Regulated protein stabilization underpins the functional interplay among basal body components in *Trypanosoma brucei*

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The basal body in the human parasite *Trypanosoma brucei* is structurally equivalent to the centriole in animals and functions in the nucleation of axonemal microtubules in the flagellum. *T. brucei* lacks many evolutionarily conserved centriolar protein homologs and constructs the basal body through unknown mechanisms. Two evolutionarily conserved centriole/basal body cartwheel proteins, TbSAS-6 and TbBLD10, and a trypanosome-specific protein, BBP65, play essential roles in basal body biogenesis in *T. brucei*, but how they cooperate in the regulation of basal body assembly remains elusive. Here using RNAi, endogenous epitope tagging, immunofluorescence microscopy, and 3D-structured illumination super-resolution microscopy, we identified a new trypanosome-specific protein named BBP164 and found that it has an essential role in basal body biogenesis in *T. brucei*. Further investigation of the functional interplay among BBP164 and the other three regulators of basal body assembly revealed that BBP164 and BBP65 are interdependent for maintaining their stability and depend on TbSAS-6 and TbBLD10 for their stabilization in the basal body. Additionally, TbSAS-6 and TbBLD10 are independent from each other and from BBP164 and BBP65 for maintaining their stability in the basal body. These findings demonstrate that basal body cartwheel proteins are required for stabilizing other basal body components and uncover that regulation of protein stability is an unusual control mechanism for assembly of the basal body in *T. brucei*.

Centrioles/basal bodies nucleate the formation of the centrosome and cilia/flagella in eukaryotes and are characterized by a 9-fold radial array of triplet microtubules surrounding a cartwheel-like structure located at the proximal end of the centriole/basal body. The centriole/basal body cartwheel is constructed by nine homodimers of the SAS-6 protein, which, through a so-called pinhead structure formed by the BLD10/CEP135 protein, connect to the A-microtubules of the nine microtubule triplets (1–4). SAS-6, BLD10/CEP135, and another centriole/basal body protein SAS-4/CPAP are considered to be the core ancestral centriole/basal body module involved in the assembly of the centriole/basal body (5). SAS-6 forms the cartwheel structure of the centriole/basal body (6–9), BLD10/CEP135 stabilizes the cartwheel (8), and SAS-4/CPAP promotes the elongation and stabilization of the triplet microtubules (10–13). A comprehensive bioinformatics analysis of 45 diverse eukaryotes identified 14 ancient centriolar/basal body proteins and a cohort of proteins that are restricted to the centrosome and suggested that basal body function is ancestral, but the centrosome function is specific to the Holozoa (14).

The molecular process for centriole assembly in animals starts with the construction of a suitable scaffold, then the assembly of the centrosome, and finally the construction of a microtubule-based organelle (15). The first molecular module for centriole assembly consists of four proteins, CEP192, CEP152, CEP63, and CEP57, which plays a scaffolding function to recruit the polo-like kinase PLK4/ZYG-1 (16–22). The second module implicated in centriole assembly comprises the PLK4-STIL complex, which promotes the recruitment of SAS-6 for assembly of the centrosphere (15). PLK4 phosphorylates STIL, relieving an auto-inhibited conformation of STIL and, consequently, promoting the binding of STIL to SAS-6 (23–25). The third module involved in centriole assembly comprises BLD10/CEP135, SAS-4/CPAP, CEP120, and SPICE1, which function in connecting the centriole to the microtubule triplets and promoting the elongation of the centriolar microtubules (8, 10–13, 26, 27). BLD10/CEP135 interacts with SAS-6 (28) and SAS-4/CPAP (11–13), which additionally associates with CEP120 (26, 27) and SPICE1 (26). The latter three proteins are required to localize BLD10/CEP135 to the pro-centriole (26).

The basal body in the flagellated eukaryote *Trypanosoma brucei* nucleates the assembly of the flagellum that is essential for cell motility, cell morphogenesis, and cell division (29). In a G1-phase cell, the basal body comprises a mature basal body (mBB)2, which nucleates the flagellar axoneme, and an adjacent pro-basal body (pBB), which, upon entry into the S phase of the cell cycle, develops into mBB and nucleates the assembly of a new flagellum. Subsequently, two new pBBs are assembled next to the two mBBSs, and the new flagellum-associated mBB-pBB

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1The abbreviations used are: mBB, mature basal body; pBB, pro-basal body; SIM, structured illumination microscope; N, nucleus; K, kinetoplast.

