SAS-4 Protein in Trypanosoma brucei Controls Life Cycle Transitions by Modulating the Length of the Flagellum Attachment Zone Filament*

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The evolutionarily conserved centriole/basal body protein SAS-4 regulates centriole duplication in metazoa and basal body duplication in flagellated and ciliated organisms. Here, we report that the SAS-4 homolog in the flagellated protozoan Trypanosoma brucei, TbSAS-4, plays an unusual role in controlling life cycle transitions by regulating the length of the flagellum attachment zone (FAZ) filament, a specialized cytoskeletal structure required for flagellum adhesion and cell morphogenesis. TbSAS-4 is concentrated at the distal tip of the FAZ filament, and depletion of TbSAS-4 in the trypanomastigote form disrupts the elongation of the new FAZ filament, generating cells with a shorter FAZ associated with a longer unattached flagellum and repositioned kinetoplast and basal body, reminiscent of a short epimastigote-like morphology. Further, we show that TbSAS-4 associates with six additional FAZ tip proteins, and depletion of TbSAS-4 disrupts the enrichment of these FAZ tip proteins at the new FAZ tip, suggesting a role of TbSAS-4 in maintaining the integrity of this FAZ tip protein complex. Together, these results uncover a novel function of TbSAS-4 in regulating the length of the FAZ filament to control basal body positioning and life cycle transitions in T. brucei.

The centrosome in animals constitutes the cell’s microtubule-organizing center that nucleates spindle assembly, and is characterized by a symmetrical array of nine microtubule triplets emanating from a cartwheel structure in the very proximal region of the centriole. Biogenesis of centrioles requires many regulatory proteins, including SAS-6, SAS-4/CPAP, and BLD10/CEP135, which are thought to constitute the core ancestral module involved in centriole assembly (1). The three proteins have distinct functions in centriole biogenesis. SAS-6 assembles to the cartwheel (2, 3) and BLD10/CEP135 forms the pinhead that connects the cartwheel spokes to the A-microtubules of the microtubule triplets (4, 5), whereas SAS-4/CPAP controls the elongation of centriolar microtubules (6–9).

Trypanosoma brucei, a flagellated protozoan and the causative agent of human sleeping sickness, does not have centrosomes, but it possesses the basal body as its microtubule-organizing center to nucleate the assembly of a motile flagellum. The basal body is characterized by an array of nine microtubule triplets, with the conserved SAS-6 protein forming the cartwheel structure (10), similar to that of the centrosome in metazoa. The flagellum is attached, along most of its length, to the cell body via a specialized cytoskeletal structure termed the flagellum attachment zone (FAZ),2 which consists of three main domains: the structure connecting the paraflagellar rod (PFR) to the flagellar membrane, the structure connecting the flagellar membrane and the cell body membrane, and a cytoplasmic filament (often referred to as the FAZ filament) and its associated microtubule quartet (11–17). In addition to mediating flagellum-cell body attachment, the FAZ filament also defines the cytokinesis cleavage plane (11, 12), with the distal tip of the new FAZ filament marking the site of cytokinesis initiation (18–20). Moreover, during the cell cycle in the procyclic form, the elongation of the FAZ filament positions the newly assembled basal body toward the cell posterior (21), which is crucial for organelle segregation and cell division (10, 22–25).

Trypanosomatids, a group of kinetoplastid parasites consisting of T. brucei, Trypanosoma cruzi, and Leishmania spp., appear in a variety of different morphological forms during their life cycle and are distinguished by the relative position of the kinetoplast (mitochondrial DNA) and the nucleus and by the position, length, and cell body attachment of the flagellum (26). In the tsetse fly vector, T. brucei differentiates from the trypanomastigote form to the epimastigote form, which undergoes asymmetrical cell division to produce a long epimastigote cell and a short epimastigote cell (26). How life cycle transitions in T. brucei are regulated is largely unknown, but recent work has begun to uncover the regulators involved in this process. ALBA3 and ALBA4, two closed related RNA-binding proteins, are the first factors found to be involved in life cycle transitions in T. brucei (27). Another RNA-binding protein, RBP6, is also required for driving the transition from trypanomastigotes to epimastigotes (28). These discoveries highlight the regulation of life cycle transitions via posttranscriptional mechanisms. Recently, two flagellar proteins, ClpGM6 and FLAM3 (15, 29), and a FAZ filament protein, FAZ9 (30), were also reported to be required for life cycle transitions in T. brucei, suggesting that

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2 The abbreviations used are: FAZ, flagellum attachment zone; PFR, paraflagellar rod; TbSAS-4, Trypanosoma brucei SAS-4; TbPLK, T. brucei Polo-like kinase; BioID, biotin identification; CPC, chromosomal passenger complex; N, nuclear DNA; K, kinetoplast DNA.
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modulation of flagellum-associated cytoskeletal structures also drives life cycle transitions.

