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TbPIF8, a *Trypanosoma brucei* protein related to the yeast Pif1 helicase, is essential for cell viability and mitochondrial genome maintenance

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Summary

The trypanosome mitochondrial genome, kinetoplast DNA (kDNA), is a massive network of interlocked DNA rings, including several thousand minicircles and dozens of maxicircles. The unusual complexity of kDNA would indicate that numerous proteins must be involved in its condensation, replication, segregation and gene expression. During our investigation of trypanosome mitochondrial PIF1-like helicases, we found that TbPIF8 is the smallest and most divergent. It lacks some conserved helicase domains, thus implying that unlike other mitochondrial PIF1-like helicases, this protein may have no enzymatic activity. TbPIF8 is positioned on the distal face of kDNA disk and its localization patterns vary with different kDNA replication stages. Stem-loop RNAi of TbPIF8 arrests cell growth and causes defects in kDNA segregation. RNAi of TbPIF8 causes only limited kDNA shrinkage but the networks become disorganized. Electron microscopy of thin sections of TbPIF8-depleted cells shows heterogeneous electron densities in the kinetoplast disk. Although we do not yet know its exact function, we conclude that TbPIF8 is essential for cell viability and is important for maintenance of kDNA.

Keywords

trypanosomes; DNA replication; kDNA; DNA helicase; DNA segregation

Introduction

Trypanosoma brucei is an early-branching protozoan parasite that causes human African trypanosomiasis (sleeping sickness) and other diseases in livestock (nagana). Trypanosomes are important because of their pathogenicity, and they also attract interest because of their unusual biological properties. One striking example is their mitochondrial genome, known as kinetoplast DNA (kDNA), which is a massive planar network of interlocked DNA rings [reviewed in (Liu *et al.*, 2005a; Shlomei, 2004)]. These include several thousand minicircles (1 kb and heterogeneous in sequence) and a few dozen maxicircles (23 kb that encode typical mitochondrial gene products). The minicircles encode guide RNAs (usually 3 per minicircle) that serve as templates for RNA editing of maxicircle transcripts. In editing, uridylate residues are inserted into or deleted from specific sites in the coding region. Multiple minicircle classes are required to encode the entire repertoire of essential guide RNAs, thus explaining the minicircle heterogeneity [see (Simpson *et al.*, 2003) for review on editing].

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There is one kDNA network within the matrix of the trypanosome's single mitochondrion. The network is condensed into a disk-shaped nucleoid that is held in position by a transmembrane filament system known as the tripartite attachment complex (TAC). TAC, also connected to the flagellar basal body (outside the mitochondrion), mediates the segregation of daughter kinetoplasts after replication (Robinson and Gull, 1991; Ogbadoyi *et al.*, 2003). In contrast to mitochondrial DNAs in higher eukaryotes that replicate randomly throughout the cell cycle, kDNA replication in trypanosomatids occurs in one part of the cell cycle nearly in synchrony with nuclear S phase [reviewed in (Liu *et al.*, 2005a; Shlomai, 2004)].

As befitting its structure, the kDNA replication mechanism is complex [reviewed in (Liu *et al.*, 2005a; Shlomai, 2004)]. For minicircles, the replication starts with a topoisomerase II (Topo II)-mediated release of monomeric, covalently closed minicircles from the kDNA network into the kinetoflagellar zone (KFZ) (Drew and Englund, 2001). The KFZ is a specialized region of the mitochondrial matrix between the kDNA disk and the flagellar basal body. These free minicircles then replicate unidirectionally as theta structures and probably segregate within the KFZ (several proteins involved in replication are known to be in the KFZ) (Klingbeil *et al.*, 2002; Abu-Elneel *et al.*, 2001). The minicircle progeny then migrate to the antipodal sites, two protein assemblies positioned on opposite sides of the kDNA disk (Melendy *et al.*, 1988; Ferguson *et al.*, 1992). Here enzymes are present that carry out the final stages of replication (primer removal and filling and closing most but not all gaps) (Engel and Ray, 1999; Liu *et al.*, 2009b; Saxowsky *et al.*, 2003; Downey *et al.*, 2005). Then, a Topo II, also positioned in the antipodal sites, reattaches the gapped circles to the opposite ends of the network adjacent to the antipodal sites (Melendy *et al.*, 1988; Wang and Englund, 2001). As replication continues, the polar regions with newly-replicated gapped minicircles expand and the central region with covalently-closed unreplicated minicircles shrinks. Finally, when all minicircles are replicated, the double-size network splits into two sister networks and then all minicircle gaps are repaired (Hoeijmakers and Weijers, 1980; Ferguson *et al.*, 1994; Robinson and Gull, 1994). At cytokinesis the sisters, each identical to the parent, move into the progeny cells. Compared to minicircles, much less is known about the replication of maxicircles. Like minicircles, the maxicircles replicate unidirectionally via theta intermediates starting at a unique origin. Unlike minicircles, they replicate while attached to the network (Hajduk *et al.*, 1984; Carpenter and Englund, 1995).

