Introduction

Kinesins are usually plus end–directed microtubule motors that move vesicles, organelles, or chromosomes (Sharp et al., 2000; Hirokawa and Noda, 2008). Members of this superfamily are defined by a conserved motor domain that binds to microtubules and transforms the chemical energy of nucleotide triphosphate into mechanical force, resulting in motility. Kinesins have been grouped into families depending on the position of their motor domain, the type and number of subunits composing their active form, and their motility. Recent classifications of superfamilies are based on the large datasets available from genome projects (Miki et al., 2005; Wickstead and Gull, 2006).

Cilia and flagella perform essential functions such as motility, sensing, or morphogenesis. Their conserved architecture is a cylinder of nine doublet microtubules that form the outer circumference of the axoneme. At least five kinesin superfamilies are limited to flagellated species (kinesin 2, 9, 13, and probably 16 and 17). Kinesin 2 and 13 participate in flagellum formation by controlling intraflagellar transport (IFT) and microtubule depolymerization (Scholey, 2008). KLP1 (kinesin–like protein 1) is the founding member of the kinesin 9 family (KIF9) that is characterized by a specific neck domain, which is downstream from the catalytic core domain (Miki et al., 2005). First described in the green algae *Chlamydomonas reinhardtii*, CrKLP1 (*C. reinhardtii* KLP1) is localized to the central pair of singlet microtubules within the axoneme (Bernstein et al., 1994) and is involved in motility, possibly by regulating flagellar dynein activity (Yokoyama et al., 2004).

Trypanosoma brucei, the protist that causes sleeping sickness, is an amenable model to study the formation and function of flagella (Ralston and Hill, 2008). Its motile flagellum contains a classically structured “9 + 2” axoneme and is attached along the cell body for most of its length. It also possesses a paraflagellar rod (PFR), which is a large, lattice-like structure that runs parallel to the axoneme from where it emerges from the cell body to the distal tip. The PFR is composed of two major proteins, PFR1 and PFR2, and contains at least 20 other proteins (Portman et al., 2009). Association of the PFR within the flagellum is required for cell motility (Bastin et al., 1998).
demonstrated the existence of two subfamilies of KIF9 in all flagellated species analyzed (Fig. 1 A). The KIF9A family includes CrKLP1 and human KIF9, whereas the KIF9B family includes the so-called KIF6 human protein. Nevertheless, the kinesin 9 gene family was clearly separate from the kinesin 6 family (Fig. 1 A). Trypanosome KIF9A and KIF9B possess the typical kinesin motor domain and ATP-binding domain signatures (P-loop, Switch1, and Switch2). Trypanosome KIF9A is characterized by a unique 35–amino acid insertion in its N-terminal domain, whereas KIF9B is marked by at least seven insertions in its C-terminal domain (Fig. S1 A).

To determine the cellular location of KIF9A and KIF9B, a fragment of each of the divergent C-terminal domains (KIF9A, amino acids 479–891; KIF9B, amino acids 490–1,041; Fig. S1 A) was expressed as His-tagged fusion proteins in Escherichia coli and used to immunize mice. The resulting polyclonal sera recognized a single band on trypanosome whole cell extracts, 100 kD for KIF9A (calculated 96.9 kD) and 115 kD for KIF9B (calculated 113.3 kD), which is in contrast to preimmune sera that gave no detectable signal (Fig. 1 B and Fig. S1 B). Both proteins were tightly associated with the cytoskeletal fraction, as shown after removal of the membrane by detergent treatment (Fig. 1 C). This is in agreement with the presence of KIF9A in a proteomic analysis of the structural components and is essential for survival at the bloodstream stage of the parasite (Branche et al., 2006; Broadhead et al., 2006).

In this study, we demonstrate that the two members of the T. brucei kinesin 9 family, KIF9A and KIF9B, are strongly associated with the flagellar skeleton and participate in flagellar motility. However, their individual contributions are distinct because only inhibition of KIF9B effects construction of the PFR, thus revealing the first kinesin involved in the formation of an extra-axonemal structure.

