex in the BOH2 RNAi cells was significantly shorter than that in the control cells (Fig. 4C).

Because BOH2 knockdown caused the loss of the shank part of the new hook complex, we examined whether TbSmee1, which localizes to the shank part of the hook complex (21), was affected. Immunofluorescence microscopy showed that knockdown of BOH2 inhibited the localization of TbSmee1 at the new FAZ tip (Fig. 4D). Quantitative analysis showed that cells with strong TbSmee1 signal in the new hook complex were decreased by ~84 and

Figure 4. Knockdown of BOH2 disrupts the assembly of the hook complex. A, immunofluorescence microscopic analysis of the hook complex in control and BOH2 RNAi cells. TbMORN1 was detected by anti-TbMORN1 antibody, and endogenously 3HA-tagged TbLRRP1 was detected by anti-HA antibody. Scale bar, 5 μm. B, 3D-SIM superresolution microscopic analysis of the hook complex in control and BOH2 RNAi cells. Cells were co-immunostained with anti-TbMORN1 antibody and 20H5 antibody. BB, basal body; CA, centrin arm; HC, hook complex. Scale bar, 1 μm. C, measurement of the length of the old and the new hook complexes in control and BOH2 RNAi cells. 100 cells were counted for each time point. ***: p < 0.001; ns, no significance. D, effect of BOH2 knockdown on TbSmee1 localization. Cells expressing endogenously 3HA-tagged TbSmee1 were co-immunostained with anti-HA antibody and anti-TbMORN1 antibody. The open arrowheads indicate TbSmee1 signal in the new hook complex, the arrows indicate TbSmee1 signal in the old hook complex, and the solid arrowhead indicates TbSmee1 signal at the new FAZ tip. Scale bar, 5 μm. E, quantitation of cells with different TbSmee1 signal in the new hook complex. 200 cells were counted for each time point and each cell type. Error bars, S.D. from three independent experiments (n = 3).
showed that the average length of the new centrin arm was significant by measuring the length of the centrin arm of the 1N2K and 2N2K cells, respectively (Fig. 5). The striking effect of BOH2 RNAi on TbSme1 localization prompted us to test whether BOH2 interacts with TbSme1 by co-immunoprecipitation. The results showed that the two proteins did not interact in vivo in trypomastigotes (Fig. S1). Thus, the disrupted localization of TbSme1 in BOH2 RNAi cells was very likely due to the loss of the shank part of the hook complex, with which TbSme1 associates. Taken together, these results suggest that BOH2 is essential for maintaining the integrity of the hook complex and the localization of proteins that associate with the shank part of the hook complex.

**BOH2 is required for centrin arm assembly**

The anterior part of BOH2 fluorescence signal overlaps with the centrin arm (Fig. 2), implying that BOH2 might be involved in centrin arm formation. We thus examined this possibility by immunofluorescence microscopy using anti-centrin antibodies to stain the centrin arm. These antibodies, such as the pan-centrin antibody 20H5 (15) and the anti-LdCen1/TbCentrin4 antibody (25), additionally label the basal body; therefore, to distinguish the basal body from the centrin arm, we performed co-immunofluorescence microscopy using antibodies that label the basal body, such as the YL 1/2 antibody (26, 27) and the anti-TbSAS-6 antibody (1). In the noninduced control cells, the centrin arm was detected by both 20H5 and anti-LdCen1/TbCentrin4 antibodies as a small bar-shaped structure next to the basal body, which was labeled by YL 1/2 and anti-TbSAS-6 antibodies (Fig. 5A). In BOH2 RNAi cells, the old centrin arm was still detected by 20H5 and anti-LdCen1/TbCentrin4 antibodies, but the new centrin arm was missing in ~85% and ~96% of the 1N2K and 2N2K cells, respectively (Fig. 5, A–C). Quantitative analysis by measuring the length of the centrin arm showed that the average length of the new centrin arm was significantly reduced (~1.1 μm in control cells versus ~0.4 μm in the RNAi cells that still had a new centrin arm) after BOH2 RNAi (Fig. 5D), confirming that BOH2 knockdown disrupted the assembly of the new centrin arm.