In this study, we continue our investigation of trypanosome proteins related to the yeast nuclear/mitochondrial helicase known as ScPif1, the founding member of the Pif1 family. The nuclear isoform of ScPif1 inhibits de novo telomere formation and telomere elongation. Its mitochondrial isoform plays important roles in the maintenance, recombination, and replication of mtDNA [reviewed in (Bochman *et al.*, 2010)]. We previously surveyed the eight PIF1-like genes in the *T. brucei* genome (Liu *et al.*, 2009a). Using ectopic expression of each TbPIF tagged at its C-terminus with GFP, we found that six of the eight helicases are mitochondrial. The others, TbPIF3 and TbPIF6, localize to the cytoplasm and nucleus, respectively. Knockout or RNAi of TbPIF1 (Liu *et al.*, 2010) and TbPIF2 (Liu *et al.*, 2009a) showed that both proteins are essential for trypanosome viability. Overexpression of TbPIF5 (Liu *et al.*, 2009b) reduced cell growth rate after 2–3 days of RNAi. These three proteins are all ATP-dependent DNA helicases as determined by assay of recombinant proteins. However, they function very differently in kDNA replication. TbPIF1, together with mitochondrial Topo II, is needed for segregation of minicircle progeny (Liu *et al.*, 2010), TbPIF5 is involved in primer removal from minicircle Okazaki fragments (Liu *et al.*, 2009b), and TbPIF2 is involved in maxicircle replication (Liu *et al.*, 2009a). Although located in the mitochondrion (Liu *et al.*, 2009a), TbPIF4 and TbPIF7 appear not to be essential for cell viability as both alleles of these genes can be knocked out. In this paper, we

focus for the first time on TbPIF8 (GenBank accession number XP_845724, GeneDB accession number Tb927.7.1000).

In comparison with other TbPIFs (88 kDa to 115 kDa), TbPIF8 is the smallest (78 kDa) and most divergent (Fig. S1) (Liu *et al.*, 2009a). As we will mention in the Discussion, the sequence of TbPIF8 has features that make it highly unlikely to have helicase activity. RNAi knockdown of TbPIF8 using the pZJM vector revealed no effect on viability, although we could not recover viable cells after the deletion of both alleles (Liu *et al.*, 2009a). The latter finding raised the possibility that TbPIF8 may be essential. Here we report that stem-loop RNAi of TbPIF8 arrested cell growth as early as day 3 after induction. Although TbPIF8 RNAi caused only limited shrinkage or loss of the kinetoplast, several studies showed that depletion of TbPIF8 caused striking effects on kDNA structure and segregation. TbPIF8 also showed an unusual distribution on the distal face of the kinetoplast that varied with the stage of kDNA replication. Overall, we conclude that TbPIF8 is essential for cell viability and is important for kDNA maintenance.

Results

RNAi shows that TbPIF8 is essential for viability of trypanosomes

To evaluate the importance of TbPIF8, we used a tetracycline-inducible RNAi vector that produces intramolecular stem-loop dsRNA (Wang *et al.*, 2000). Stem-loop vectors are widely believed to be more effective than those, like the pZJM vector with dual opposing T7 promoters, we used previously for *TbPIF8*. We inserted into the stem-loop vector two opposing copies of the first 500 bp of the *TbPIF8* coding sequence, a sequence that has no homology with other *T. brucei* sequences that could cause RNAi knockdown of other proteins. The final construct (pSL8) was linearized and transfected into *T. brucei* 29–13 cells that constitutively express the tetracycline (tet) repressor and T7 RNA polymerase (Wirtz *et al.*, 1999). We recovered 8 clonal cell lines with similar properties and chose one for all further analyses. Fig. 1A shows that the cells underwent growth arrest starting 3 days after induction of RNAi. Then the cells gradually became less motile and the population did not recover during the following 8 days. The inset graph shows a dramatic drop in mRNA level during the first 2 days of RNAi. To assess the effect of RNAi on TbPIF8 protein level, we transfected pSL8 into cells in which one allele of TbPIF8 had been tagged with the Myc-epitope. Probing a Western blot with c-Myc antibody, we found that the tagged TbPIF8-Myc protein was reduced >90% by day 2 and was virtually undetectable by day 3 (Fig. 1B). Thus we conclude that TbPIF8 is essential for viability and that its intracellular level, at least of the tagged protein, can be greatly reduced by RNAi using a tet-inducible stem-loop vector.

TbPIF8-Myc localizes on the distal face of the kDNA disk

The import of nuclear encoded mitochondrial matrix proteins is usually directed by their N-terminal mitochondrial targeting sequence (Neupert, 1997). While many kinetoplastid mitochondrial targeting sequences are as short as 9 amino acids (Clayton *et al.*, 1995; Hausler *et al.*, 1997), Mitoprot II predicts the targeting sequence of TbPIF8 to be abnormally long (63 amino acids out of 693). In our previous study, overexpression of an ectopic TbPIF8-GFP fusion protein, driven by the strong procyclin promoter, showed colocalization with the kDNA disk (Liu *et al.*, 2009a). After several unsuccessful attempts to use polyclonal antibody to PIF8, we instead examined the distribution of the tagged TbPIF8-Myc protein. Immunofluorescence revealed that TbPIF8-Myc is present in the mitochondrion of >90% of cells and always associated with the kinetoplast (Fig. 2A). There was no detectable TbPIF8-Myc signal above background in the nucleus. Since a log phase asynchronous trypanosome culture contains only ~30% of cells in S phase, TbPIF8-Myc must therefore be present during non-replicative phases of the cell cycle. The overlay of

Myc and DAPI signals in Fig. 2A(panels 1–4), showed that TbPIF8-Myc localizes primarily to one face of the kinetoplast disk. To determine which face of the kDNA was labeled, we examined the distribution of TbPIF8-Myc with respect to basal bodies (Fig. 2B). TbPIF8-Myc signal clustered on the distal face of the kDNA disk. We designate the face of the disk that faces the basal body and associates with TAC as the ‘proximal face’ and the opposite face of the kDNA as the ‘distal face’. In Fig. S2A, two enlarged images of kinetoplasts in cells marked ‘a’ and ‘b’, with the TbPIF8-Myc-stained face of the kDNA in opposite orientations in the field of view, showed that the distal-face localization was not due to misalignment of the red and green channels (e.g., a ‘pixel shift’ problem), but truly represents a unique pattern for TbPIF8. In contrast to the asymmetric distribution of TbPIF8, we found a virtually complete overlap of the staining for the mitochondrial DNA polymerase β -PAK (Saxowsky et al., 2003) and kDNA (Fig. S2B).