Results and discussion

Trypanosome KIF9 proteins display different characteristics and locations

Searching the T. brucei genome database (http://www.genedb.org/genedb/tryp/blast.jsp) with the CrKLP1 protein sequence (P46870) identified two candidate members for the KIF9 family, which were termed KIF9A (NCBI Protein Database accession no. XP_846252) and KIF9B (NCBI Protein Database accession no. XP_846346). Reciprocal Blastp analysis showed that both KIF9A and KIF9B sequences recognized the CrKLP1 (expectancy [e]: KIF9A-CrKLP1 = 9 e − 66; KIF9B-CrKLP1 = 9 e − 67) as well as members of the KIF9 family from numerous flagellated species. Phylogenetic analyses demonstrated the existence of two subfamilies of KIF9 in all flagellated species analyzed (Fig. 1 A). The KIF9A family includes CrKLP1 and human KIF9, whereas the KIF9B family includes the so-called KIF6 human protein. Nevertheless, the kinesin 9 gene family was clearly separate from the kinesin 6 family (Fig. 1 A). Trypanosome KIF9A and KIF9B possess the typical kinesin motor domain and ATP-binding domain signatures (P-loop, Switch1, and Switch2). Trypanosome KIF9A is characterized by a unique 35–amino acid insertion in its N-terminal domain, whereas KIF9B is marked by at least seven insertions in its C-terminal domain (Fig. S1 A).

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of flagella purified upon detergent and high salt treatment (Broadhead et al., 2006).

Indirect immunofluorescence assays (IFAs) of detergent-extracted cytoskeletons revealed the presence of KIF9A and KIF9B on the flagellum, but KIF9B also localized to the basal body (BB; Fig. 1, D and E; and Fig. S1 G). The preimmune sera did not produce any signal (unpublished data). These results were confirmed by IFA on nonextracted cells using KIF9A (Fig. S1 C) or in live cells by expression of a GFP::KIF9B fusion protein (Fig. S1 H). Double staining with L8C4 (PFR marker; Kohl et al., 1999) or MAb25 (axoneme marker; Pradel et al., 2006) showed that both KIF9A and KIF9B are associated with the axoneme and not the PFR. The initiation of PFR construction happens only after the flagellum has emerged from the cell body (Fig. S1, D, E, I, and J). Double IFA with BB markers showed that KIF9A staining was absent from the BB area in contrast to KIF9B (Fig. S1, F and K). Unfortunately, immunogold electron microscopy using antibodies against KIF9A, KIF9B, or GFP in the case of GFP::KIF9 failed to produce a significant signal, whereas a clear positive signal was obtained for the control L3B2 monoclonal antibody (Kohl et al., 1999).

The axonemal localization and the association with the cytoskeleton are similar to CrKLP1 (Bernstein et al., 1994), but KIF9B localization at the BB was more surprising. Dual localization to the axoneme and BB is typical for kinesin 2 and IFT proteins, but localization at the BB was more surprising. Dual localization to the axoneme and BB is typical for kinesin 2 and IFT proteins, but localization at the BB was more surprising.

Silencing of KIF9A and KIF9B causes different motility defects

Plasmids allowing tetracycline-inducible expression of KIF9A or KIF9B double-stranded RNA were used to generate KIF9ARNAi or KIF9BRNAi cell lines. Western blotting demonstrated knockdown efficiency in both cases as well as absence of cross RNAi (Fig. 2 A; and Fig. S2, A and D). Observation of live KIF9ARNAi cells revealed a significant reduction in flagellum and cell movement, resulting in cell sedimentation (Fig. 2 B and Video 2), which is in agreement with the proposed function for KLP1 in Chlamydomonas (Yokoyama et al., 2004). The motility phenotype was more pronounced in KIF9BRNAi cells, leading to cell paralysis (Fig. 2 B and Video 3) and growth arrest (Fig. 2 C) in contrast to KIF9ARNAi. This growth phenotype could be a result of extremely reduced motility, as it has been shown that motility contributes to the completion of cytokinesis (Branche et al., 2006; Broadhead et al., 2006; Ralston et al., 2006). Because kinetics of RNAi can be variable from one target to another, KIF9ARNAi cells were induced for up to 8 d, but this did not modify the intensity of the motility phenotype (Fig. S2, B and C). IFA with the anti-KIF9A antibody showed the same signal pattern as KIF9ARNAi cells (compare Figs. S2, B and C).