We next asked whether the localization of centrin arm–associated proteins to the new centrin arm was affected in BOH2 RNAi cells. To date, only one protein (Tb927.10.1450) has been found to localize to the centrin arm besides the two centrin proteins (23), and we named this protein CAAP1 for centrin arm–associated protein 1. CAAP1 was endogenously tagged with a triple HA epitope in the BOH2 RNAi cell line and was detected by immunofluorescence microscopy and 3D-SIM superresolution microscopy. In all of the BOH2 RNAi cells that had lost the new centrin arm, CAAP1 fluorescence signal in the new centrin arm was much weaker than that in the control cells (Fig. 5 (E and F) and Movies S8 and S9). This suggests that disruption of the new centrin arm by depletion of BOH2 impairs the localization of the centrin arm–associated protein CAAP1.

**Disruption of the centrin arm by depleting TbCentrin2 impairs BOH2 localization**

The striking effects of BOH2 knockdown on the formation of the new centrin arm (Fig. 5) and the morphogenesis of the new hook complex (Fig. 4) prompted us to investigate whether disruption of the centrin arm or the hook complex might affect BOH2 localization. We first investigated the potential effect of centrin arm disruption on BOH2 localization. To date, three centrin arm–localizing proteins, TbCentrin2, TbCentrin4, and CAAP1, have been identified, but none of them have been reported to be involved in centrin arm duplication. TbCentrin2 is required for Golgi duplication (15), TbCentrin4 participates in the coordination between karyokinesis and cytokinesis (16), and CAAP1 appears to be nonessential (28). We first tested whether knockdown of TbCentrin2 affected centrin arm duplication. Because knockdown of TbCentrin2 inhibits basal body segregation and, hence, caused pleiotropic effects on the segregation of multiple flagellum-associated structures after 48 h of RNAi induction (15), we chose to analyze the effect at 24 h of RNAi induction. We found that knockdown of TbCentrin2 reduced the 1N2K and 2N2K cells with two centrin arms (2CA or new and old CAs) by ~24% and ~20% (Fig. 6A and B). Consequently, it generated ~17% of 1N2K cells and ~15% of 2N2K cells with a single centrin arm (1CA or old CA only), and increased the 1N2K cells and 2N2K cells with a short, new centrin arm (snCA) and an old centrin arm (1CA + 1snCA) by ~7% and ~5%, respectively (Fig. 6A and B). This result suggests that TbCentrin2 is required for the formation of the new centrin arm.

Having demonstrated that knockdown of TbCentrin2 disrupted centrin arm formation, we examined the localization of BOH2 in those TbCentrin2 RNAi cells that did not form the new centrin arm or had a short, new centrin arm. Among the ~28% of the 1N2K cells and the ~22% of the 2N2K cells containing a single centrin arm or a short, new centrin arm and an old centrin arm (Fig. 6A and B), ~19% of the 1N2K cells and ~16% of the 2N2K cells did not contain BOH2 fluorescence signal in the new hook complex, and ~9% of the 1N2K cells and ~6% of the 2N2K cells contained weak BOH2 signal in the new hook complex (Fig. 6, A and C). These results suggest that disruption of the centrin arm by depletion of TbCentrin2 impaired BOH2 localization to the hook complex.

We next tested whether disruption of the hook complex might affect BOH2 localization. To date, two hook complex proteins, TbmORN1 and TblRRP1, have been functionally characterized (8, 18), and only TblRRP1 was found to be essential in the procyclic form of T. brucei (8). Knockdown of TblRRP1 generated 2N1K cells, the majority (~72%) of which contained a single centrin arm, and it was thus concluded that TblRRP1 knockdown inhibited the duplication of the centrin arm (8). However, it is unclear whether the single centrin arm in these 2N1K cells actually was a cluster of two centrin arms that failed to segregate or was the old centrin arm. Additionally, there was no analysis of the centrin arm and the hook complex in normal cell types (1N1K, 1N2K, and 2N2K cells) after TblRRP1 RNAi (8), making it difficult to evaluate the effect of TblRRP1 depletion on the duplication of the centrin arm and
The hook complex. We used TbLRRP1 RNAi cells to analyze the effect on BOH2 localization and focused on the TbLRRP1-deficient 2N2K cells to assess the effect on the hook complex and the centrin arm and to examine BOH2 localization in the new hook complex. We found that the morphology of hook complex in these TbLRRP1-deficient 2N2K cells appeared to be distorted, but both the hook complex and the centrin arm were duplicated, albeit the duplicated structures failed to segregate (Fig. 6, D and E). Despite the distortion of hook complex morphology, however, BOH2 remained to localize to the proximal portion of the new hook complex and to overlap with the proximal end of the new centrin arm (Fig. 6, D and E). These results suggest that disruption of hook complex morphology by depletion of TbLRRP1 exerted no effect on BOH2 localization.