2 The abbreviations used are: mBB, mature basal body; pBB, pro-basal body; SIM, structured illumination microscope; N, nucleus; K, kinetoplast.
Functional interplay among basal body components

pair undergoes a rotational move toward the posterior of the old flagellum-associated mBB-pBB pair. Elongation of the new flagellum pushes the new mBB-pBB pair to move further toward the posterior portion of the cell (30, 31). The trypanosome basal body adopts the canonical 9 × 3 microtubule array (31), and it contains homologs of two (SAS-6 and BLD10) of the three core ancestral centriolar/basal body proteins (32–34) (see Fig. 1A) and homologs of all but 1 of the remaining 11 ancestral centriolar/basal body proteins (14, 34, 35). Although T. brucei expresses a close homolog of the third core ancestral centriole/basal body protein SAS-4, this homolog (TbSAS-4) is not a basal body component but instead localizes to the distal tip of the flagellum attachment zone (FAZ) and plays a role in promoting FAZ elongation and cell morphology transitions (33). Among the evolutionarily conserved basal body proteins and the trypanosome-specific basal body identified in trypanosomes so far (32–44), three proteins, TbSAS-6 and TbBLD10 and BBP65, have been demonstrated to play essential roles in pBB biogenesis and flagellum assembly (32, 34). However, the mechanisms underlying basal body assembly in T. brucei remains elusive.

In this report we identify another trypanosome-specific basal body component, named BBP164, which plays an essential role in pBB biogenesis and flagellum assembly. Through genetic and cell biological approaches, we further investigate the functional interplay among the four essential regulators of pBB biogenesis, TbSAS-6, TbBLD10, BBP65, and BBP164. We show that regulated protein stabilization among these basal body components constitutes the primary control scheme, highlighting a distinct mechanism for basal body assembly in this early divergent microbial eukaryote.

Results

BBP164 is a new basal body component in T. brucei

A previous biochemical screen had identified a number of hypothetical proteins as candidate basal body components in T. brucei (34). Through epitope tagging of candidates on this list, we identified another new trypanosome-specific basal body protein, which we named BBP164 (Basal Body Protein of 164 kDa). This protein is encoded by Tb927.6.2760, and it contains only a coiled-coil motif in the C-terminal portion of the protein (Fig. 1A). A close homolog of BBP164 was identified in other kinetoplastid parasites, including Trypanosoma cruzi (TcCLB. 507925.20) and Leishmania major (LmjF.30.1260), but not in any eukaryotes outside of the kinetoplastid parasites, suggesting that BBP164 is a kinetoplastid-specific protein.

Immunofluorescence microscopy using cells expressing endogenously triple HA-tagged BBP164 protein showed that BBP164 localizes to both mBB and pBB, as demonstrated by the co-localization with the basal body cartwheel proteins TbSAS-6 and TbBLD10 and the kinetoplastid-specific basal body protein BBP65 (Fig. 1B). To determine the relative localization of BBP164 to the basal body cartwheel and to the BBP65 protein at higher resolutions, we carried out 3D-SIM super-resolution microscopy. BBP164 is located in the anterior part of the basal body barrel toward the flagellum and overlaps partly with the cartwheel proteins TbSAS-6 and TbBLD10 (Fig. 1C).

BBP164 also overlaps partly with BBP65, but BBP65 is located more anterior of the basal body cartwheel than BBP164 (Fig. 1C). Thus, BBP164 appears to localize to a subdomain of the basal body between BBP65 and the basal body cartwheel proteins TbSAS-6 and TbBLD10 (Fig. 1D).

BBP164 is required for basal body biogenesis and flagellum assembly

The function of BBP164 was investigated by inducible RNAi in the procyclic form of T. brucei. Knockdown of BBP164 was confirmed by Western blotting, which showed that the level of BBP164 protein, which was endogenously tagged with a triple HA epitope, was reduced to ~30% of the control level after 24 h and was undetectable after 48 h of RNAi induction (Fig. 2A). Depletion of BBP164 inhibited cell proliferation after 24 h of RNAi induction (Fig. 2B). To characterize the potential cell cycle defect caused by BBP164 RNAi, cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) to label the nucleus (N) and the kinetoplast DNA (K), the cell’s unique mitochondrial DNA complex, and then counted for the numbers of nuclei and kinetoplasts. The cell cycle stage of a trypanosome cell can be readily determined by the number of nuclei and kinetoplasts. G1 and S-phase cells contain one nucleus and one kinetoplast (1N1K); G2-phase and early mitotic cells contain one nucleus and two kinetoplasts (1N2K); and late mitotic cells, post-mitotic cells, and cells undergoing cytokinesis contain two nuclei and two kinetoplasts (2N2K). Knockdown of BBP164 caused a gradual decrease of 1N1K, 1N2K, and 2N2K cells, followed by an initial increase of 2N1K cells and a subsequent increase of cells containing multiple (>2) nuclei and one kinetoplast (XN1K) (Fig. 2C). This result suggests that depletion of BBP164 inhibited kinetoplast segregation and cell division. The emergence of 2N1K cells could also be attributed to defective duplication of the kinetoplast; however, given that BBP164 is a basal body protein, its depletion is unlikely to inhibit kinetoplast duplication.