Here, we report that the SAS-4 homolog in *T. brucei*, TbSAS-4, plays a surprising role in life cycle transitions. Unlike its orthologs in metazoa and other flagellated and ciliated organisms, TbSAS-4 is not detectable in the basal body but, instead, is concentrated at the distal tip of the FAZ filament, where it associates with multiple FAZ tip proteins. Consistent with its localization to the FAZ tip, TbSAS-4 promotes the elongation of the newly synthesized FAZ filament during the cell cycle, thereby positioning the newly assembled basal body toward the cell posterior and maintaining trypanomastigote cell morphology.

**Experimental Procedures**

Trypanosome Cell Culture and RNAi—The procyclic 29-13 cell line (31) was grown in the SDM-79 medium containing 10% fetal bovine serum (Atlanta Biologicals, Inc.), 15 µg/ml G418, and 50 µg/ml hygromycin, whereas the procyclic 427 cell line was cultured at 27 °C in SDM-79 medium containing 10% fetal bovine serum. Cells were routinely diluted when the cell density reaches 5 × 10^6 cells/ml.

For RNAi of TbSAS-4, a 464-bp DNA fragment from the coding region of *TbSAS-4* was cloned into the pZJM vector (32), and the resulting plasmid was electroporated into the 29-13 cell line according to the previous procedure (10). Successful transfectants were selected under 2.5 µg/ml phleomycin. Cells were cloned by limiting dilution in a 96-well plate in SDM-79 medium containing 20% fetal bovine serum and all three antibiotics. At least three clonal cell lines were selected for further analysis. To induce RNAi, the clonal cell lines were induced with 1.0 µg/ml tetracycline. Cell growth was monitored daily by counting the number of cells with a hemocytometer and plotted against the time of RNAi induction.

Purification of Recombinant *TbSAS-4* Protein and Antibody Production—A 948-bp DNA fragment corresponding to the C-terminal coding region (amino acids 617–932) of *TbSAS-4* was PCR-amplified from the genomic DNA, and then cloned into the pET26 vector for expressing a hexahistidine-fused C-terminal coding region (amino acids 617–932) of *TbSAS-4* truncation protein was induced with 1 mM isopropyl-1-thio-

Expression of *TbSAS-4*-BirA*-HA* for Proximity-dependent Biotin Identification (BioID)—The full-length coding sequence of *TbSAS-4* was PCR-amplified from genomic DNA, and then cloned into the pLew100-BirA*-HA vector, which was generated by cloning the BirA*-HA into the pLew100 vector for expressing BirA*-HA-tagged *TbSAS-4*. The construct was linearized with NotI and electroporated into the 29-13 cell line. Transfectants were selected with 2.5 µg/ml phleomycin and then cloned by limiting dilution as described above. Expression of *TbSAS-4*-BirA*-HA* was induced by incubating the cells with 0.5 µg/ml tetracycline, and then verified by Western blotting with anti-HA antibody and anti-*TbSAS-4*-antibody and by immunofluorescence microscopy with FITC-conjugated anti-HA antibody.

Affinity Purification of Biotinylated Proteins and LC-MS/MS—Affinity purification of biotinylated proteins was carried out essentially as described previously (35). Briefly, *TbSAS-4*-BirA*-HA* was overexpressed by induction with 0.5 µg/ml tetracycline for 24 h, and cells (~5 × 10^9) were incubated with 50 µM biotin for an additional 24 h. Cells were collected by centrifugation, washed three times with PBS, and then treated with PEME buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 0.1 mM EDTA, 1 mM MgSO4) containing 0.5% Nonidet P-40. Cytosolic (soluble) and cytoskeletal (pellet) fractions were separated by centrifugation at 3,200 g for 10 min. 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) at room temperature for 30 min, and solubilized materials were collected by centrifugation. Both the cytosolic extract and the cytoskeletal extract were incubated with 500 µl of streptavidin-coated Dynabeads (Invitrogen), which were pre-washed with PBS, at 4 °C for 4 h. The Dynabeads were then washed extensively with PBS. The non-induced
control cells \((\sim 2.5 \times 10^9)\) were similarly treated and included as the negative control.