We noticed some striking variations of TbPIF8-Myc localization among cells. For example, the image in panel 1 of Fig. 2A shows TbPIF8-Myc was distributed in a single elongated zone covering the entire face of the kDNA disk, while in panel 2 the Myc signal was mainly concentrated in the middle of the face. The V-shaped, double-sized kinetoplast in the lower cell of panel 3 shows TbPIF8-Myc covering the entire side of the disk. For the two daughter kinetoplasts in panel 4, the TbPIF8-Myc signal concentrated at one corner of each sister kinetoplast. We speculated that these variations might correlate with the stage of kDNA replication.

TbPIF8-Myc localization patterns vary with different kDNA replication stages

From an asynchronous TbPIF8-Myc culture, we labeled cells with the c-Myc antibody and then used terminal deoxynucleotidyl transferase (TdT) with fluorescein-dUTP to determine stage of kDNA replication. For this analysis we characterized 104 cells (Fig. 3). As mentioned in the Introduction, kDNA replication involves first the release and replication of free minicircles and then reattachment of free minicircle progeny to the network adjacent to the antipodal sites. These newly-synthesized minicircles contain one or more nicks or gaps (in this paper we will refer to these discontinuities as gaps). By targeting 3'-OH groups of gapped minicircles, TdT can label the regions of the kinetoplast with newly-synthesized minicircles and the information reveals the stage of network replication (Guilbride and Englund, 1998; Johnson and Englund, 1998). Gapped maxicircles will also be labeled, but due to their low abundance they contribute little to the overall TdT signal. In wild type cells, kDNAs progressing through the replication pathway have increasing amounts of TdT labeling. Examples are shown in Fig. 3. Cell 1 is a pre-replication kinetoplast without TdT labeling. In the early stages of kDNA replication, the TdT signal is found only in the polar regions adjacent to the antipodal sites (cell 2). In later stages, these polar regions enlarge (cell 3) and the central region, containing covalently-closed unreplicated minicircles, shrinks. When replication is complete, the kinetoplast is uniformly labeled with TdT and the double-size networks assume a dumbbell-shaped structure (cell 4). They then divide into two daughter kinetoplasts still having uniform TdT labeling (cell 5). Then the gaps are repaired and TdT fluorescence disappears (cell 6).

The cells in Fig. 3 were also labeled with c-Myc antibody, and the results provide strong evidence that TbPIF8-Myc localization varies during the different stages of kDNA replication. On a pre-replication kDNA (Fig. 3, cell 1), TbPIF8-Myc mainly resided in the central part of distal face. In an early stage of replication with TdT labeling only at the network poles, TbPIF8-Myc appeared to spread from the middle region to cover nearly the entire distal face (cell 2). Later stages of replication show kinetoplasts elongating (cell 3) and approaching a dumbbell shape (cell 4). In cell 3, TbPIF8-Myc apparently distributed over the entire distal face of the disk with two diffused zones. These zones then spread out again to cap the whole distal face of a V-shaped kDNA at a later stage (cell 4). When a

double-size kDNA was segregating, TbPIF8-Myc seemed to partition with the two daughter kinetoplasts, still covering the entire face of both (cell 5). Very quickly, minicircle gaps are repaired, thereby causing the TdT signal to disappear (cell 6). At this time, TbPIF8 clustered predominantly on one corner of the daughter kinetoplasts. We conclude that variations of TbPIF8-Myc localization correlate with the stage of kDNA replication.

TbPIF8 RNAi causes disorganization of kDNA but has only a modest effect on kinetoplast size

There are many examples in which RNAi of a replication enzyme causes rapid and extensive kDNA shrinkage and loss (Bruhn *et al.*, 2010; Downey *et al.*, 2005; Wang and Englund, 2001). Therefore, we checked the *in vivo* effects of TbPIF8 RNAi on kDNA first by DAPI staining and then by electron microscopy. Inspection of kinetoplasts in RNAi cells showed kDNA shrinkage and loss only in a small percentage of TbPIF8 RNAi cells (see Fig. 4A for examples of normal K, small K, and no K cells). Although images of cell populations at each day are not shown, the graph in Fig. 4B shows that by day 6, only about one-fourth of the cells seemed to be affected. Reduction of kDNA size was confirmed by DAPI-staining of kDNA networks isolated from uninduced cells and from cells undergoing RNAi for 1 to 6 days (Fig. 4C). We measured the surface area of about 500 kDNA networks at each time point (Fig. S3). Average area of the isolated networks progressively declined by almost 50% over the 6-day experiment (Fig. 4D). Because the kDNA network isolation included a centrifugation step, very small kDNAs may have been selectively lost. Thus, our surface area measurements might be overestimates.

EM of isolated networks from uninduced and RNAi cells confirmed the reduction of kDNA size caused by RNAi (Fig. 5A and more examples in Fig. S4; A-G). Images of thin sections from RNAi cells also revealed abnormalities in kinetoplast structure (Fig. 5B). Some kinetoplasts appeared to have normal disk shape, but contained regions with low electron density, perhaps suggesting the depletion of minicircles from this region. Others lost normal shape, and, for example, had bulges in the middle of the disk. The abnormalities at day 6 appeared even more extreme than those at day 4.

TbPIF8 RNAi has only a small effect on minicircle replication

To evaluate whether TbPIF8 RNAi affects replication of minicircles or maxicircles or both, we performed Southern blotting of total DNA isolated from a 6-day course of RNAi. Probing the blot for minicircle and maxicircle fragments (Fig. 6A) showed that the minicircle abundance gradually declined to about 50% by day 6. In contrast, there was little change in maxicircle abundance (Fig. 6B). These data are consistent with the EM data in Fig. 5A where most networks from RNAi cells appear by visual inspection to have a maxicircle/minicircle ratio higher than normal.