Kinetoplast segregation depends on basal body separation (46); therefore, the defective kinetoplast segregation caused by BBP164 RNAi likely was attributed to defects in basal body duplication or segregation. To test this possibility, we performed immunofluorescence microscopy with the anti-TbSAS-6 antibody, which detects both the mBB and the pBB (32), and the YL 1/2 antibody, which stains the TbRP2 protein in the transition fibers radiating from the mBB (37). We focused on 2N1K cells from the BBP164 RNAi, as these cells increased significantly (Fig. 2C). Upon BBP164 RNAi induction, the majority of the 2N1K cells contained 1mBB-0pBB (~38%) or 1mBB-1pBB (~40%) (Fig. 2, D and E). In contrast, in the non-induced control, the majority of the 2N2K cells contained 2mBB-2pBB (~60%) or 2mBB-1pBB (~20%) or 2mBB-0pBB (~10%) (Fig. 2, D and E). Because in WT cells each mBB associates with a pBB (31), all (100%) of the 2N2K cells should contain 2mBB-2pBB. However, because of the various viewing angles in the immunofluorescence microscopy, the pBB and the mBB might overlap in some cells, making it difficult to distinguish them. Therefore, these cells were counted as 2mBB-0pBB or 2mBB-1pBB (Fig. 2E). Similarly, in BBP164 RNAi cells, the 2N1K cells containing 1mBB-0pBB or 2mBB-0pBB (Fig. 2E)
were actually cells containing 1mBB-1pBB or 2mBB-2pBB, respectively. Therefore, 78% of the 2N1K cells contained 1mBB-1pBB (Fig. 2E). These results demonstrated that BBP164 is required for formation of the new mBB-pBB pair.

Because the new flagellum is nucleated from the newly matured pBB during S phase of the cell cycle in T. brucei, we investigated whether flagellum formation was impaired by BBP164 RNAi. We used the pan-centrin antibody 20H5 to label the flagellum by immunofluorescence microscopy. In the control binucleate (2N2K) cells, all of them contained two full-length flagella (Fig. 2E). These results demonstrated that BBP164 is required for formation of the new mBB-pBB pair.

**Figure 1. BBP164 localizes to both the pro-basal body and the mature basal body.** A, essential components of the centriole cartwheel in animals and of the basal body cartwheel in trypanosomes. The trypanosome-specific BBP65 and BBP164 proteins are also shown. B, BBP164 localizes to both the mBB and the pBB in procyclic trypanosomes. Endogenously 3HA-tagged BBP164 was detected by anti-HA mAb, TbSAS-6 by anti-TbSAS6 pAb, Tbd10 by anti-TbBLD10 pAb, and BBP65-PTP by anti-Protein A pAb. DIC, differential interference contrast. Scale bar, 5 μm. C, 3D-SIM super-resolution microscopic analysis of BBP164 localization relative to TbSAS-6, Tbd10, and BBP65, and of BBP65 localization relative to TbSAS-6. BBP164 was endogenously tagged with a triple HA epitope and detected by anti-HA mAb, whereas BBP65 was endogenously tagged with a PTP epitope and detected by anti-Protein A pAb. TbSAS-6 and Tbd10 were detected by anti-TbSAS-6 pAb and anti-TbBLD10 pAb, respectively. Scale bar, 1 μm. D, schematic illustration of the relative locations of BBP164, BBP65, and the cartwheel proteins TbSAS-6 and Tbd10 in the basal body in T. brucei.

**TbSAS-6 and Tbd10 maintain BBP164 protein stability in the basal body**

The phenotypes caused by BBP164 RNAi are similar to that caused by depletion of TbSAS-6, Tbd10, or BBP65 (32 34), suggesting that they may cooperate with each other in regulating basal body biogenesis. We therefore investigated the functional interplay among these basal body proteins by examining the effect of depletion of one of them on the subcellular localization and protein stability of the others. As RNAi of these basal body proteins abolished the formation of the new mBB-pBB pair, we focused the localization of these proteins in the existing (old) basal body.

We first investigated the functional relationship between TbSAS-6 and BBP164. Immunofluorescence microscopy showed that induction of TbSAS-6 RNAi for 48 h disrupted the localization of BBP164 to the existing basal body in ~80% of the...
cells, but when the TbSAS-6 RNAi cells were treated with the proteasome inhibitor MG-132, the cells with BBP164 localized to the existing basal body restored to ~90% of the total population (Fig. 3, A and B). Western blotting showed that upon TbSAS-6 depletion BBP164 protein level gradually decreased to ~10% after 48 h (Fig. 3C). However, treatment of the TbSAS-6 RNAi cells with MG-132 stabilized BBP164 protein (Fig. 3C), demonstrating that BBP164 protein was destabilized by TbSAS-6 depletion. Taken together, these results suggest that TbSAS-6 is required for maintaining the stability of BBP164 in the existing basal body. Conversely, we investigated the effect of BBP164 depletion on the subcellular localization and stability of TbSAS-6 by similar approaches. Immunofluorescence microscopy showed that TbSAS-6 remained in the existing basal body in almost all the cells after BBP164 was knocked down, and Western blotting showed that TbSAS-6 protein was