For mass spectrometry, the Dynabeads were washed five times with 50 mM ammonium bicarbonate before resuspended in 100 mM ammonium bicarbonate. 10% DTT was then added to reduce the disulfide bond, and subsequently 50% iodoacetamide was added for alkylation. 5% DTT was then added to the solution, and proteins were digested with trypsin overnight at 37 °C. Digestion was stopped by adding trifluoroacetic acid to approximately pH 2.0. The digests were desalted, and then analyzed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) interfaced with an Eksigent nano-LC 2D plus cHiPLC system (Eksigent Technologies) at the Proteomics Core Facility of the University of Texas Health Science Center at Houston.

Data analysis was performed according to our published methods (17, 36). 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.

### Results

The \(T. brucei\) SAS-4 Homolog Is Concentrated at the Distal Tip of the FAZ Filament—Previous comprehensive analyses of centriole/basal body components conserved in eukaryotes (1, 37) showed that the \(T. brucei\) genome encodes a conserved SAS-4 homolog (Tb927.11.3300), which we named TbSAS-4, but the function of TbSAS-4 is unknown. TbSAS-4 possesses several coiled coil motifs at its N terminus and a conserved TCP10-like domain and a highly conserved G-box motif at its C terminus, which are in the same order as in its human ortholog CPAP (Fig. 1A). Alignment of the G-box motif between TbSAS-4 and human CPAP (HsCPAP) identified 47% identity and 77% similarity in this motif between the two proteins (Fig. 1B). TbSAS-4 exhibits sequence similarity to the SAS-4 proteins from many organisms, but is closely related to
the putative SAS-4 protein from *T. cruzi* and *Leishmania major* (1, 37).

To determine the subcellular localization of TbSAS-4, a polyclonal antibody against TbSAS-4 was raised in rabbit and used for immunofluorescence microscopy. Surprisingly, TbSAS-4 was not detected in the basal body at any cell cycle stages. Instead, it was concentrated at the distal tip of the FAZ filament, which was labeled by the anti-FAZ1 antibody L3B2 (Fig. 1C). The localization of TbSAS-4 to the distal tip of the FAZ filament was further confirmed by co-immunostaining with the 1B41 antibody (34), which detects the microtubule quartet that associates with the FAZ filament (Fig. 1D). The unusual localization of TbSAS-4 to the FAZ tip suggests that TbSAS-4 may play roles in the FAZ filament.

Depletion of TbSAS-4 Generates Epimastigote-like Cells—
To investigate the function of TbSAS-4, we carried out RNAi in the procyclic form of *T. brucei*. Western blotting with anti-TbSAS-4 antibody showed that upon RNAi induction, TbSAS-4 was reduced to ~15% of the control level at day 2 of RNAi induction (Fig. 2A). This knockdown caused severe growth defects (Fig. 2B), suggesting that TbSAS-4 is essential for cell proliferation in the procyclic form. To further characterize the defects, we tabulated the cells for the numbers of nucleus and kinetoplast, and the results showed that depletion of TbSAS-4 resulted in the accumulation of cells of various karyotypes, including anucleate cells containing only the kinetoplast (0N1K), bi-nucleate cells with a single kinetoplast (2N1K), multi-nucleate (>2 nuclei) cells with a single kinetoplast...
T. brucei NbSAS-4 deficiency disrupts the elongation of the new FAZ filament—The generation of epimastigote-like cells upon NbSAS-4 RNAi indicates that the FAZ filament may be shortened, as in the cases of ClpGm6 RNAi (15) and FLAM3 RNAi (29). To test this possibility, we immunostained the control and NbSAS-4 RNAi cells with anti-CC2D antibody (12), the 1B41 antibody (34), and the L8C4 (anti-PFR2) antibody (33) to label the FAZ filament, the FAZ-associated quartet microtubules, and the flagellum, respectively (Fig. 2, A and B). We then measured the length of the FAZ filament, the flagellum, and the unattached (free) flagellum in 1N1K cells, and the length of the new FAZ filament, the new flagellum, and the unattached (free) new flagellum in 2N2K cells. The results showed that the length of the FAZ filament of the NbSAS-4-deficient 1N1K cells was shorter than that in the control 1N1K cells (average length of 6.7 μm versus 14.0 μm) (Fig. 3, A–C). However, the length of the flagellum of the NbSAS-4 RNAi cells (average length of 20.2 μm) appeared similar to that of the control cells (average length of 20.1 μm) (Fig. 3, A–C). These 1N1K cells with a short FAZ filament also possessed a longer unattached (free) flagellum than the control cells (average length of 6.7 μm versus 4.3 μm) (Fig. 3, A, B, and D). In NbSAS-4-deficient 2N2K cells, the length of the new FAZ filament was also shorter than that of the control 2N2K cells (average length of 9.6 μm versus 11.6 μm) (Fig. 3, A, B, and E). The new flagellum in the NbSAS-4-deficient 2N2K cells was partially detached, likely attributed to the formation of a shorter FAZ filament (Fig. 3, A, B, and F). However, the old flagellum and the old FAZ filament in the 2N2K cells appeared to be unaffected after NbSAS-4 knockdown (Fig. 3). These results suggest that NbSAS-4 depletion impaired the elongation of the new FAZ filament, causing partial detachment of the new flagellum at the distal region. Therefore, the 1N1K cells with a shorter FAZ filament and a longer unattached flagellum were likely derived from the 2N2K cells with a shorter new FAZ filament.