Localization of BOH1 to the hook complex requires BOH2

Although BOH2 and BOH1 do not interact in vivo, they both localize to the hooked part of the hook complex (Fig. 1). We thus asked whether knockdown of BOH2 might affect BOH1 localization or vice versa. To this end, we epitope-tagged BOH1 in BOH2 RNAi cell line and BOH2 in BOH1 RNAi cell line and

Figure 5. Depletion of BOH1 inhibits centrin arm formation. A, immunofluorescence microscopic analysis of the centrin arm by anti-LdCen1/TbCentrin4 antibody in control and BOH2 RNAi cells. Cells were co-immunostained with anti-LdCen1/TbCentrin4 to label the centrin arm and YL 1/2 antibody to label the mature basal body (BB). Arrows, old centrin arm; open arrowheads, new centrin arm; solid arrowheads, basal body. Scale bar, 5 μm. B, immunofluorescence microscopic analysis of the centrin arm by the pan-centrin antibody 20H5 in control and BOH2 RNAi cells. Cells were co-immunostained with 20H5 to label the centrin arm and with anti-TbSAS-6 to label the basal body. Arrows, old centrin arm; open arrowheads, new centrin arm; solid arrowheads, basal body. Scale bar, 5 μm. C, quantitation of cells with different numbers of the centrin arm in control and BOH2 RNAi cells based on the immunofluorescence microscopy data in A and B. 200 cells were counted for each time point and each cell type, and error bars indicate S.D. from three replicates. CA, centrin arm. D, measurement of the length of the centrin arm in control and BOH1 RNAi cells. 100 cells for each cell type were used for measurement. *** p < 0.001; ns, no significance. E, BOH2 knockdown impaired the localization of CAAP1 to the new centrin arm. Cells expressing endogenously 3HA-tagged CAAP1 were co-immunostained with anti-HA antibody and anti-LdCen1/TbCentrin4 antibody. Open arrowheads, CAAP1 signal in the new centrin arm; arrows, CAAP1 signal in the old centrin arm. The insets in the CAAP1-3HA panel show the CAAP1 signal in the new centrin arm. Scale bar, 5 μm. F, 3D-SIM superresolution microscopy to analyze the effect of BOH2 RNAi on the localization of CAAP1 to the new centrin arm. Open arrowheads, CAAP1 signal in the new centrin arm; arrows, CAAP1 signal in the old centrin arm. Scale bar, 1 μm.
then performed immunofluorescence microscopy to examine the localization of BOH1 in the new hook complex in BOH2 RNAi and BOH2 in the new hook complex in BOH1 RNAi cells. We focused on the 1N2K and 2N2K cells, as these cells have duplicated the hook complex. In cells induced for BOH2 RNAi for 24 h, BOH1 fluorescence signal in the new hook complex was either lost in 18% of the 1N2K cells and 11% of the 2N2K cells, respectively, or became weaker than that in the old hook complex in 72% of the 1N2K cells and 71% of the 2N2K cells, respectively (Fig. 7, A and B). These results suggest that BOH2 is required for BOH1 localization to the hook complex. Conversely, in cells induced for BOH1 RNAi for 72 h, BOH2 fluorescence signal in the new hook complex was still detectable in all of the cells examined (Fig. 7C), indicating that BOH1 is not required for BOH2 localization to the hook complex. It appears that BOH2 is likely to be assembled onto the hook complex independently of BOH1 and that BOH2 may be involved in the recruitment of BOH1 to the hook complex.