Figure 2. BBP164 is required for basal body and flagellum biogenesis. A, RNAi-mediated knockdown of BBP164 in procyclic trypanosomes. Endogenously 3HA-tagged BBP164 was detected by anti-HA mAb. TbPSA6 served as the loading control. B, BBP164 is required for cell proliferation. Shown are the growth curves of control and BBP164 RNAi cell lines. Error bars indicate S.D. from three replicates. C, depletion of BBP164 inhibited kinetoplast segregation. 200 cells for each time point were counted. Error bars indicate S.D. from three replicates. D, co-immunofluorescence microscopy to detect the mBB and the pBB in control and BBP164 RNAi cells. The mBB was labeled with YL 1/2 mAb, whereas the mBB and the pBB were labeled with anti-TbSAS-6 pAb. Scale bar: 5 μm. E, quantitation of mBB and pBB in binucleated cells from control (2N2K cells) and BBP164 RNAi (2N1K cells). 200 binucleated cells for each cell line were counted. Error bars indicate S.D. calculated from three replicates. F, BBP164 RNAi inhibited the biogenesis of the new flagellum. The flagellum was immunostained with the pan-centrin antibody 20H5, which additionally labels the basal bodies and the centrin arm structure. The mBB was labeled with YL 1/2 mAb. The white arrow in the DIC panels indicates a short flagellum (sF). Scale bar: 5 μm. G, quantitation of the numbers of flagella and their associated mBB in binucleated cells from the control (2N2K cells) and BBP164 RNAi cells (2N1K cells). 150 cells for each cell line were counted, and the error bars indicate S.D. calculated from three replicates.

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not significantly affected by BBP164 depletion (Fig. 3, D–F). These results demonstrated that TbSAS-6 localization and stability in the existing basal body are not dependent on BBP164.

We next examined the effect of TbBLD10 knockdown on the localization and stability of BBP164. Immunofluorescence microscopy showed that after 48 h of TbBLD10 RNAi induction, BBP164 protein became undetectable in the existing basal body in ∼70% of the cells, but when the RNAi cells were treated with MG-132, BBP164 protein was detected in the existing basal body in ∼80% of the cells (Fig. 4, A and B). Western blotting showed that BBP164 protein level was reduced to <5% of the control level after 48 h of TbBLD10 RNAi, and treatment with MG-132 stabilized BBP164 protein (Fig. 4C). Together, these results suggest that TbBLD10 is required to maintain BBP164 stability in the existing basal body. In the reciprocal experiment, knockdown of BBP164 did not affect the localization of TbBLD10 in the existing basal body (Fig. 4, D and E), despite that depletion of BBP164 caused slight decrease in TbBLD10 protein level after 48 h of RNAi induction (Fig. 4F). These results suggest that BBP164 is not required for localizing TbBLD10 in the existing basal body.

**BBP164 and BBP65 are interdependent for their stability in the basal body**

We investigated the effect of BBP65 knockdown on the localization and stability of BBP164. Immunofluorescence microscopy showed that in BBP65-depleted cells, BBP164 signal was not detectable in the existing basal body in ∼80% of the cells. When cells were treated with MG-132, BBP164 was detected in the existing basal body in ∼85% of the cells (Fig. 5, A and B). Western blotting showed that when BBP65 RNAi was induced for 48 h, BBP164 protein level was reduced to <5% of the control level and was restored by treatment with MG-132 (Fig. 5C). These results demonstrated that BBP65 is required for the maintenance of BBP164 stability in the existing basal body.
We next investigated the effect of BBP164 depletion on the subcellular localization and stability of BBP65 protein. Immunofluorescence microscopy showed that BBP65 protein was not detectable in the existing basal body in ~85% of the total cell population after BBP164 RNAi was induced for 48 h. In the presence of MG-132, the localization of BBP65 to the existing basal body was restored in ~90% of the cells (Fig. 5, D and E). These results suggest that BBP164 maintains BBP65 stability in the existing basal body. Altogether, these results demonstrated that BBP65 and BBP164 are interdependent for maintaining their protein stability in the basal body.

**Functional interplay among basal body components**

Figure 4. **TbBLD10 maintains BBP164 protein stability in the basal body.**

A, effect of TbBLD10 depletion on the localization of BBP164. Cells were co-immunostained with FITC-conjugated anti-HA mAb to detect endogenously 3HA-tagged BBP164 and YL 1/2 mAb to stain the mBB. Scale bar: 5 µm. B, quantitation of cells with BBP164 localization at the basal body in control, TbBLD10 RNAi cells, and TbBLD10 RNAi cells treated with MG-132. 200 cells were counted for each cell line, and error bars indicate S.D. calculated from three replicates.

We next investigated the effect of BBP164 depletion on the localization and stability of BBP65 protein. Immunofluorescence microscopy showed that BBP65 protein was not detectable in the existing basal body in ~85% of the total cell population after BBP164 RNAi was induced for 48 h. In the presence of MG-132, the localization of BBP65 to the existing basal body was restored in ~90% of the cells (Fig. 5, D and E). Furthermore, Western blotting showed that BBP65 protein level was significantly reduced after BBP164 RNAi for 48 h, but treatment with MG-132 stabilized BBP65 (Fig. 5F). These results suggest that BBP164 maintains BBP65 stability in the existing basal body. Altogether, these results demonstrated that BBP65 and BBP164 are interdependent for maintaining their protein stability in the basal body.