Electron microscopy analysis of cross sections of KIF9ARNAi flagella failed to show any striking modifications: the nine doublets, the central pair, the outer or inner dynein arms, and the PFR all appear normal, as was the orientation of the central pair relative to the PFR (Fig. 3, C and D), which is a common feature of many motility mutants in trypanosomes (Branche et al., 2006; Gadella et al., 2006; Ralston et al., 2006). KIF9A could be affected in mutants lacking some of the flagellar substructures. Therefore, we examined the abundance and location of KIF9A by Western blotting (Fig. 3 E) or IFA (not depicted) in various RNAi mutants where dynein arm (DNAI1RNAi or ODA7RNAi) or central pair (PF16RNAi) components were affected. No significant modifications were observed.

In Western blots, the MAb25 signal, an axoneme marker, remained unchanged in both KIF9ARNAi and KIF9BRNAi cells. However, an unexpected decrease in the amount of PFR2 was...
observed in KIF9B\textsuperscript{RNAi} induced cells (Fig. 2 A). By IFA, MAb25 showed the expected labeling from the proximal to the distal tip of the flagellum in both cases. L8C4 showed a uniform labeling of the flagellum on KIF9A\textsuperscript{RNAi} cells (Fig. 2 D) but not in the KIF9B\textsuperscript{RNAi} mutants where it produced a discontinuous signal, alternating regions of intense labeling with regions where the signal was negative (Fig. 2 E). This observation was reproduced using antibodies that recognize both PFR1 and PFR2 proteins (Fig. S3 A) or a component of the distal PFR domain (Fig. S3 B), indicating global defects in PFR organization.

KIF9B\textsuperscript{RNAi} cells fail to assemble a normal PFR

When a new flagellum is built, the PFR is only added to the axoneme as the flagellum exits the flagellar pocket and the two structures are built almost simultaneously (Sherwin and Gull, 1989). The PFR abnormalities in KIF9B\textsuperscript{RNAi} cells could result from either its partial assembly or postassembly destabilization. Cells were carefully analyzed at early time points of RNAi induction, revealing that only the new flagellum showed discontinuous labeling, whereas the old flagellum was normal (Fig. 4 A and Fig. S2 F). We quantified the emergence of the PFR-related phenotype in cells with either one or two flagella at various time points after RNAi-mediated knockdown (n > 100). In cells with one flagellum, an irregular PFR labeling was first detected after 24 h. The abundance of these cells then increased steadily to reach a plateau at ~80% (Fig. 4 B). In billagellated cells (Fig. 4 C), the proportion of cells with both new and old normal PFR labeling decreased rapidly: they were replaced by siblings with an abnormal new flagellum (up to 42% at 48 h). After 24 h, most cells possessed two flagella that were both affected. This can be explained if one daughter cell inherits the flagellum with a normal PFR structure at cytokinesis, and the other daughter exhibits the abnormal PFR. Further division of the cell with abnormal PFRs results in irregular labeling of both the old and new flagellum.

PFR structure in KIF9B\textsuperscript{RNAi} cells

Although both old and new flagellum of wild-type (WT) trypanosomes have a constant diameter of ~300 nm all along their length, induced KIF9B\textsuperscript{RNAi} cells exhibit a highly variable diameter (150–450 nm) along the length of the flagellum (Fig. S3, D and E). To better visualize flagellar structure, cells were treated with cold triton to remove the pellicular and flagellar membrane (Absalon et al., 2008b). In WT trypanosomes, the characteristic lattice-like PFR structure connected to the axoneme is clearly visible with a constant diameter of ~300 nm (Fig. 4 D). In contrast, the flagellum of induced KIF9B\textsuperscript{RNAi} cells shows regions with one or more PFR-like structures (Fig. 4 E, arrows), alternating with portions containing only the axoneme (Fig. 4 E, arrowhead). The locally excessive PFR material is still connected to the axoneme but is not resistant to extraction with 1M NaCl (Fig. S3, F and G), indicating that these segments are not as stably connected to the axoneme as in WT cells.

In transmission electron microscopy of WT cells, the PFR has a diameter of ~150–200 nm and is anchored to the axoneme via connections to doublets 4–7 (Fig. 4 F, asterisks). In KIF9B\textsuperscript{RNAi} cells, several situations are encountered: a naked axoneme (36%), one PFR-like structure (15%), or two or more PFR-like structures (49%; n = 89). Several connections were visible between the multiple PFR-like structures and the axoneme (Fig. 4, G–I). The axoneme appeared normal, with the 9+2 organization and the dynein arms, but the orientation of the central pair could not be determined as a result of the presence of multiple PFR-like structures. In addition to these structural modifications, induced KIF9B\textsuperscript{RNAi} trypanosomes display a flagellum that is partially detached from the cell body (Fig. S3, H and I), which is in contrast to KIF9A\textsuperscript{RNAi} cells. The flagellum of cells with normal PFR labeling was always attached, whereas ~35% of cells with interrupted PFR labeling exhibited a detached flagellum (Fig. S3 J). This could be a consequence of the motility defect as described in other mutants with decreased flagellar beating (Absalon et al., 2007; Li and Wang, 2008).