Discussion

Proper positioning and attachment of the motile flagellum are critical for cell proliferation in T. brucei and depend on the faithful duplication and/or segregation of multiple flagellum-associated cytoskeletal structures. One of these cytoskeletal structures that play essential roles in maintaining flagellum inheritance is the hook complex, a new terminology for its initial name, the bilobe structure (15), based on its fishhook-like morphology (20). The first evidence to demonstrate the involvement of the hook complex in flagellum inheritance was obtained from the functional characterization of a hook complex–localizing protein named TbLRRP1, which showed that knockdown of TbLRRP1 abolished FAZ assembly and caused...
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flagellum detachment (8). Further evidence to support this notion came from the study of BOH1, which localizes to the hooked part of the hook complex and maintains hook complex morphology to control flagellum inheritance (22). In the current work, we identified BOH2, which co-localizes with BOH1 to the hooked part of the hook complex, and demonstrated its essential role in flagellum inheritance, thus providing additional evidence for the involvement of the hook complex in flagellum inheritance. Whereas the underlying mechanisms remain to be fully explored, we postulate that the hook complex controls flagellum inheritance by promoting FAZ nucleation and elongation based on the following evidence. First, knockdown of TbLRRP1 (8), BOH1 (22), or BOH2 (Fig. 3) all disrupted the nucleation and elongation of the new FAZ. Second, the proximal end of the FAZ is tethered into the hook complex–centrin arm structure between the shank part of the hook complex and the centrin arm (19). Third, assembly of the FAZ occurs at its proximal end (10, 29); therefore, newly synthesized FAZ components are targeted and assembled within the compartment surrounded by the hook complex and the centrin arm. Finally, proper assembly of the FAZ contributes to flagellum attachment and positioning (9, 10). In these regards, one of the major functions of the hook complex is likely to coordinate FAZ nucleation and elongation.

BOH2 is the second protein found localized to the hooked part of the hook complex besides BOH1 (Fig. 2), but the function of BOH2 is likely to be different from that of BOH1. Like BOH1, BOH2 also appears to bridge the hook complex, the centrin arm, and the proximal end of the FAZ filament (Fig. 2), but the two proteins do not form parts of a protein complex, suggesting that they function independently to promote flagellum inheritance. This notion is supported by the distinct defects on the hook complex and the centrin arm exerted by the knockdowns of BOH1 and BOH2. Depletion of BOH1 distorted the overall morphology of the hook complex and impaired the elongation of the centrin arm (22), whereas RNAi of BOH2 disrupted the integrity of the hook complex and inhibited the formation of the centrin arm (Figs. 4 and 5). Additionally, knockdown of BOH2 exerted faster and more severe impacts on cell proliferation and FAZ assembly than BOH1 knockdown (Fig. 3). Furthermore, depletion of BOH2 impaired the localization of BOH1, but not vice versa (Fig. 7), indicating that BOH2 either acts upstream of BOH1 in a yet-known regulatory pathway or is assembled onto the hook complex independently of BOH1 and helps recruit BOH1 through an indirect means. Nonetheless, due to the participations of BOH1 and BOH2 in the morphogenesis of the hook complex, the formation of the centrin arm, and the assembly of the FAZ, we postulate that both proteins might function to maintain the integrity and/or morphology of the hook complex–centrin arm assembly, thereby promoting the assembly of the FAZ filament from its proximal end that is embedded in the hook complex–centrin arm assembly.

The defects in FAZ assembly caused by BOH2 knockdown (Fig. 3, E and F), TbLRRP1 knockdown (8), and BOH1 knockdown (22) are strikingly different from that caused by knockdown of the FAZ filament proteins CC2D and FAZ2 (9, 10). Depletion of these hook complex–associated proteins generated cells containing only the old FAZ (Fig. 3, E and F) (8, 22), whereas knockdown of the two FAZ filament proteins did not generate such cells, but instead generated cells containing a snFAZ and the old FAZ (9, 10). The snFAZ observed in the CC2D RNAi cells and FAZ2 RNAi cells is believed to represent the FAZ root that is nucleated before the formation of the new flagellum (6, 30). These observations support the hypothesis that the hook complex is involved in the nucleation of the FAZ and that FAZ nucleation requires some of those hook complex–associated proteins. However, it should be pointed out that knockdown of BOH2 (Fig. 3, E and F), as well as knockdown of BOH1 (22) and knockdown of TbLRRP1 (8), also generated cells containing a snFAZ plus the old FAZ. We speculate that the snFAZ or the FAZ root had already formed in these cells prior to RNAi induction, but the elongation of the FAZ was inhibited. In this scenario, it suggests that FAZ elongation...
also requires the hook complex and some of those hook complex–associated proteins.