**TbSAS-6 and TbBLD10 maintain BBP65 protein stability in the basal body**

We also investigated the effect of TbSAS-6 knockdown on the localization and stability of BBP65. Immunofluorescence microscopy of TbSAS-6 RNAi cells expressing endogenously triple HA-tagged BBP65 showed that after TbSAS-6 RNAi induction for 48 h, BBP65 was no longer detectable in the existing basal body in ~70% of the total cell population (Fig. 6, A and B). Further treatment of the TbSAS-6 RNAi cells with MG-132 allowed the detection of BBP65 in the existing basal body in ~90% of the cells (Fig. 6, A and B). Western blotting showed that BBP65 protein was reduced to undetectable level after TbSAS-6 RNAi for 48 h, but it was stabilized in the presence of MG-132 (Fig. 6C). These results demonstrated that TbSAS-6 is also required for maintaining BBP65 stability. Conversely, knockdown of BBP65 did not affect TbSAS-6 protein localization and stability (Fig. 6, D–F), suggesting that BBP65 is not required for TbSAS-6 stabilization in the existing basal body.

The functional relationship between TbBLD10 and BBP65 was similarly investigated. Immunofluorescence microscopy showed that BBP65 signal in the existing basal body was lost in ~50% of the TbBLD10 RNAi cells, whereas it was restored
when cells were treated with MG-132 (Fig. 7, A and B). Western blotting showed that BBP65 protein was reduced to undetectable level after 48 h of TbBLD10 RNAi, but it was stabilized by MG-132 treatment (Fig. 7C). These results suggest that TbBLD10 is required to maintain BBP65 stability in the existing basal body. Conversely, we investigated the effect of BBP65 knockdown on TbBLD10 protein stability. Endogenously 3HA-tagged BBP164 and BBP65 were detected by anti-HA mAb. TbPSA6 served as the loading control (Fig. 7D). Western blotting showed that TbBLD10 depletion did not affect the protein level of TbBLD10 (Fig. 7F). These results suggest that TbSAS-6 and TbBLD10 are independent from each other for maintaining their stability in the existing basal body.

Discussion

The basal body is one of the microtubule-organizing centers in T. brucei and is responsible for biogenesis of the motile flagellum. Although the T. brucei basal body is structurally similar to other eukaryotic basal bodies, the precise roles of its components in maintaining and stabilizing the basal body have not been fully elucidated. Our study provides insights into the functional interplay among basal body components, particularly focusing on the interdependence between BBP164 and BBP65.

Figure 5. BBP164 and BBP65 are interdependent for maintaining protein stability in the basal body. A, effect of BBP65 depletion on the localization of BBP164. Endogenously 3HA-tagged BBP164 was detected by FITC-conjugated anti-HA mAb. YL 1/2 staining of the mBB served as the basal body marker. Scale bar: 5 μm. B, quantitation of cells with BBP164 localization in the basal body in control, BBP65 RNAi cells, and BBP65 RNAi cells treated with MG-132. 200 cells were counted for each cell line, and error bars indicate S.D. calculated from three replicates. C, effect of BBP65 knockdown on BBP164 protein stability. Endogenously 3HA-tagged BBP164 and BBP65 were detected by anti-HA mAb. TbPSA6 served as the loading control. D, effect of BBP164 depletion on the localization of BBP65. Endogenously 3HA-tagged BBP65 was detected by FITC-conjugated anti-HA mAb. YL 1/2 staining of the mBB served as the basal body marker. Scale bar: 5 μm. E, quantitation of cells with BBP65 localization in the basal body in control, BBP164 RNAi cells, and BBP164 RNAi cells treated with MG-132. 200 cells were counted for each cell line, and error bars indicate S.D. calculated from three replicates. F, effect of BBP164 knockdown on BBP65 protein stability. Endogenously 3HA-tagged BBP65 and BBP164 were detected by anti-HA mAb. TbPSA6 served as the loading control.
to the centriole in animals, the mechanism for its assembly might be significantly different from that for centriole assembly in animals. Such a notion is based on the lack of homologs of numerous key regulators of centriole biogenesis in *T. brucei* (5, 47). Among the key regulators that are missing in *T. brucei* are PLK4/SAK, CP110, SPD2/CEP192, and Asterless/CEP152, which appear to have been added in a taxon-specific manner throughout evolution (47). Other regulators of centriole biogenesis, including PLK1 and SAS-4, have a close homolog in *T. brucei*, but these homologs are not involved in basal body biogenesis (33, 48).