Separate functions for KIF9A and KIF9B in flagellum beating and construction

Our data demonstrate that the two kinesin 9 proteins are performing distinct functions in the trypanosome flagellum. KIF9A is required for motility but does not play a role in PFR assembly. In C. reinhardtii, CrKLP1 regulates flagellar dynein activity (Yokoyama et al., 2004) and interacts with Hydin, a central pair protein essential for motility in algae (Lechtreck and Witman, 2007) and trypanosomes (Dawe et al., 2007). It has been shown recently that Hydin is also essential for ciliary motility and has a similar effect on central pair structure in mice (Lechtreck et al., 2008). In contrast to algae or ciliates, the central pair does not twist during beating in trypanosomes (as in humans), and this functional difference could be associated with discrete structural variations of the axoneme.
KIF9B displays a conserved motor domain in its N-terminal region and a divergent C-terminal region with multiple insertions. We postulate that these insertions could be interacting either directly with PFR proteins or with proteins ensuring the junction between axoneme and PFR. During PFR assembly, PFR1 and PFR2 subunits are actively transported to their assembly site at the distal tip of the growing flagellum (Bastin et al., 1999a,b). This transport does not require pressembly, as shown in the mutant snl-1, where in the absence of PFR2, PFR1 is still transported to the distal tip (Bastin et al., 1999a). This transport could be ensured by the IFT machinery as reported for radial spokes or dynein arms in the C. reinhardtii axoneme (Qin et al., 2004). This is supported by the absence of a PFR in mutants where IFT has been abolished (Kohl et al., 2003). In this case, KIF9B would be deposited on the growing axoneme by the IFT machinery and would serve as a linker protein with the growing PFR structure or in the incorporation of PFR proteins in the PFR structure. However, the PFR is added to the axoneme only after the emergence of the flagellum from the flagellar pocket, i.e., when the axoneme is already 2–µm long. Therefore, the absence of PFR in IFT mutants could be caused by the lack of an axoneme that is needed as support for the PFR.

The intriguing localization of KIF9B to the BB region could also be significant for the role of this protein in flagellar assembly and motility. BBs appear ninefold symmetric but have asymmetric accessory structures. KIF9B could be important in either recognizing or determining this asymmetry, thereby allowing proper PFR assembly at defined microtubule doublets 4–7, whereas its absence would lead to patches of PFR that assemble with random radial orientation.

**Figure 4.** Characterization of the KIF9BRNAi mutant. KIF9B is involved in PFR assembly. (A) IFA on 48 h–induced KIF9BRNAi cells. (left) Cell with one nucleus, an old flagellum (OF), and a short new flagellum (NF) are shown. The old flagellum shows a normal axoneme/PFR labeling, whereas the new flagellum shows a normal axoneme but a disrupted PFR. (right) Cells with two nuclei and two flagella exhibit the same phenotype. Detached flagella are shown by arrows. The experiment was reproduced more than three times, and at least 100 biflagellated cells were analyzed. (B and C) KIF9BRNAi cells with a normal PFR labeling (black lines) are progressively replaced by cells with an interrupted labeling (red lines) both in uniflagellated (B) and biflagellated cells (C). In biflagellated cells, the phenotype is first visible in the new flagellum (purple line). For both populations, at least 100 cells were analyzed per induction time. (D and E) Scanning electron micrograph of a WT cell (D) and a 72 h–induced KIF9BRNAi cell (E) extracted with cold Triton X-100. The white rectangle indicates the position of the magnified area. Representative images were chosen from at least 20 cells. (F–I) Transmission electron micrographs showing cross sections of flagella from WT (F) or 72 h–induced KIF9BRNAi cells (G–I). Representative images were chosen from >50 sections of WT and 89 sections of KIF9BRNAi. Asterisks indicate doublets 4–7, arrows point toward excessive PFR, and arrowheads show a naked axoneme.
KIF9B could be involved in a specific machinery constructing the PFR, associating with kinesin 2, like kinesin 2 and osm-3 do in sensory cilia of Caenorhabditis elegans (Snow et al., 2004). KIF9B could transport PFR and associated proteins to the tip for assembly, allowing coordinated elongation of PFR and axoneme. In the absence of KIF9B, kinesin 2 still drives IFT and axoneme formation, whereas PFR elongation fails with proteins being dropped along the way. However, no movement of KIF9B could be detected in live cells, and the protein is tightly attached to the axoneme. Using its polar movement, KIF9B could translocate PFR material from one KIF9B molecule to its neighbor toward the distal tip. The extremely divergent C-terminal domain of KIF9B could be used as an adapter to interact with different partners according to the cell type where KIF9B is expressed. In mammals, KIF9A and KIF9B are expressed in a variety of tissues, such as testis, brain, and lung, but their function is unknown. Expression in testis could be related to the presence of extraxonemal structures in the flagella of spermatozoa (Escalier, 2003), and investigation of their function in the construction of these elements holds promises. In conclusion, this work illustrates the diversification of the KIF9 family with contribution at the axoneme and the PFR level. Although sharing the motor domain, they have diversified their C-terminal tail to suit specific cytoskeletal requirements.