The specific effects of BOH2 knockdown on the shank part of the hook complex (Fig. 4, A–C) and the centrin arm (Fig. 5, A–D) and their associated proteins, such as TbSme1 (Fig. 4, D and E) and CAAP1 (Fig. 5, E and F) led us to hypothesize that BOH2 participates in the construction of the anterior part of the hook complex–centrin arm assembly by assisting the formation of the shank part of the hook complex and its associated centrin arm (Fig. 8). The underlying mechanisms for this function of BOH2 are, however, still unknown, but given that BOH2 overlaps, for most of its length, with the shank part of the hook complex (Fig. 2B) and with the centrin arm (Fig. 2C), we speculate that BOH2 might constitute an integral part of the shank–centrin arm structure whose components are assembled in a highly coordinated manner. Thus, depletion of BOH2 might disrupt this coordinated assembly process, thereby abolishing the assembly of the components of both the centrin arm and the shank part of the hook complex (Fig. 8). In support of this hypothesis, disruption of the centrin arm by knockdown of TbCentrin2 impaired BOH2 localization (Fig. 6, A–C), suggesting that depletion of TbCentrin2 might also disrupt that coordinated assembly process, thus affecting BOH2 localization to the hook complex–centrin arm structure. Knockdown of TbLRRP1, which appeared to distort hook complex morphology but did not inhibit the duplication of the hook complex and the centrin arm in binucleated cells, exerted no effect on BOH2 localization (Fig. 6, D and E). Because no hook complex proteins have been shown to inhibit the duplication of the hook complex so far, it is impossible to test whether the disruption of the hook complex would affect BOH2 localization.

In summary, we have identified a kinetoplastid-specific protein that maintains hook complex integrity and promotes centrin arm formation to facilitate FAZ nucleation and elongation and flagellum positioning and attachment. These findings highlight the crucial role of the hook complex in regulating flagellum inheritance, reveal the functional distinction among hook complex–associated proteins, and provide insights into the understanding of the mechanistic functions of these unique flagellum-associated cytoskeletal structures in regulating T. brucei cell proliferation.

Experimental procedures

Trypanosoma cell culture

The procyclic form of T. brucei 29-13 strain, which expresses T7 RNA polymerase and tetracycline repressor (31), was cultured in the SDM-79 medium containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma–Aldrich), 15 μg/ml G418, and 50 μg/ml hygromycin at 27 °C. The procyclic form of T. brucei Lister 427 strain was cultured in the SDM-79 medium supplemented with 10% heat-inactivated FBS at 27 °C. Cells were diluted with fresh medium once the cell density had reached 5 × 10⁶ cells/ml.

Proximity-dependent biotin identification (BioID) and MS

The full-length coding sequence of BOH1 was cloned into the pLew100-BirA*-HA vector (32), and the resulting plasmid was linearized by restriction digestion with NotI and transfected into the 29-13 strain. Successful transfectants were selected with 2.5 μg/ml phleomycin and cloned by limiting dilution in a 96-well plate containing SDM-79 medium supplemented with 20% heat-inactivated FBS and appropriate antibiotics. Expression of BOH1-BirA*-HA was induced with 0.5 μg/ml tetracycline and confirmed by Western blotting and immunofluorescence microscopy. Cells harboring the pLew100-BOH1-BirA*-HA plasmid were incubated with 0.5 μg/ml tetracycline for 24 h and then with 50 μM biotin for an additional 24 h. Cells were harvested by centrifugation and treated with PEME buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM MgSO₄, 0.1 mM EDTA) containing 0.5% Nonidet P-40. Cell lysates were spun to separate the cytosolic (soluble) and cytoskeletal (pellet) fractions, and the cytoskeletal fraction was further extracted with lysis buffer (0.4% SDS, 500 mM NaCl, 5 mM EDTA, 1 mM DTT, 50 mM Tris-HCl, pH 7.4) to solubilize
BOH2 promotes flagellum inheritance
cytoskeletal proteins. Subsequently, the cytosolic extract and the
cytoskeletal extract were combined and incubated with 500 μl of prewashed streptavidin-coated Dynabeads (Invitrogen)
for 4 h at 4°C. The Dynabeads were then washed with PBS five
times and with 50 ml ammonium bicarbonate five times and
resuspended in 100 mM ammonium bicarbonate. Finally, 10% DTT, 50% iodoacetamide, and 5% DTT were sequentially
added to the resuspension. Proteins bound on the Dynabeads
were digested with trypsin overnight at 37°C, and the trypsin
digestion reaction was stopped by adding TFA to the solution
until the pH had reached 2.0. Trypsin-digested peptides were
then desalted and analyzed on an LTQ Orbitrap XL mass spec-
trometer (Thermo Fisher Scientific) interfaced with an Eksgent
nano-LC 2D plus chipLC system (Eksigent Technologies). Raw
data files were searched against the T. brucei genome database
using the Mascot search engine. The search conditions used
peptide tolerance of 10 ppm and MS/MS tolerance of 0.8 Da
with the enzyme trypsin and two missed cleavages. MS and
data analysis were performed at the Clinical and Translational
Proteomics Service Center of the University of Texas Health
Science Center at Houston.