Knockdown of NbSAS-4 causes asymmetrical cell division—The length of the new FAZ filament is known to determine the cytokinesis cleavage plane in T. brucei (12). Therefore, a 2N2K cell with a short, new FAZ filament can undergo asymmetrical cytokinesis to produce a smaller-sized 1N1K daughter cell with a shorter FAZ filament (12). Because NbSAS-4 RNAi disrupted new FAZ elongation (Fig. 3), we reasoned that the NbSAS-4-deficient 2N2K cells might also be able to divide asymmetrically to produce smaller-sized 1N1K cells with a shorter new FAZ filament. Indeed, after induction of NbSAS-4 RNAi for 2 days, ~55% of the 1N1K cells and ~98% of the anucleate (0N1K) cells were smaller in size (Fig. 4, A and B), and all of these smaller-sized 1N1K cells were of epimastigote-like morphology (Fig. 4B). Moreover, NbSAS-4-deficient cells undergoing an asymmetrical cytokinesis could be easily detected (Fig. 4, C and D). Among the 2N2K cells that were undergoing cytokinesis in NbSAS-4 RNAi-induced cells, ~77% of them were dividing asymmetrically (Fig. 4C). This asymmetrical cytokinesis could either produce a smaller-sized 1N1K daughter cell with repositioned basal body and a normal-sized 1N1K cell with or without repositioned basal body or generate a smaller-sized anucleate daughter cell and a 2N1K daughter cell (Fig. 4D). This result suggests that the 2N1K cells that accumulated upon NbSAS-4 RNAi (Fig. 2C) were very likely to be derived from asymmetrical cytokinesis of the 2N2K cells.

Affinity purification of biotinylated proteins was carried out for both the non-induced control and NbSAS-4–BirA*-HA induced cells (Fig. 5C), and the purified biotinylated proteins from the cytosolic fraction and the cytoskeletal fraction were digested with trypsin and analyzed by LC-MS/MS. A total of 21 proteins, in addition to NbSAS-4 itself, were detected in the NbSAS-4–BirA*-HA overexpressed cells only, which contained four known FAZ proteins, FAZ6 (16), FAZ9 (16), FAZ10 (35), and FAZ11 (35); five known bilobe proteins (35); the tripartite attachment complex component p197 (39); KMP-11 (40); the flagellum targeting protein khar01 (KH1) homolog (41); the putative centriole protein POCl homolog (Tb927.10.2860); and eight hypothetical proteins (Table 1). The identification of five bilobe proteins as NbSAS-4–binding partners and near neighbors likely was due to the fact that the new FAZ filament is assembled from the bilobe region (42) and, therefore, during the early S-phase when the FAZ filament just starts to
elongate, TbSAS-4 at the tip of the newly assembled FAZ may make contact with these bilobe proteins.