Materials and methods

Trypanosome cell lines and cultures
All cell lines used for this study were derivatives of strain 427 of Trypanosome cell lines and cultures. Cell lines KIF9BRNAi and KIF9A were expressed in P. falciparum and P. berghei, respectively. DNA was amplified using the Superscript OneStep RT-PCR (Invitrogen) enzyme mix. Quantification was performed from ethidium bromide gels using the Image Quant software (version 5.2; Molecular Dynamics).

Immunofluorescence
Trypanosomes were settled onto poly-L-lysine–coated slides, and cytoskeletons were prepared by extraction with 1% NP-40 in 100 mM Pipes, pH 6.9, 2 mM EGTA, 1 mM MgSO4, and 0.1 mM EDTA. After fixation in methanol at −20°C for 5 min followed by rehydration (PBS for 10 min), the slides were incubated with primary antibodies. As PFR markers, we used the monoclonal antibodies LBC4 (Kohl et al., 1999), L13D6 (Kohl et al., 1999), 2E10 (Ismach et al., 1989), and ROD-1 (Woods et al., 1989). Monoclonal antibodies MAb25 and MAb22 were used as markers for the axoneme (MaB25; Pradel et al., 2000) and the BB (MaB22; Bonhivers et al., 2008).

Subcellular-specific antibodies coupled to Alexa Fluor 488 (Invitrogen), Cy3 (Jackson ImmunoResearch Laboratories, Inc.), or Cy5 (Jackson ImmunoResearch Laboratories, Inc.) were used for double labeling. Slides were stained with DAPI for visualization of kinetoplast and nuclear DNA content and mounted with antifade Vectashield medium (Vector Laboratories).

IFA was performed as described previously (Abasolun et al., 2007). The primary antibodies LBC4 (Kohl et al., 1999), MAb25 (Pradel et al., 2006), L13D6 (Kohl et al., 1999), 2E10 (Ismach et al., 1989), and ROD-1 (Woods et al., 1989) were used.

For IFA with the anti-KIF9A and the anti-KIF9B antibodies, cells were treated with 1% Triton X-100 for 10 min at 4°C and fixed for 15 min at room temperature in 4% paraformaldehyde and 0.1% glutaraldehyde. After blocking by incubation for 45–60 min in PBS containing 1% BSA, the slides were incubated for 45–60 min with the antisera diluted 1:200 in PBS/0.1% BSA and treated as described previously (Abasolun et al., 2007). Slides were mounted in Vectashield medium.

Slides were observed at room temperature with a microscope (DMR; Leica) using a 100x NA 1.3 Plan Fluor objective, and images were captured with a camera (Cool Snap HQ; Roper Industries). Alternatively, slides were viewed on a microscope (DM14000; Leica) using a 100x NA 1.4 Plan Apo objective, and images were acquired with a camera (Retiga-SRV; Q-Imaging). Images were analyzed using the IPLab Spectrum software (version 3.9; BD) or ImageJ (National Institutes of Health) and processed with Photoshop (CS2; Adobe). For live cell imaging, cells were filmed at room temperature on a microscope (DM14000) using the aforementioned settings.