Tetracycline-inducible RNAi

The BOH2 RNAi cell line was generated by cloning a 722-bp
DNA fragment (nucleotides 5190–5912) from the coding
region of BOH2 gene into the pZJM vector (33), and the
TbCentrin2 RNAi cell line was generated by cloning the full-
length coding sequence of TbCentrin2 gene into the pZJM vec-
tor (33). The resulting plasmids were each linearized by restriction
digestion with NotI and then electroporated into the 29-13
cell line. Successful transfectants were selected with 2.5 μg/ml phleomycin and cloned by limiting dilution as described above.
The TbLRPP1 RNAi cell line (8) and the BOH1 RNAi cell line
(22) have been reported previously. RNAi was induced with 1.0
μg/ml tetracycline, and cell growth was monitored daily.

Endogenous epitope tagging of proteins

Endogenous tagging of BOH2, BOH1, TbLRPP1, TbSme1,
and CAAP1 with a C-terminal triple HA epitope and endoge-
nous tagging of BOH1 and TbSme1 with an N-terminal PTP
epitope were carried out using the PCR-based one-step epi-
tope-tagging method (34). PCR products were purified and
electroporated into either BOH1 RNAi cell line or BOH2 RNAi
cell line. Successful transfectants were selected with 1 μg/ml puromycin and cloned by limiting dilution in a 96-well plate
containing SDM-79 medium supplemented with 20% heat-
inactivated FBS and appropriate antibiotics.

For co-tagging of BOH2 and BOH1 in the same cell line and
c-o-tagging of BOH2 and TbSme1 in the same cell line for co-
localization and co-immunoprecipitation, BOH1 and TbSme1
were tagged with an N-terminal PTP epitope, and BOH2 was
tagged with a C-terminal HA epitope in the Lister 427 strain by sequential transfections using the PCR-based one-
step epitope-tagging method described above. Successful trans-
flectants were selected with 10 μg/ml blastcidin in addition to
1.0 μg/ml puromycin and were further cloned by limiting dilu-
tion as described above.

Co-immunoprecipitation and Western blotting

T. brucei cells co-expressing PTP-BOH1 and BOH2-3HA
were washed once with PBS and resuspended in 500 μl of
immunoprecipitation buffer (25 mM Tris-HCl, pH 7.6, 150 mM
NaCl, 1 mM DTT, 1% Nonidet P-40, and protease inhibitor
mixture). Cells were lysed by sonication, and cell lysate was
cleared by centrifugation. Cleared cell lysate was incubated with
30 μl of settled IgG-Sepharose beads (GE Healthcare) for 1 h at 4°C with gentle rotation. The IgG-Sepharose beads
were washed five times with the immunoprecipitation buffer,
and bound proteins were eluted with 30 μl of 10% SDS.
Immunoprecipitated proteins were separated by SDS-PAGE,
transferred onto a polyvinylidene difluoride membrane, and
immunoblotted with Horseradish Peroxidase-conjugated
anti-HA antibody (1:5000 dilution) and anti-Protein A polyclonal
antibody (1:5000 dilution) to detect BOH2-3HA and
PTP-BOH1, respectively. Cells expressing PTP-BOH1 alone
and BOH2-3HA alone were included as controls.

To prepare cytosolic and cytoskeletal fractions for Western
blotting, cells expressing 3HA-tagged BOH1 and BOH2 were
treated with PEME buffer containing 1% Nonidet P-40 for 5
min at room temperature and then centrifuged to separate cy-
tosolic and cytoskeletal fractions. Western blotting was carried
out with Horseradish Peroxidase-conjugated anti-HA anti-
body, anti-TbPSA6 antibody, which detects the α6 subunit of
the 26S proteasome (35), as the cytosol marker, and anti-tubu-
lin antibody as cytoskeleton marker.