The apparent distinct mechanism of basal body biogenesis in *T. brucei* suggests that *T. brucei* might employ trypanosome-specific proteins to compensate for the absence of those evolutionarily conserved regulators. We previously identified 25 trypanosome-specific basal body proteins, and 2 of them, BBP65 and BBP46, have been functionally characterized to control basal body duplication and segregation, respectively (34). In the current work, we characterized a new trypanosome-specific basal body protein BBP164 and demonstrated that it plays a crucial role in basal body biogenesis (Fig. 2).

The RNAi-based gene ablation experiments demonstrated that BBP164 is essential for basal body biogenesis in *T. brucei* (Fig. 2), but the mechanistic role of BBP164 remains largely unknown. The 3D-SIM super-resolution microscopy localized BBP164 to the distal region of the basal body cartwheel proteins TbSAS-6 and TbBLD10 and to the proximal region of the essential basal body protein BBP65 (Fig. 1). BBP164 might

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**Figure 6.** TbSAS-6 maintains BBP65 stability in the basal body. 
A, effect of TbSAS-6 depletion on the localization of BBP65. Cells were co-immunostained with FITC-conjugated anti-HA mAb to detect endogenously 3HA-tagged BBP65 and YL1/2 mAb to stain the mBB. Scale bar: 5 μm. B, quantitation of cells with BBP65 localization in the basal body in control, TbSAS-6 RNAi cells, and TbSAS-6 RNAi cells treated with MG-132. 200 cells were counted for each cell line, and error bars indicate S.D. calculated from three replicates. C, effect of TbSAS-6 knockdown on BBP65 protein stability. Endogenously 3HA-tagged BBP65 was detected by anti-HA mAb, and TbSAS-6 was detected by anti-TbSAS-6 pAb. TbPSA6 served as the loading control. D, effect of BBP65 depletion on the localization of TbSAS-6. Cells were co-immunostained with anti-TbSAS-6 pAb and YL1/2 mAb. Scale bar: 5 μm. E, quantitation of cells with TbSAS-6 localization in the basal body in control, BBP65 RNAi cells, and BBP65 RNAi cells treated with MG-132. 200 cells were counted for each cell line, and error bars indicate S.D. calculated from three replicates. F, effect of BBP65 knockdown on TbSAS-6 protein stability. TbSAS-6 was detected by anti-TbSAS-6 pAb, endogenously 3HA-tagged BBP65 by anti-HA mAb, and TbPSA6 by anti-TbPSA6 pAb, which served as the loading control.
interact with one or some of these three proteins, but because of the insolubility nature of these proteins, co-immunoprecipitation experiments cannot be carried out to test this possibility. The interdependency on protein stability between BBP164 and BBP65 (Fig. 5) suggests that these two proteins likely are correlated both functionally and physically. Because RNAi of BBP65 impaired basal body biogenesis (34), the destabilization of BBP65 by BBP164 RNAi might contribute, at least partly, to the basal body biogenesis defect observed in BBP164 RNAi cells. However, the mechanistic role of BBP65 in basal body biogenesis is also unknown (34). Both BBP65 and BBP164 contain only coiled-coil motifs (Fig. 1A) and localize to the close proximity of the basal body cartwheel structure (Fig. 1, C and D), suggesting that they might be components of a large protein complex and act to recruit other basal body components.

An evolutionarily conserved pathway for the recruitment of centriolar components has been established using *Caenorhabditis elegans* and human cells as model systems, which places SPD-2/CEP192 at the most upstream of the pathway, followed by the Polo-like kinase ZYG-1/PLK4, the cartwheel protein SAS-6, and then SAS-4 (15, 50). This order of recruitment of centriolar proteins was determined by investigating the localization of individual centriolar proteins in cells depleted of other centriolar proteins. In *T. brucei*, the order of recruitment of basal body components has not been determined. Based on the functions of their counterparts in animals (51–53), *Chlamydomonas* (6, 8, 9), and *Leishmania major* (54), TbSAS-6 constructs the basal body cartwheel structure, and TbBLD10 forms the pinhead connecting the cartwheel structure to the A-microtubules of the microtubule triplets. Therefore, TbSAS-6 and
TbBLD10 are most likely the first set of basal body components recruited to the basal body during basal body assembly. Using the approaches similar to that used in *C. elegans* and humans, we found that knockdown of TbSAS-6 and TbBLD10 caused the disappearance of BBP65 and BBP164 fluorescence signal at the existing basal body, but the two BBP proteins re-appeared at the existing basal body when the proteasome inhibitor MG-132 was added to the RNAi cells (Figs. 3, 4, 6, and 7). This result suggests that TbSAS-6 and TbBLD10 are both required for maintaining the stability of BBP65 and BBP164 in the existing basal body. It also implies that TbSAS-6 and TbBLD10 likely are needed to be present for the recruitment of BBP65 and BBP164 to the basal body. This assumption is consistent with the hierarchical organization of the four proteins along the z axis of the basal body (Fig. 1D). However, for the recruitment of BBP65 in BBP164 RNAi cells and the recruitment of BBP164 in BBP65 RNAi cells (Fig. 5), the interdependence for stability between the two proteins in the existing basal body made it difficult to determine their order of recruitment to the basal body. In the case of TbSAS-6 and TbBLD10, they are independent from each other for maintaining stability in the existing basal body (Fig. 8), which suggests that their recruitment to the basal body is likely also independent.