Electron microscopy
For transmission electron microscopy, WT and induced KIF9BRNAi samples were washed in 0.1 M cacodylate buffer, pH 7.2, and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.5% tannic acid solution for 2 h at room temperature. Fixed samples were washed three times in 0.1 M cacodylate buffer, pH 7.2, and postfixed in 1% OsO4 in 0.1 M sodium cacodylate solution, pH 7.2, for 2 h in the dark under agitation. Samples were stained en block with 2% aqueous uranyl solution at 4°C overnight, gradually dehydrated in ethanol, and embedded in Spurr resin followed by polymerization for 3 d at 70°C. For detergent extraction of cytoskeletons, cells were treated with 100 mM Pipes, 2 mM EGTA, and 1 mM MgSO4 containing 1% Nonidet P40 solution for 10 min (Sherwin and Gil, 1989). Washed samples were fixed in 2.5% glutaraldehyde, 0.5% tannic acid solution for 2 h at room temperature, and processed as described for whole cells (see previous paragraph). 60-nm-thick sections were cut with an Ultracut E microtome (Reichert S; Leica) and picked up on copper rhodium–coated grids. Grids were analyzed on an electron microscope (JEM 1010; Jeol), and images were captured with a digital camera (C4742-95; Hamamatsu Photonics). Fixation, embedding, and sectioning for transmission electron microscopy of whole cells from WT and induced KIF9BRNAi samples were performed as described previously (Branche et al., 2006).

For scanning electron microscopy, samples were washed in PBS, fixed for at least 1 h with 2.5% glutaraldehyde in PBS or 0.2 M cacodylate buffer, washed, and postfixed in 1% osmium tetroxide. After dehydration, samples were critical point dried (K550; Emitech) or Union-QCD30 (Balzers) and coated with gold (JFC-1200; Jeol) or Ion Beam Coater 681 [Gatan]). Samples were visualized with a scanning microscope (JMM-700 F; Jeol). For detergent-extracted cytoskeleton preparations, cells were treated with 1% Triton X-100 at 4°C in PBS for 10 min to strip the plasma membrane. After two washes in PBS, the samples were processed for scanning electron microscopy as described for whole cells (see previous paragraph).
Cell fractionation

5 x 10^7 cells were harvested by centrifugation and washed once in PBS. The pellet was resuspended in 100 mM Pipes, 2 mM EGTA, and 1 mM MgSO4 containing 1% Nonidet P40 and incubated for 2 min at room temperature to provide a cytoskeleton preparation (Sherwin and Gull, 1989). After centrifugation (at 1,000 g for 5 min), the pellet was resuspended in either 60 mM CaCl2 or 1 M NaCl to depolymerize the subpellicular microtubules, which were incubated for 15 min at room temperature and centrifuged (Kohl et al., 1999). The pellet was resuspended in PBS. Equal cell equivalents of each fraction were resolved by SDS-PAGE and subjected to Western blot analysis.

Western blotting

1–2 x 10^7 cells were loaded per well on 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Hybond ECL Plus; GE Healthcare) and after blocking with 1% TBS-BSA, were incubated with anti-KIF9B antibody (1:500), anti-KIF9A antibody (1:500), L8C4 (1:50), or MAb25 (1:50). The secondary antibody used was an anti-mouse HRP (1:5,000; GE Healthcare).

Multiple alignment and tree inference of kinesin 9 family sequences

The kinesin multiple alignment made by Wickstead and Gull (2006) was updated with new sequences of kinesin 9 family members: Trypanosoma cruzi [NCBI Protein Database accession no. XP_810642.1 and XP_808244.1], Micromonas pusilla [NCBI Protein Database accession no. ACA632662], Mus musculus [NCBI Protein Database accession nos. AA115766, NP_799602.6, and NP_032472], Leishmania major [NCBI Protein Database accession no. XP_001682276], and Homo sapiens [NCBI Protein Database accession no. NP_651938].