Minicircle replication intermediates can be resolved by electrophoresis of total DNA in the presence of ethidium bromide (Kitchin *et al.*, 1985; Kitchin *et al.*, 1984). Since *TbPIF8* RNAi caused about a 50% decline in total minicircle abundance, we used this method to determine if any of the free minicircle species were also reduced. We anticipate two extreme scenarios after depletion of essential kDNA replication enzymes: (1) If RNAi blocks minicircle release from the network, there should be a complete loss of free minicircles; (2) If RNAi allows release but blocks replication, then there should be an increase in covalently-closed minicircles and no gapped minicircles. As shown in the gel (Fig. 6C), our RNAi cells did not yield either scenario and instead revealed only a modest effect on kDNA replication. There was a small decline of gapped minicircle intermediates, with almost no change in abundance of covalently-closed free minicircles (Fig. 6D). Although these results could be explained by the partial inhibition of both release and replication by TbPIF8 RNAi, this

conclusion is not compelling. Another less likely possibility to explain the decline in gapped minicircles is that RNAi increased the rate of their attachment to the network. Note that RNAi also caused a very small increase in free minicircle multimers (marked by * in Fig. 6C).

EdU labeling indicates that TbPIF8 RNAi causes inhibition of kDNA replication

We used a novel method to further assess kDNA replication during TbPIF8 RNAi. Our procedure used metabolic labeling with the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU). This precursor, when incorporated into DNA during nuclear and kDNA replication, can be detected by a 'click chemistry' reaction with a fluorescently-labeled azide. After a 6-day induction of TbPIF8 RNAi, we harvested cells and then metabolically labeled the cells with EdU for 1 h. After fixing and permeabilizing the cells, we conducted the click reactions, and measured the percentage of cells with EdU fluorescence in the nucleus and kinetoplast. Fig. 7A shows examples of EdU labeling of cells undergoing RNAi for 4 and 6 days. We found at day 0 (before the RNAi induction) about 40% of cells were labeled in the nucleus, but only 25% were labeled in the kinetoplast. Since the S phase of the nucleus and kinetoplast are comparable in length, we expected these percentages to be closer in value. The difference is probably due to the difficulty in visualizing weak EdU fluorescence in kinetoplasts at early stages of replication. In cells undergoing RNAi, the percentage of cells with EdU-labeled kinetoplasts dramatically decreased between day 2 and day 4, when the cell growth was arrested (See Fig. 1). By day 5, very few kinetoplasts were EdU-labeled (Fig. 7B). Examination of nuclear DNA shows that EdU labeling also declined (See Discussion). This result indicates that RNAi indeed had an effect on kDNA replication although it may not be direct. We also note that our results with EdU differ from those above (see Fig. 6C) that showed little effect of TbPIF8 RNAi on kDNA replication. In the discussion we address this apparent paradox.

In uninduced cells (day 0), EdU labeling for one hour resulted in a polar labeling pattern (marked by arrows in Fig. 7A, row 1). This pattern is due to the attachment to the network of EdU-labeled gapped free minicircles adjacent to the antipodal sites. Following RNAi for 4 days most networks still had polar labeling, but we also observed cells with only one spot (see example marked by an arrowhead in Fig. 7A, row 2). We could not examine the effect of RNAi at later timepoints because there were so few EdU-labeled cells. In Fig. 7C, we show examples of isolated kDNA networks isolated from the same EdU-labeled cells used in Fig. 7A, which showed the percentage of EdU-labeled kinetoplasts also dramatically declined between day 2 and day 4 (Fig. 7D).

TdT labeling also indicates that TbPIF8 RNAi inhibits kDNA replication and alters network structure

We next used TdT labeling, as mentioned above, to assess network structure in RNAi cells. While EdU (in Fig. 7) revealed only the part of network containing minicircles synthesized during the 1 h pulse, TdT labeling detected all the gapped minicircles on the network. With TdT, polar labeling and uniform labeling of double-sized dumbbell-shaped networks were two frequently observed patterns in wild-type cells, or in isolated networks from wild-type cells (Day 0 in Fig. 8A and D). TdT labeling is useful to identify changes in kDNA structure in RNAi cells. TdT labeling of uninduced cells (Fig. 8A, upper row) or induced RNAi cells for 6 days (Fig. 8A lower row) showed that RNAi caused a dramatic decrease in the percentage of TdT-labeled cells from 45% at day 0 to 26% at day 6 (Fig. 8B), providing more evidence for inhibition of kDNA replication. At the same time, there was a major change in the pattern of labeling. Polar labeling declined by almost 45% and a new species appeared-- a uniform labeling of unit-sized networks, which increased to 60% at day 4 (Fig. 8C). In wild type cells, uniform labeling was detected only on double-sized, dumbbell-

shaped networks. Examination of isolated networks (Fig. 8D) from TbPIF8 RNAi cells also showed a marked change in labeling pattern, with a decline in the conventional polar labeling and uniform labeling of double-sized, dumbbell-shaped networks and an increase in the uniform labeling of unit-sized or smaller networks (Fig. 8F). We also detected an increase in a ring-shaped labeling pattern (Fig. 8F). Ring labeling has been detected following RNAi of other *T. brucei* replication proteins such as SSE1 (Liu *et al.*, 2005b) and p38 (Liu *et al.*, 2006) and its significance is not clear. The appearance of cells lacking kDNA (Fig. 5A) during RNAi may explain the discrepancy between the percentages of TdT-positive cells and isolated kDNA networks (Fig. 8B and 8E). Consistent with our EdU labeling results, the appearance of unit-sized, uniform labeling and ring TdT labeling patterns showed that some kDNA network structures changed during TbPIF8 RNAi. We will comment on these results in the Discussion.