The most intriguing findings made in this work are the requirement of TbSAS-6 and TbBLD10 for maintaining the stability of BBP65 and BBP164 and the interdependence between BBP65 and BBP164 for maintaining stability (Figs. 3–7). Notably, a similar phenomenon, with regard to the regulation of the stability of basal body or centriolar proteins, has not been observed in any other organisms previously. It suggests that *T. brucei* uses an unusual mechanism in regulating its basal body components. Because TbSAS-6 and TbBLD10 build the cartwheel, which can be considered as the foundation of the basal body, during the formation of the new basal body, it suggests that once the cartwheel of the new basal body is not properly formed when either TbSAS-6 or TbBLD10 is depleted, BBP65 and BBP164 become unstable. However, it is interesting to note that BBP65 and BBP164 were undetectable in the existing basal body in TbSAS-6 RNAi cells and TbBLD10 RNAi cells (Figs. 3A, 4A, 6A, and 7A). This finding suggests that in WT trypanosome cells, BBP65 and BBP164 in the existing basal body likely are constantly being exchanged with the cytoplasmic pool of the two proteins (Fig. 9). When either TbSAS-6 or TbBLD10 is depleted by RNAi, the cytoplasmic pool of BBP65 and BBP164 proteins becomes unstable, and the BBP65 and BBP164 proteins dissociated from the existing basal body are also degraded, thereby causing the lack of BBP65 and BBP164 proteins in the existing basal body (Fig. 9). Nonetheless, it remains unclear how TbSAS-6 and TbBLD10 maintain the stability of BBP65 and BBP164 proteins, but it is possible that it is the integrity of the basal body that helps maintain the stability of basal body proteins other than the cartwheel proteins TbSAS-6 and TbBLD10. Another possibility is that TbSAS-6 and TbBLD10 might interact with BBP65 and BBP164 to stabilize the latter two proteins. Like BBP65 and BBP164, TbSAS-6 and TbBLD10 likely are also constantly being exchanged with the cytoplasmic pool of TbSAS-6 and TbBLD10 proteins (Fig. 9). However, depletion of BBP65 or BBP164 exerted no significant effect on the stability of TbSAS-6 and TbBLD10 (Figs. 3F, 4F, 6F, and 7F). Therefore, TbSAS-6 and TbBLD10 remained at the existing basal body in BBP65 RNAi cells and BBP164 RNAi cells (Fig. 9). In the case of the interdependence between BBP65 and BBP164 (Fig. 9), it is possible that the two proteins might interact with each other and thus become interdependent, as
are the cases of other protein complexes, such as the TbKin-C-TbKin-D complex (55), TbCentrin3-Tb1AD5–1 complex (56), the CRK9-CYC12 complex (57), and the CIF1-CIF2 complex (58) in *T. brucei* and the various protein complexes in other organisms (59–62). It appears that such a control of protein stability provides a means to regulate the function of the protein complex. Alternatively, if BBP65 and BBP164 do not interact with each other, the observed interdependence between them indicates that the depletion of either of the two proteins might disrupt the integrity of the basal body structure, thereby leading to the destabilization of other basal body proteins. Nonetheless, the findings in this work suggest that regulation of protein stability likely represent a primary control mechanism in basal body assembly in *T. brucei*.

**Experimental procedures**

**Trypanosoma cell culture**

The procyclic form of *Trypanosoma brucei* Lister 427 strain was cultured in the SDM-79 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) at 27 °C. The procyclic form of *T. brucei* 29-13 cell line (63), which expresses the T7 RNA polymerase and the tetracycline repressor protein (63), was cultured in the SDM-79 medium containing 10% fetal bovine serum, 15 μg/ml G418, and 50 μg/ml hygromycin. Cells were subcultured routinely by 10-fold dilutions with fresh medium once the cell density reaches 5 × 10⁶ cells/ml. Cells were monitored daily under a light microscope.

**Tetracycline-inducible RNAi**

To generate the BBP164 RNAi cell line, a 1024-bp DNA fragment (nucleotides 3354 to 4358) corresponding to the C-terminal portion of the coding sequence of BBP164 was amplified by PCR from the genomic DNA and cloned into the pZJM vector (64). The resulting plasmid, pZJM-BBP164, was linearized by restriction digestion with NotI and used to transfect the 29-13 cell line by electroporation. Successfully transfected cells were selected with 2.5 μg/ml phleomycin and subsequently cloned by limiting dilution in a 96-well plate containing SDM-79 medium supplemented with 20% heat-inactivated fetal bovine serum and 2.5 μg/ml phleomycin in addition to 15 μg/ml G418 and 50 μg/ml hygromycin. The TbSAS-6 RNAi cell line (32), the Tbd10 RNAi cell line (34), and the BBP65 RNAi cell line (34) have been described previously. RNAi was induced with 1.0 μg/ml tetracycline, and cell growth was monitored by daily counting of cells with a hemacytometer under a light microscope.