All new sequences were incorporated and aligned using the ED program of the package MUST (Management Utilities for Sequences and Trees; Philippe, 1993). Ambiguously aligned regions and gaps were excluded in phylogenetic analyses. Maximum likelihood phylogenetic analyses were performed with the program Treefinder (Jobb et al., 2004), and included in phylogenetic analyses. Maximum likelihood phylogenetic analyses were performed with the program Treefinder (Jobb et al., 2004), and included in phylogenetic analyses. Maximum likelihood phylogenetic analyses were performed with the program Treefinder (Jobb et al., 2004), and included in phylogenetic analyses. Maximum likelihood phylogenetic analyses were performed with the program Treefinder (Jobb et al., 2004), and included in phylogenetic analyses. Maximum likelihood phylogenetic analyses were performed with the program Treefinder (Jobb et al., 2004), and included in phylogenetic analyses.

Online supplemental material

Fig. S1 shows the characterization of the kinesin 9 proteins, Fig. S2 shows Online supplemental material in substitution rate approximated to four discrete categories. bootstrap values were calculated from 1,000 replicates. The Whelan and Goldman (2001) substitution matrix was used with a γ-distributed variation in substitution rate approximated to four discrete categories.

References


Figure S1. Characterization of kinesin 9 proteins. (A) Trypanosome KIF9 proteins display a conserved motor domain as well as specific insertions. Schematic drawing of kinesin 9 proteins from Cr. reinhardtii (CrKLP1; Protein Data Bank accession no. 1BCL1), trypanosome KIF9A (TbKIF9A; GeneID accession no. Tb927.7.6290) and KIF9B (TbKIF9B; GeneID accession no. Tb927.7.7260), and human KIF9 (HsKIF9; NCBI Protein Database accession no. NP_071737) and KIF6 (HsKIF6; NCBI Protein Database accession no. NP_659464). Positions of important residues for kinesin motor activity, P-loop (P; green), Switch1 (S1; blue), and Switch2 (S2; blue), are indicated in each sequence. Trypanosome-specific insertions are shown in red and are indicated by asterisks. The fragments used for antibody production are shown in purple underneath the corresponding proteins. (B) Western blot on T. brucei whole cell extracts (10^7 cells/lane) probed with preimmune sera (Pre), anti-KIF9A (left; 1:500), or anti-KIF9B (right; 1:500) antisera. (C–F) Localization of KIF9A in the flagellar axoneme. Values on blots are given in kilodaltons. (C) IFA staining on WT trypanosomes with the anti-KIF9A antibody (1:400). Combined phase-contrast and DAPI image (left), IFA with anti-KIF9A (middle), and combined anti-KIF9A and DAPI staining (right) are shown. Insets show magnification of the BB region. The arrows indicate the beginning of the PFR labeling. (D–F) IFA staining on WT detergent-extracted trypanosomes combining the anti-KIF9A antibody (1:400) with the PFR marker L8C4 (D), the axoneme marker MAb25 (E), or the BB region marker anticentrin (F; 1:3,000). Insets show magnification of the BB region (200%). The arrows indicate the beginning of the PFR labeling. (G–I) Localization of KIF9B in the flagellar axoneme and the BBs. (G) Localization of KIF9B during the trypanosome cell cycle. IFA staining on WT detergent-extracted trypanosomes at different phases of the cell cycle. Combined phase-contrast and DAPI images (top) and IFA with anti-KIF9B (bottom) are shown. Insets show magnification of the BB region. The anti-KIF9B clearly labels both pro- and mature BB (two to four spots visible in the inset, depending on the stage in the cell cycle) in both new (NF) and old (OF) flagellum. (H) Localization of GFP::KIF9B in live cells shows staining in the flagellum and both BB (arrows) as well as cytoplasmic staining. (I–K) IFA staining on GFP::KIF9 detergent-extracted cells combining the anti-GFP antibody with the PFR marker L8C4 (I), the axoneme marker MAb25 (J), or the BB marker MAb22 (K). Insets show magnification of the BB region (200%). The arrows indicate the beginning of the PFR labeling.