We next tagged the eight hypothetical proteins at their C terminus with a triple HA epitope at their respective endogenous locus. Immunofluorescence microscopy showed that three proteins were localized to the FAZ tip, and thus we named them FAZ12 (Tb927.11.2590), FAZ13 (Tb927.3.1020), and FAZ14 (Tb927.8.6890); one protein (Tb927.5.4340) was localized to the basal body; one protein (Tb927.11.15140) was localized in the bilobe region, and the rest of them were localized to the cytoskeleton (Table 1). Although FAZ12 appeared to be detected only at the FAZ tip of 1N1K cells, FAZ13 and FAZ14 were localized to the distal tips of both the new and the old FAZ filaments (see below).

Given that TbSAS-4 is enriched at the FAZ tip (Fig. 1), we examined its co-localization with FAZ9 and the six FAZ tip-enriched proteins, which were each tagged with a triple HA epitope at their respective endogenous locus. Immunofluorescence microscopy showed that TbSAS-4 was partly co-localized with all of these FAZ proteins at the FAZ tip (Fig. 5, D–J).
except FAZ1, with which TbSAS-4 co-localized (Fig. 5G). TbSAS-4 was located at the distal side of FAZ6 (Fig. 5D), FAZ10 (Fig. 5F), FAZ12 (Fig. 5H), and FAZ14 (Fig. 5J), but was located at the posterior side of FAZ13 (Fig. 5J). FAZ9 was not enriched at the old FAZ tip, but was concentrated at the new FAZ tip, where it partially co-localized with TbSAS-4 (Fig. 5E). Together, these results suggest that TbSAS-4 may constitute a component of a protein complex at the distal tip of the FAZ filament.

Depletion of TbSAS-4 Disrupts the Enrichment of Several FAZ Tip Proteins at the New FAZ Tip—We next investigated the effect of TbSAS-4 knockdown on the localization of these FAZ tip proteins. Four of the six FAZ tip proteins, FAZ6, FAZ11, FAZ13, and FAZ14, were each endogenously tagged in cells harboring the TbSAS-4 RNAi construct. After RNAi induction for 2 days, all four proteins were still enriched (as a bright dot) at the distal tip of the old FAZ filament in the RNAi-induced 2N2K cells, similar to that in the control 2N2K cells (Fig. 6, A–D, arrowheads), but were spread along about one-third to one-half of the length of the new FAZ filament at the distal region (Fig. 6, A–D, brackets). This defect was further investigated by measuring the length of the fluorescence signal of these FAZ tip proteins at the new FAZ tip (Fig. 6E). The average length of the fluorescence signal of the four FAZ tip proteins at the new FAZ tip was measured to be between 0.8 and 1.0 μm in the control 2N2K cells, but was measured to be between 3.8 and 5.1 μm in TbSAS-4-depleted 2N2K cells, an ~5-fold increase over the control (Fig. 6, A–E). This result suggests that TbSAS-4 depletion disrupted the enrichment of these FAZ tip proteins at the new FAZ tip.

Discussion

In this study, we investigated the localization and function of the SAS-4 homolog in T. brucei, an early branching parasitic protozoan. The finding that TbSAS-4 is concentrated at the FAZ tip throughout the cell cycle (Fig. 1) is surprising, as its orthologs in other eukaryotes have been localized to the centrosome or the basal body. At the FAZ tip, TbSAS-4 appears to constitute an essential component of a large protein complex that is composed of TbSAS-4 and six FAZ tip-enriched proteins, FAZ6 and FAZ10 to FAZ14 (Fig. 5). Given that FAZ12 is only detected at the FAZ tip of G1 (1N1K) cells (Fig. 5H) and the
other five FAZ tip-enriched proteins are localized at the distal tip of the FAZ filament in G₁ cells and the distal tips of both the new and the old FAZ filaments in S-phase, G₂ phase, and mitotic cells (Fig. 5, D, F, G, I, and J and data not shown), this suggests that there are two distinct TbSAS-4-containing complexes at the FAZ tip. The complex with FAZ12 is present only in G₁ cells, and following cell cycle progression to S-phase and later cell cycle stages, the complex without FAZ12 emerges at...
the tips of both the new and the old FAZ filaments. Up to now, only a few proteins have been localized to the FAZ tip, i.e. the Polo-like kinase TbPLK (43, 44) and the chromosomal passenger complex (CPC) consisting of the Aurora B kinase TbAUK1 and two trypanosome-specific chromosomal passengers TbCPC1 and TbCPC2 (19, 20). All of these proteins are concentrated only at the new FAZ tip, and are required for cytokinesis initiation, without any involvement in life cycle transitions. We thus postulate that there are at least two functionally distinct protein groups at the new FAZ tip: regulators of cytokinesis furrow ingression, which include TbPLK and the CPC, and regulators of life cycle form transitions, which include TbSAS-4 (and potentially other unidentified proteins). These two protein groups may reside at distinct locations in the new FAZ tip, and are likely to be distantly separated from each other, because BioID with TbSAS-4 as the bait did not identify TbPLK and CPC subunits as its near neighbors (Table 1).