**Endogenous epitope tagging of proteins**

Endogenous tagging of BBP164 and BBP65 with a C-terminal triple HA epitope at one of the endogenous loci was carried out by using the PCR-based one-step epitope tagging approach (65). The PCR primers used for epitope tagging each contains a 100-bp sequence overlapped with the sequences flanking the last codon and the 3′-UTR (the stop codon was excluded). The PCR product was purified from agarose gel and used to transfect the Lister 427 cell line, the BBP164 RNAi cell line, the TbSAS-6 RNAi cell line, the Tbd10 RNAi cell line, and the BBP65 RNAi cell line. Successfully transfected cells were selected with 1 μg/ml puromycin and then cloned by limiting dilution as described above. Correct *in situ* tagging of one of the two alleles of BBP164 or BBP65 genes was confirmed by PCR amplification of purified genomic DNA from the transfected cells.

**Immunofluorescence microscopy**

Immunofluorescence microscopy was performed as described previously (34). Cells were harvested by centrifugation at 5000 × g for 5 min, washed once with PBS, and adhered onto glass coverslips at room temperature for 30 min. Immunofluorescence was carried out with either methanol-fixed intact cells or cytoskeletons. To prepare methanol-fixed intact cells, the cells on the coverslip were incubated with cold methanol at −20 °C for 30 min and then rehydrated with PBS for 5 min at room temperature. To prepare cytoskeleton, the cells on the coverslip were incubated with the PEME buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 0.1 mM EDTA, 1 mM MgSO₄) plus 0.1% Nonidet P-40 at room temperature for 5 min. The methanol-fixed intact cells or the cytoskeleton samples were incubated with blocking buffer (3% BSA in PBS) for 20 min at room temperature. Immunostaining was performed by incubating the cells or cytoskeletons on the coverslip with a primary antibody for 1 h at room temperature. After washing the cells on the
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coverslip three times with PBS, cells were incubated with a secondary antibody. Cells were washed three times with PBS and one time with distilled water before permanently mounted in the 4',6-diamidino-2-phenylindole–containing VectaShield mounting medium (Vector Laboratories) and observed with an inverted fluorescence microscope (Olympus IX71). The primary antibodies used were the FITC-conjugated anti-HA monoclonal antibody (mAb) (Sigma-Aldrich), anti-TbSAS6 polyclonal antibody (pAb) (32), anti-TbBLD10 pAb (34), YL1/2 mAb (EMD Millipore) (66). The secondary antibodies used are the FITC-conjugated anti-rat IgG, FITC-conjugated anti-mouse IgG, FITC-conjugated anti-rabbit IgG, Cy3-conjugated anti-rabbit IgG, and Cy3-conjugated anti-rat IgG, all of which were from Sigma-Aldrich. Images were acquired and processed with the SlideBook5 software.

3D-structured illumination microscopy (3D-SIM) super-resolution microscopy

*T. brucei* cells were washed once with PBS, adhered to the No. 1.5 high-precision glass coverslip, and treated with PEME buffer containing 1% Nonidet P-40 for 1 min at room temperature. Subsequently, cells were fixed in cold methanol (−20 °C) for 30 min and incubated in blocking buffer (1% BSA in PBS) at room temperature for 1 h. Cells were co-immunostained with FITC-conjugated anti-HA mAb (Clone HA-7, H7411, Sigma-Aldrich, 1:400 dilution) and anti-TbSAS6-6 pAb (1:400 dilution) (32), anti-TbBLD10 pAb (1:400 dilution) (34), or anti-Protein A pAb (Sigma-Aldrich, 1:400 dilution). Cells were washed three times with PBS, and then incubated with Cy3-conjugated anti-rabbit IgG (Sigma-Aldrich, AP132C, 1:400 dilution). Slides were viewed under Nikon Super Resolution Microscope n-SIM E (Nikon Instruments Inc.). The acquired SIM images were applied to Stack 3D-SIM reconstruction and analyzed by using the NIS-Elements AR software.

Western blotting

Cells were collected by centrifugation at 5000 × g for 10 min, washed once with PBS, re-suspended in PBS, and lysed by boiling the cells in SDS sampling buffer for 10 min. Proteins were separated in a 10% polyacrylamide SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was incubated in 4% milk for 45 min at room temperature and subsequently incubated with an appropriate primary antibody for 1 h at room temperature. After washing the membrane three times with TBS-T buffer (20 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20), the membrane was incubated with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG. The anti-TbPSA6 pAb, which detects the α-6 subunit of the 26S proteasome (45), was used as the control for equal loading of protein samples. Protein band intensity was measured by ImageJ and then normalized by that of the loading control.

Data analysis

The error bar represents S.D. from the mean of three independent biological replicates. For immunofluorescence microscopy experiments, images were taken randomly, and all cells in the images were used for counting and analysis.

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