Figure S2. Efficiency of RNAi in the KIF9RNAi mutants. (A–C) Efficiency of RNAi silencing in the KIF9A RNAi mutant. (A) Knockdown of KIF9A does not affect the expression of KIF9B. Western blot on T. brucei whole cell extracts (5 x 10^6 cells/lane) probed with anti-KIF9A antibody (1:500) or with the antialdolase antibody (1:3,000) as loading control. (B and C) Longer induction does not increase the efficiency of KIF9A knockdown. (B) Western blot on T. brucei whole cell extracts (5 x 10^6 cells/lane) induced over a 2-, 4-, 6-, and 8-d period probed with the anti-KIF9A antibody (1:500). The white line indicates that intervening lanes have been spliced out. (C) Sedimentation assay. Trypanosomes were incubated in cuvettes, and optical density was measured before and after mixing. Cell lines KIF9A RNAi and KIF9B RNAi were induced for 4 d (KIF9A RNAi, dark blue), for 8 d (KIF9A RNAi, light blue), or for 3 d (KIF9B RNAi, black). (D–F) Efficiency of RNAi silencing in the KIF9B RNAi mutant. (D) Knockdown of KIF9B does not affect the expression of KIF9A. Western blot on T. brucei whole cell extracts (10^7 cells/lane) probed with anti-KIF9B antibody (1:500) or with the antialdolase antibody (1:3,000) as loading control. (E) The GFP::KIF9 fusion protein is correctly expressed in the WT and KIF9B RNAi cell lines and is absent in the 3 d–induced KIF9B RNAi cells. T. brucei whole cell extracts (10^7 cells/lane) were probed with anti-KIF9B (1:500) or antialdolase (1:3,000). (F) Absence of PFR2 is correlated to a reduction in KIF9B at the individual cell level as shown by IFA staining on GFP::KIF9-expressing KIF9B RNAi detergent-extracted trypanosomes double labeled with the anti-KIF9B antibody (1:400) and the anti-PFR antibody L8C4. Values on blots are given in kilodaltons.
Figure S3. Analysis of the KIF9B<sup>RNAi</sup> mutant. (A and B) Knockdown of KIF9B shows the same phenotype using different PFR markers labeling with ROD-1 (A) and 2E10 (B). Combined phase/DAPI images (left) and anti-PFR antibodies (right) are shown. The signal observed with these antibodies corresponds to the one seen with L8C4. (C and D) Scanning electron microscopy analysis of WT (C) and 72 h–induced KIF9B<sup>RNAi</sup> cells (D). A cell with two flagella is shown in both panels. In induced cells, thin regions (arrowhead) alternate with thicker regions (arrows). (E) The diameter of the flagellum varies in KIF9B<sup>RNAi</sup>-induced cells. The diameter of the flagellum was measured on scanning electron microscopy images of 75 cells either in WT or 72 h–induced KIF9B<sup>RNAi</sup> cells. The results were divided into three classes: diameters from 150 to 250, from 250 to 350, and from 350 to 450 nm. In noninduced cultures, in the large majority, the flagellar diameter is between 250 to 350 nm. In contrast, it varies enormously (from 150 to 450 nm) in induced cells. (F and G) Evaluation of the strength of the attachment of the PFR-like structure to the axoneme upon 1 M NaCl, a treatment that depolymerizes the corset microtubules but does not affect the flagellar microtubules. (F) In WT trypanosomes, the PFR stays connected to the axoneme after treatment with NaCl. (G) In contrast, the PFR-like structures of 72 h–induced KIF9B<sup>RNAi</sup> cells are detached by NaCl treatment, showing reduced stability. (H) Merged image showing phase contrast, DAPI (white), and double-IFA staining of 72 h–induced KIF9B<sup>RNAi</sup> cells with MAb25 (red) and L8C4 (green). Arrows indicate detached flagella. (I) Scanning electron microscopy of a 72 h–induced KIF9B<sup>RNAi</sup> cell showing a detached new flagellum (NF) with an irregular diameter (arrow) and an attached old flagellum (OF) with a regular diameter. (J) Detachment of the flagellum in the course of induction of KIF9B<sup>RNAi</sup> cells.

Video 1. <i>T. brucei</i> expressing GFP::KIF9B. The fluorescence signal for GFP-tagged KIF9B is present in the area near the BB and in the flagellum where no clear movement could be detected.
Video 2. **Knockdown of KIF9A in T. brucei reduces flagellar beating and cell motility.** *KIF9A<sup>RNAi</sup>* cells were induced for 4 d. Sequences show different individual cells with clearly reduced motility and evidence for apparently normal cell division despite reduced movement.

Video 3. **Knockdown of KIF9B in T. brucei dramatically affects flagellar beating and cell behavior.** The first sequence shows noninduced cells that swim rapidly, as expected. The following sequences show *KIF9B<sup>RNAi</sup>* cells induced for 3 d. Two individual cells with a detached flagellum and one individual cell with an attached flagellum all show severe reduction in flagellar beating. This motility phenotype results in failure to complete cytokinesis (last sequence).