Immunofluorescence microscopy

T. brucei cells were washed with PBS, settled on glass cover-
slips for 30 min at room temperature, and then fixed with cold
methanol for 30 min at −20°C. For immunofluorescence micro-
scopy using T. brucei cytoskeletons, cells were settled on
glass coverslips and then treated with the PEME buffer (see
above) supplemented with 1% Nonidet P-40 for 5 min at room
temperature. Cytoskeletons were then fixed with 4% parafor-
maldehyde for 10 min at room temperature. The fixed intact
cells or cytoskeletons were incubated with 3% BSA in PBS for
20 min at room temperature. Immunostaining was performed
by incubating the fixed cells or cytoskeletons with a primary
antibody for 60 min at room temperature. The following prim-
ary antibodies were used: FITC-conjugated anti-HA mAb
(Sigma–Aldrich; 1:400 dilution), anti-TbMORN1 polyclonal
antibody (18) (1:400 dilution), anti-LdCen1/TbCentrin4 polyclonal
antibody (25) (1:1000 dilution), anti-TbBILBO1 polyclonal
antibody (36) (1:1000 dilution), anti-CC2D polyclonal anti-
body (9) (1:1000 dilution), anti-TbSAS6 polyclonal antibody (1)
(1:1000 dilution), 20H5 mAb (37) (1:400 dilution), YL 1/2 mAb
(26) (1:1000 dilution), and anti-Protein A polyclonal antibody
(Sigma–Aldrich, 1:400 dilution). After washing the cells or
cytoskeletons on the coverslip three times with PBS, cells or
cytoskeletons were incubated with appropriate secondary anti-
bodies for 60 min at room temperature. The following second-
ary antibodies were used: FITC-conjugated anti-rabbit IgG,
Alexa Fluor 594–conjugated anti-rat IgG, Cy3-conjugated anti-
rabbit IgG, and Cy3-conjugated anti-mouse IgG. Cells or cyto-
skeletons were washed three times with PBS, mounted in the
DAPI-containing VectaShield mounting medium (Vector Laboratory), and observed with an inverted fluorescence microscope (Olympus IX71) equipped with a cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and a PlanApo N ×60 1.42 numerical aperture oil lens. Images were acquired with Slidebook 5 software.

**3D-SIM superresolution microscopy**

*T. brucei* cells were settled on No. 1.5 high-precision glass coverslips for 30 min at room temperature before treating with the PEME buffer containing 1% Nonidet P-40 for 1 min at room temperature. Cells were fixed in cold methanol (−20 °C) for 30 min and incubated in blocking buffer (1% BSA in PBS) for 20 min at room temperature. Cells were co-immunostained with FITC-conjugated anti-HA mAb (Sigma–Aldrich), anti-TbMORN1 polyclonal antibody (18), anti-LdCen1/TbCentrin4 polyclonal antibody (25), anti-CC2D polyclonal antibody (9), 20H5 antibody (37), anti-Protein A polyclonal antibody (Sigma–Aldrich) for 60 min at room temperature. After three washes with PBS, cells were incubated with Cy3-conjugated anti-rabbit IgG (Sigma–Aldrich) or Cy3-conjugated antimouse IgG (Sigma–Aldrich) for 60 min at room temperature. Cells on the coverslip were viewed under the Nikon Super Resolution Microscope n-SIM E instrument (Nikon Instruments Inc.) with a ×100 lens equipped with 488- and 592-nm lasers. The image stack thickness was taken with 0.1-µm z-steps, and 20–30 images/Z-section were taken. The acquired SIM images were applied to Stack 3D–structured illumination microscopy reconstruction and analyzed by NIS-Elements AR software.

**Data analysis and statistical analysis**

ImageJ software was used to measure the length of the hook complex and the length of the centrin arm, and data were exported to GraphPad Prism5 for analysis. Statistical analysis was conducted using the t test in GraphPad Prism5 and Microsoft Excel. Error bars represent S.D. from the mean of three independent experiments. For immunofluorescence microscopy experiments, images were taken at random, and all cells were used for counting and analysis.

**Data availability**

All data are contained within the article.

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**Abbreviations**—The abbreviations used are: FAZ, flagellum attachment zone; aa, amino acids; 3D-SIM, three-dimensional structured illumination microscopy; K, kinetoplast(s); N, nucleus/nuclei; snFAZ, short, new FAZ; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; FBS, fetal bovine serum; HA, hemagglutinin.

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