TbPIF8 RNAi causes kDNA segregation defects without affecting flagellar basal body duplication and separation

When we first examined TbPIF8 RNAi cells by DAPI staining, we noted that RNAi also caused kDNA segregation defects. The most common defect was asymmetric division, where the two sister kinetoplasts differ in size (Fig. 9A). Asymmetric division occurred in ~20% of 2K cells at day 4 and in more than 80% of cells by day 6. A small fraction of RNAi cells must have had a complete inhibition of kDNA segregation, leading to cells without kinetoplast (Fig. 4A) and cells with more than a double-sized kinetoplast. Inhibition of kDNA segregation would also explain the presence of 2N1K cells.

In cells undergoing TbPIF8 RNAi, we observed, on one hand, that the percentage of 2K cells decreased. On the other hand, the number of 2K cells undergoing asymmetric division increased (Fig. 9B). Since some networks resulting from asymmetric division may be too small to be detected by DAPI-staining, the percentage of asymmetric division might even be underestimated. We confirmed the asymmetrical division of the kDNA networks by EM of isolated kDNA, and Fig. 9C shows two examples of segregating networks in the early or late stage of division in which the two developing progeny networks significantly differ in size. Thin sections of uninduced cells and cells undergoing RNAi for 4 days provided further evidence of asymmetrical division in TbPIF8-depleted cells (Fig. 9D). While a longitudinal section through an uninduced cell showed two daughter networks in almost equal size (the left panel in Fig. 9D), a cross section through an RNAi cell shows two segregated kDNA networks that differ significantly in size (the right panel in Fig. 9D). The presence of two flagella and flagellar pockets in a single cell suggests both daughter networks could have been cut in the same plane and therefore the size comparison of the two daughter kinetoplasts seemed reliable.

Since kDNA positioning and segregation are mediated by flagellar basal bodies in trypanosomes (Ogbadoyi *et al.*, 2003), we used immunofluorescence microscopy to assess the duplication and separation of basal bodies in uninduced cells (Fig. S5A) and cells undergoing TbPIF8 RNAi for 4 days (Fig. S5B and S5C). In Fig. S5A, cells uninduced for RNAi (cells 1 – 6) show the temporal coordination of duplication and segregation of both kDNA and flagellar basal bodies during the cell cycle. Cells '1' and '2' in Fig. S5B were 4-day RNAi cells undergoing asymmetric division of the kinetoplast and in these cells the duplication and segregation of basal bodies and flagella occurred normally. Fig. S5C shows a normal flagellar basal body in RNAi cells with a small kDNA (cells 1 and 2), without kDNA (cell 3) and in an RNAi cell lacking both kDNA and nucleus (0N0K cell) (cell 4). These images provide more evidence that TbPIF8 RNAi did not inhibit the duplication and separation of basal bodies and flagella.

Discussion

We have studied TbPIF8, one of six trypanosome mitochondrial proteins related to the yeast ScPif1 helicase. RNAi studies indicate that TbPIF1 and TbPIF2 are required for viability and overexpression of TbPIF5 reduces the growth rate. Recombinant TbPIF1, TbPIF2, and TbPIF5 all have helicase activity (Liu *et al.*, 2010; Liu *et al.*, 2009a; Liu *et al.*, 2009b). and we demonstrated that these three helicases have distinct roles in kDNA replication (See Introduction). In this study, we showed that TbPIF8 is essential because stem-loop RNAi abruptly arrests growth (Fig. 1). Of the eight PIF-like proteins in *T. brucei*, TbPIF8 is the most divergent. It is the smallest member of the family, missing at least two of seven conserved helicase motifs (Fig. S1) (Bochman *et al.*, 2010). A 21-residue Pif1 family signature sequence located between motifs II and III (Bochman *et al.*, 2010) is very degenerate in TbPIF8. Moreover, since TbPIF8 lacks essential residues in its Walker A motif (motif I, involved in ATP binding), it almost certainly does not have ATPase or helicase activity. If not a helicase, what could TbPIF8's function be? One possibility is that TbPIF8 is not directly involved in kDNA metabolism and its effects on kDNA are secondary. Consistent with this possibility is the fact that we detected no kDNA-related phenotypes prior to growth arrest at day 3, except a small amount of asymmetric division at day 2 (Fig. 9B). Another fact is that the shrinkage of the kinetoplast in cells undergoing RNAi was modest (Fig. 4A and 4B) in comparison with that found in many RNAi studies of other replication proteins. Another minor effect of TbPIF8 RNAi on free minicircle replication intermediates is that there was only a gradual loss of gapped minicircles and a slight increase of oligomeric species (Fig. 6C). However, with regard to total kDNA loss, other experiments [surface area measurements on isolated networks (Fig. 4C, 4D and Fig. S3), blot quantitation (Fig. 6B and 6D), and EM of isolated networks (Fig. 5A and Fig. S4)] demonstrated that about 50% percent of the kDNA, primarily minicircles, is lost during the 6 days of RNAi.

Metabolic EdU labeling of *TbPIF8* RNAi cells showed a more dramatic effect on kDNA replication. The typical "polar EdU labeling" pattern in uninduced cells results from the reattachment of minicircles to the network periphery adjacent to the antipodal sites. Newly synthesized minicircles are detected in this experiment because they have been metabolically labeled with EdU for 1 h. We evaluated EdU fluorescence in both intact cells (Fig. 7A) and networks isolated from them (Fig. 7C). In both cases, we observed that EdU labeling stopped at day 4–5. During the course of RNAi, the frequency of normal polar labeling pattern declined and irregular labeling (one spot on periphery or two spots not positioned symmetrically) increased to at least 20%. These patterns could be due to inactivation of one of the antipodal sites or to the attachment of EdU-labeled minicircles at other regions of the periphery. The decline in EdU labeling (Fig. 7B) could be due either to inhibition of minicircle replication and/or a block in attachment of newly synthesized minicircles to the network. The latter seems less likely because gapped free minicircles do not increase (Fig. 6C). As for the inhibition of nuclear labeling by EdU (Fig. 7B), this effect could be secondary to that on kDNA or it could be due to a nuclear isoform of TbPIF8. TdT labeling of RNAi cells also revealed a decline in polar labeling and an increase in uniform labeling of unit-sized kDNA. This decline could be also due to a block in replication and/or reattachment and the uniform labeling of unit-sized networks could be caused by imperfect segregation or interruption of gap repair.