Our results demonstrated that at the distal tip of the new FAZ filament, TbSAS-4 promotes elongation of the new FAZ filament and, consequently, positions the basal body toward the posterior part of the cell (Figs. 2 and 3). Thus, TbSAS-4 functions to maintain trypomastigote cell morphology. Given that depletion of TbSAS-4 generates epimastigote-like cells (Fig. 2), we speculate that either TbSAS-4 is not expressed or its level is significantly reduced in the epimastigote cells differentiated from trypomastigote cells in the tsetse fly vector. Although the expression levels of TbSAS-4 in the two developmental forms are not known, TbSAS-4 mRNA is among the most highly
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down-regulated transcripts during the differentiation to epimastigote cells by ablation of ALBA3/4 (45), RNA-binding proteins that are also involved in cell morphology transitions (27). Similarly, ClpGM6 mRNA was also down-regulated by depletion of ALBA3/4 (15, 45). These results suggest that life cycle form transitions in T. brucei may be regulated at the posttranscriptional level by RNA-binding protein-mediated modulation of the expression of certain FAZ-associated cytoskeletal proteins, such as ClpGM6 and TbSAS-4.

The epimastigote-like cells generated by depletion of TbSAS-4 (Figs. 2 and 3) appeared to be morphologically different from the epimastigote-like cells generated by knockdown of ClpGM6 and FLAM3, because the latter possesses a shorter cell body and a longer unattached flagellum than the former (15, 29). This distinction is likely attributed to the different length of the FAZ filaments of TbSAS-4 RNAi cells and of ClpGM6 and FLAM3 RNAi cells, as the length of the new FAZ filament defines the cytokinesis cleavage plane and, therefore, determines the size of the new flagellum daughter cells (12). Indeed, the new FAZ filament in TbSAS-4 RNAi cells (2N2K) appeared to be longer than that in ClpGM6 RNAi cells and in FLAM3 RNAi cells. Nevertheless, our results suggest that life cycle form transitions in T. brucei require the FAZ tip-localizing TbSAS-4, in addition to ClpGM6 and FLAM3, both of which are localized along the length of the FAZ structure inside the flagellum (15, 29), and FAZ9, which localizes along the length of the FAZ filament inside the cell body (30). It should be noted that FAZ9 was identified in TbSAS-4 BioID (Table 1), suggesting that TbSAS-4 and FAZ9 may cooperate to control life cycle transitions.

Given that all of the SAS-4 homologs characterized in other model systems were found to play an essential role in centriole or basal body duplication (46), the finding that TbSAS-4 controls trypanosome life cycle form transitions is very surprising. The basal body in T. brucei shares similar architecture as the centriole in metazoa and the basal body in other flagellated and ciliated organisms, and contains the SAS-6 protein that forms the cartwheel structure (10). Additionally, SAS-4 appears to constitute one of the three factors that are part of the ancestral module in centriole/basal body assembly (1). In this regard, it is puzzling why TbSAS-4 is not recruited for basal body biogenesis in T. brucei. It also raises an interesting question of how TbSAS-4 has evolved this novel function in controlling life cycle transitions. One may postulate that in the last common eukaryotic ancestor, SAS-4 was not recruited to the centriole/basal body, but was later acquired to control the elongation of the centriole/basal body microtubules, because it possesses microtubule binding activity (47). Paramecium tetraurelia, a ciliated protozoan that branched after T. brucei from the last common eukaryotic ancestor, contains a SAS-4 homolog that is essential for basal body duplication (48). However, Giardia lamblia, one of the earliest diverging eukaryotes following the last common eukaryotic ancestor (49), contains a conserved SAS-4 homolog that is enriched in the basal body, although its function is not known (50). Therefore, it is highly likely that the T. brucei SAS-4 homolog has evolved a novel function. Given that T. cruzi and Leishmania also undergo similar life cycle transitions as T. brucei, it would be interesting to investigate whether the SAS-4 homolog in these two organisms plays a similar role as TbSAS-4 in T. brucei.

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