The results with metabolic EdU labeling, as evaluated with isolated networks (Fig. 7C, day 0), differed significantly from those obtained previously with BrdU labeling, another thymidine analog used extensively in our lab (Liu and Englund, 2007) and others (Gluezn *et al.*, 2011) for metabolic labeling of kDNA. BrdU is detected by an antibody, but only after the DNA is denatured (usually by acid treatment). In contrast, with no denaturation required,

EdU is detected by a click chemistry reaction with a fluorescent azide that occurs under physiological conditions in the presence of Cu^{2+} . We recently reported that pulse metabolic labeling with BrdU also resulted in labeling at two peripheral sites 180° apart (Liu and Englund, 2007). However, these sites were frequently not at the network poles, but displaced from the poles. This and other results led us to propose that the kinetoplast oscillates between the antipodal sites. The pattern of BrdU labeling thus differed from that with EdU where the two labeled sites are positioned at or very near the poles (many examples are in Fig. 7C, day 0, and in other similar experiments). The EdU data fit well with the recent 3-dimensional images of the replicating kinetoplasts reconstructed from electron micrographs of serial thin sections (Gluenz *et al.*, 2011). Further work is needed to explain this discrepancy.

Other experiments strongly suggested that TbPIF8 has a novel and significant effect on kDNA. First, TbPIF8 is located exclusively on the distal face of the kDNA disk, and this unusual distribution differs from the sandwich configuration of two other proteins, DNA primase and DNA ligase k-alpha, which flank both faces of the kDNA disk (Johnson and Englund, 1999; Sinha *et al.*, 2006). TbPIF8 is found in both replicating and non-replicating cells. However, there is a small but significant variation in TbPIF8's localization according to the stages of the kDNA replication (Fig. 3). With our current level of knowledge of TbPIF8, we cannot speculate on the significance of this dynamic localization pattern. Unlike yeast ScPif1, which localizes to both the nucleus and mitochondria, we could not detect any signal in the nucleus. However, we cannot rule out the possibility that some TbPIF8 is broadly distributed in the nucleus and undetectable by our microscopy.

Another unique TbPIF8 RNAi phenotype, observed by EM of thin sections, is the presence of regions in the kinetoplasts with low electron density. This pattern also has never been detected following RNAi of other proteins, although most such investigations have not employed thin-section EM. These regions of low electron density could possibly have reduced minicircle density. It is notable that 6 of the 10 kinetoplasts from RNAi cells shown in Fig. 5B apparently have the low-density regions arranged symmetrically.

RNAi of TbPIF8 also caused a striking increase in asymmetric division of kDNA. In measurements on 2K cells (Fig. 9B), we observed only a small percentage of asymmetric division in the early stages of RNAi (day 2-day 4) but a much higher percentage, reaching more than 60% at day 5 and day 6. Although kDNA segregation is strongly affected by RNAi, duplication and separation of flagella and basal bodies appear normal.

One paradox of our data is why free minicircle analysis (Fig. 6) indicates little effect of TbPIF8 RNAi on kDNA replication whereas EdU (Fig. 7) and TdT labeling (Fig. 8) suggest that RNAi does block replication. The answer could be that free minicircle species are sensitive to RNAi of any of the multitude of enzymes involved in their release from the network, initiation of replication, propagation of the replication fork, segregating the replication products, final processing of the replication products, and reattachment of the products to the network. Knockdown of any of these enzymes should alter the free minicircle population and cause accumulation of an intermediate in the replication pathway. In contrast, TbPIF8 may not catalyze a step in the pathway. Instead it may have a more general role such as helping to organize the antipodal sites or some other complex of replication proteins (perhaps one on the distal face of the kDNA disk) or it could affect the connection of kDNA to TAC which in turn could affect replication. Nevertheless, since the growth effect of TbPIF8 RNAi (Fig. 1) precedes that of replication inhibition detected by EdU or TdT labeling, this defect cannot be the primary effect of RNAi.

Another paradox is how 40% of the minicircles can be lost during RNAi (Fig. 6B), but there is hardly any change in level of total free minicircles (the small decline in gapped free minicircles is insignificant relative to the total free minicircles, Fig. 6C). So if total minicircles decline and free minicircles do not increase, what happened to the missing minicircles? One possibility is that they were degraded to nucleotides or small oligonucleotides that are not detected in any of our experiments. Another possibility is that networks were degraded to small, catenated minicircle oligomers. These would have been lost in purification of isolated networks because they would be too small to sediment during the purification of isolated networks. They also would not show up on free minicircle gels like that in Fig. 6C. Using the same EB gel that we used in Fig. 6C, catenated minicircle tetramers migrate only 10% as fast as covalently-closed monomers (Shapiro, 1994). Therefore, larger oligomers will run even slower or even be excluded from the gel. Thus we would have missed such larger oligomers in all experiments in this paper, and this could account for the missing minicircles.

In conclusion, we have found a very interesting protein, TbPIF8 that has a unique intramitochondrial localization on the distal face of the kDNA disk, which changes during kDNA replication. TbPIF8 RNAi arrests growth and has significant effects on kDNA segregation and network structure. More experiments will be needed to reveal its function.

Experimental materials and methods

Trypanosome growth and transfection

Procyclic *T. brucei* 29-13 (a gift from Dr. George Cross, Rockefeller University), which constitutively expresses the T7 RNA polymerase and the tetracycline repressor (Wirtz *et al.*, 1999), was cultured, transfected and used for RNAi as described previously (Wang *et al.*, 2000). Cell lines were cloned by limiting dilution (Djikeng *et al.*, 2004) in SDM-79 medium containing 15% FBS and the appropriate antibiotics.

TbPIF8-myc fusion protein

3×Myc tagging of one allele of *TbPIF8* was as described (Scocca and Shapiro, 2008). Briefly, a 500 bp-fragment from the 3'-end of the *TbPIF8* ORF and another 500 bp-fragment from its 3'-UTR were amplified using *T. brucei* TREU 927 genomic DNA and the primers in Table S1. Both fragments were then cloned stepwise into pMOTag33M (Oberholzer *et al.*, 2006) and the resulting plasmid is named pMOTPIF8mycBSD. After being linearized with Acc65I and NotI, the tagging cassette from pMOTPIF8mycBSD was transfected into Tb29-13 cells. A clonal cell line, TbPIF8-Myc-C8, was used for further analyses.

RNAi and overexpression plasmid constructs

For RNAi experiments, 500 bp of *TbPIF8* were amplified using genomic DNA and the primers shown in Table S1. A stem-loop construct, named SL8, was then prepared as described (Wang *et al.*, 2000). A clonal cell line, SL8B2, was used for further analyses. For detection of TbPIF8 level during RNAi, the above SL8B2 cells were transfected with linearized 3×Myc tagging plasmid pMOTPIF8mycBSD (see above) to produce SL8B2Myc cell line. For overexpression of wild type or mutated TbPIF8, the full-length of *TbPIF8* with or without the mutation was amplified from genomic DNA using the primers in Table S1 and then inserted into pLew79-MHTAP (Jensen *et al.*, 2007).

kDNA isolation and DAPI staining

kDNA networks from uninduced and RNAi cells from a staggered induction were isolated on a 20% sucrose cushion were isolated as described (Pérez-Morga and Englund, 1993a) and stained with 2 µg/ml DAPI as described (Lindsay *et al.*, 2008).

Southern and Northern Analyses

DNA and RNA purification, Southern blotting, and Northern analyses were as described (Ryan *et al.*, 1988; Liu *et al.*, 2005b). ³²P-labeled probes for minicircles, maxicircles and the nuclear trypanosome hexose transporter (THT) genes have been described (Wang and Englund, 2001). *TbPIF8* mRNA was detected by random primer labeling of the same 500 bp fragment of *TbPIF8* used in the pSL8 construct described above.

Immunofluorescence microscopy

Immunostaining of *T. brucei* cells was as described (Scocca and Shapiro, 2008) using 1:200 rabbit anti-Myc to detect TbPIF8-myc, 1:1000 rat monoclonal antibody YL1/2 (Chemicon; anti-tyrosinated α -tubulin) for the basal body, 1:100 rabbit antiserum to *T. brucei* mitochondrial DNA polymerase β -PAK, 1:50 mouse monoclonal antibody L8C4 (anti-PFR2) (a gift from Dr. Derrick Robinson, Universite de Bordeaux II) for the paraflagellar rod and 1:200 secondary antibodies [Alexa FluorR 488-conjugated goat anti-rabbit IgG, Alexa FluorR 594-conjugated anti-rat, or anti-mouse IgG (Molecular Probes)]. TdT labelings were as described (Liu *et al.*, 2005b).

EdU labeling

Mid-log phase cells from a staggered induction of SL8B2 cells were labeled with 100 μ M 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) for 1 h. Cells were harvested and EdU was then detected with Alexa-488-azide (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 5 min and permeabilized in methanol at -20°C for 1 hr. After washing once with 3% (w/v) BSA in PBS, the cells were then incubated in 25 μ l Click-iT® reaction cocktail (1 x TBS, 2 mM CuSO₄, 1 x buffer additive, 5 μ M Alexa-488-azide) in the dark for 30 min. After washing twice with PBS for 5 min, the cells were stained with DAPI (1 μ g/ml) and examined. For *in vitro* staining, kDNA networks were isolated from trypanosomes, labeled with EdU, spotting on slides, and then incubating with Click-iTR reaction cocktail followed by DAPI staining.

Electron Microscopy (EM)

EM of isolated kDNA was performed as described previously (Perez-Morga and Englund, 1993). For thin sections, mid-log phase cells (either uninduced cells or induced for *TbPIF8* RNAi for 4 days) were harvested and washed once with PBS. Cell pellets were fixed with 2% (v/v) formaldehyde, 0.5% (w/v) glutaraldehyde in 0.07M Cacodylate buffer (pH 7.4) for 1 h, postfixed in 4%(w/v) OsO₄ (1 h, 4°C), dehydrated in graded ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Thin sections (70 nm) were cut on a Reichert-Jung Ultracut E microtome and stained with 2% uranyl acetate for 5 min. All images were taken on a Hitachi transmission electron microscope at 80 kV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. *TbPIF8* RNAi arrests growth and knocks down protein level. (A) Effect on growth. *TbPIF8* RNAi was induced by addition of tetracycline (1 μ g/ml) from day 0. Inset, Northern blot of *TbPIF8* mRNA levels without or with induction of RNAi. (B) Effect on *TbPIF8*-Myc level, analyzed by western blot using antibodies to the Myc tag. The loading control was mitochondrial DNA polymerase β (Saxowsky *et al.*, 2003).

Fig. 2. TbPIF8-Myc localizes on the distal face of the kinetoplast disk. (A) Immunostaining of DAPI-stained cells expressing TbPIF8-Myc. In the merged images, TbPIF8-Myc is green and kDNA is red. N, nucleus; k, kinetoplast. Four cells (labeled 1 – 4) are enlarged in the panels to the right. (B) Staining of TbPIF8-myc cells with DAPI and antibodies to the Myc tag and to YL1/2 (the basal body). TbPIF8-Myc is green (arrowhead), basal bodies are blue (arrows) and kDNA is red.

Fig. 3. TbPIF8-Myc distribution in the cell cycle. (A) TbPIF8-Myc localization varies with different kDNA replication stages. TbPIF8-Myc expressing cells from an asynchronous log phase culture were decorated with antibodies to the Myc tag, followed by TdT labeling and DAPI staining. In the Myc and DAPI merged images, kDNA is red and TbPIF8-Myc is green. In the Myc +DAPI+TdT merged images, kDNA is blue, TdT labeling is green, and TbPIF8-Myc is red. N, nucleus; K, kinetoplast. Bar, 5 μ m.

Fig. 4. Effect of *TbPIF8* RNAi on kDNA. (A) Examples of kDNA morphology changes following *TbPIF8* RNAi. 'Normal K' cells include 1N1K, 1N2K and 2N2K cells. (B) Kinetics of kDNA loss during *TbPIF8* RNAi. At least 400 randomly-chosen, DAPI-stained cells were analyzed at each timepoint and the results plotted. (C) Images of DAPI-stained kDNA networks isolated from uninduced cells (Day 0), and *TbPIF8* RNAi cells induced for 2, 4 and 6 days. (D) Progressive reduction in surface areas of isolated kDNA networks during *TbPIF8* RNAi (size measurements of individual networks are shown in Fig. S3).

Fig. 5.

Effect of *TbPIF8* RNAi on kDNA network structure. (A) EM of isolated kDNA networks from cells without (Day 0) or with *TbPIF8* RNAi for 4 and 6 days. Bar, 500 nm. (B) EM of thin-sections from resin-embedded uninduced cells (Day 0) or cells induced for RNAi for 4 and 6 days (Day 4, 6). FP, flagellar pocket. Arrows, region of kDNA disk with normal electron density; open arrowheads, region of kDNA disk with low electron density; filled arrowheads, a bulging region in the middle of kDNA disk.

Fig. 6.

TbPIF8 RNAi has a modest effect on kDNA abundance and free minicircle replication intermediates. (A) Effect on kDNA abundance. Total DNA (10^6 cell equivalents/lane) was isolated from cells following *TbPIF8* RNAi for different times, digested with HindIII/XbaI, Southern blotted and probed for minicircles (a 1 kb linear DNA fragment), maxicircles (a 1.4 kb DNA fragment), and the nuclear THT genes (trypanosome hexose transporter; loading control). (B) Changes of total minicircle levels and maxicircle levels during the course of *TbPIF8* RNAi. DNA levels were quantitated by phosphorimaging of the Southern blot shown in panel A and levels were corrected for load. (C) Effects of *TbPIF8* RNAi on free minicircle replication intermediates. Total DNA (10^6 cell equivalents/lane) was Southern blotted and probed for minicircles. Gapped and covalently-closed (C.C.) minicircles are indicated. Arrowheads on the upper right of gel are multimers of minicircles. THT was used as a loading control. (D) Changes in levels of covalently-closed minicircles and gapped minicircles following *TbPIF8* RNAi, determined by phosphorimaging of the Southern blot shown in panel C. Relative value is the ratio of maxicircle or minicircle abundance to that of the loading control (THT).

Fig. 7. *TbPIF8* RNAi inhibits nuclear and kDNA replication. (A) EdU labeling of cells without (Day 0) or with *TbPIF8* RNAi for 4 or 6 days. Cells were adhered to a slide, DAPI-stained, and incorporated EdU detected with Alexa-488-azide. (B) Kinetics of EdU labeling during *TbPIF8* RNAi. (C) Alexa-488-azide labeled (green) or DAPI stained (red) networks from EdU-labeled cells without (Day 0) or with *TbPIF8* RNAi for 4 days. (D) Kinetics of EdU labeling of isolated kDNAs during *TbPIF8* RNAi.

Fig. 8. Effects of *TbPIF8* RNAi on kDNA replication and structure. (A) TdT labeling of cells without (Day 0) or following *TbPIF8* RNAi for 6 days. Insets 1 and 2 are enlarged antipodal TdT labeling (cell 1) and uniform TdT labeling of unit-sized network (cell 2). (B) Kinetics of TdT labeling in cells following *TbPIF8* RNAi. (C) Pattern of TdT labeling patterns in cells following *TbPIF8* RNAi. (D) TdT labeling (green) or DAPI staining (red) of isolated kDNA network from cells without (Day 0) or with *TbPIF8* RNAi for 3 or 5 days. (E) Kinetics of TdT labeling of isolated kDNA network following *TbPIF8* RNAi. (F) Kinetics of four different TdT-labeling patterns in kDNA networks isolated from *TbPIF8* RNAi cells. The patterns are: antipodal labeling, uniform labeling of double-sized network, uniform labeling of unit-sized network, and ring labeling. For each timepoint, at least 500 networks were evaluated.

Fig. 9. Asymmetric segregation of kDNA following *TbPIF8* RNAi. **A.** Examples of cells with asymmetric segregation following *TbPIF8* RNAi. Arrows, small kDNAs. **(B)** Bar graphs showing increasing asymmetric segregation in *TbPIF8* RNAi cells. Black bar, cells with normal symmetric segregation of kDNA; white bar, cells with asymmetric segregation of kDNA. **(C)** EM of isolated kDNA networks undergoing asymmetric segregation. Arrows show developing small kDNA networks and arrowheads show its developing larger sister. **(D)** EM of thin sections showing kDNA asymmetric segregation in cells induced for *TbPIF8* RNAi for 4 days (right panel). Left panel, an uninduced cell having two sister kDNAs similar in size. Arrows, kinetoplast; FP, flagellar pocket; FL, flagellum. Bar, 500 nm.