

Conserved and specific functions of axoneme components in trypanosome motility

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Summary

The *Trypanosoma brucei* flagellum is unusual as it is attached along the cell body and contains, in addition to an apparently conventional axoneme, a structure called the paraflagellar rod, which is essential for cell motility. Here, we investigated flagellum behaviour in normal and mutant trypanosome cell lines where expression of genes encoding various axoneme proteins (PF16, PF20, DNAI1, LC2) had been silenced by RNAi. First, we show that the propulsive wave (normally used for forward motility) is abolished in the absence of outer dynein arms, whereas the reverse wave (normally used for changing direction) still occurs. Second, in contrast to *Chlamydomonas* – but like metazoa, the central pair adopts a fixed orientation during flagellum beating. This orientation becomes highly variable in central-pair- and outer-dynein-arm-mutants. Third, the

paraflagellar rod contributes to motility by facilitating three-dimensional wave propagation and controlling cell shape. Fourth, motility is required to complete the last stage of cell division in both insect and bloodstream stages of the parasite. Finally, our study also reveals the conservation of molecular components of the trypanosome flagellum. Coupled to the ease of reverse genetics, it raises the interest of trypanosomes as model organisms to study cilia and flagella.

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Key words: Flagellum, Axoneme, Central pair, Motility, Trypanosome, Paraflagellar rod

Introduction

Cilia and flagella are ancient cellular organelles encountered from protists to mammals. They are often motile and as such involved in cell propulsion or in movement of the surrounding environment. Defects in cilia construction or function are responsible for various genetic diseases such as primary ciliary dyskinesia, polycystic kidney disease, retinitis pigmentosa or the Bardet-Biedl syndrome (Eley et al., 2005; Pan et al., 2005). The basic architecture of cilia and flagella is the axoneme, which is remarkably conserved during evolution (Silflow and Lefebvre, 2001). This cylindrical structure is made of nine doublets of peripheral microtubules, with (9+2) or without (9+0) a central pair of single microtubules. The axoneme extends from the basal body, an organelle equivalent to the centriole (Beisson and Wright, 2003). Defined substructures are associated to the majority of motile 9+2 axonemes: the central-pair microtubules carry specific projections, whereas peripheral microtubules bear inner and outer dynein arms, radial spokes and nexin links. Dyneins are molecular motors and their action generates the force that causes adjacent microtubule doublets to slide past one another (Brokaw, 1972). Since the doublets are tethered to the cell via the basal body and attached to each other by nexin links, this sliding is translated into flagellar bending. However, to generate the complex waveforms observed in numerous cilia and flagella, a

precise coordination of dynein action is required. Indeed, the axoneme is a symmetric structure and if all dynein arms were active simultaneously, it would result in a rigid rigor-like state. The central-pair apparatus displays specific associated projections and is structurally and biochemically asymmetric (Mitchell, 2003b). It could therefore act as a regulator of dynein activity, for example by transmitting signals through radial spokes (Smith and Sale, 1992). In agreement with this model, mutations affecting central-pair presence, structure or components all have drastic consequences on axoneme movement (Sapiro et al., 2002; Smith and Lefebvre, 1996; Smith and Lefebvre, 1997; Tamalet et al., 2001). The existence of a dynein-regulating complex has been proposed (Piperno et al., 1992) and one of its molecular component was recently identified (Hutchings et al., 2002; Rupp and Porter, 2003).

In *Paramecium* and in the green alga *Chlamydomonas*, the central pair twists during flagellum bending in such a way that it is found parallel to the bend plane in curved areas and perpendicular in straight segments (Mitchell, 2003a; Omoto and Kung, 1979). This led to the suggestion that rotation of the central pair would define which dynein arm should be active, an assumption supported by the fact that the C1 microtubule is predominantly orientated towards the position of active dynein in re-activated axonemes of *Chlamydomonas* (Wargo and Smith, 2003). By contrast, in the flagellum of sea urchin sperm

and in cilia of other metazoa, the orientation of the central pair appears fixed and perpendicular to the bend plane (Sale, 1986; Tamm and Tamm, 1981). Nevertheless, these flagella are also actively beating, raising the question of how the central pair regulates their motility. Unfortunately, reverse and forward genetics are limited in these organisms, which restricts detailed functional analysis.

Trypanosoma brucei is a flagellated protozoan responsible for sleeping sickness in Africa. The parasite alternates between the bloodstream of a mammalian host and several tissues of its insect vector, the tsetse fly. All life cycle stages are flagellated. The single flagellum emerges from an invagination of the cell body called the flagellar pocket, which is also the unique site for endocytosis or exocytosis (Gull, 2003). The flagellum is tethered along the cell body with the exception of its distal tip. It is made of a classic 9+2 axoneme with dynein arms and radial spokes, but exhibits an original additional structure, the paraflagellar rod (PFR) (reviewed in Bastin et al., 1996; Maga and LeBowitz, 1999). The PFR has a diameter similar to that of the axoneme and is anchored via connections to doublets 4 to 7 of the latter. Intriguingly, RNA interference (RNAi) silencing of PFR main components led to strong reduction in flagellum beating and cell paralysis, despite the presence of an apparently normal axoneme structure (Bastin et al., 1998; Durand-Dubief et al., 2003). Motility was significantly reduced (although not abolished) after the corresponding genes were knocked out in the related parasite *Leishmania mexicana*, whose flagellum is free from most of the cell body (Maga et al., 1999; Santrich et al., 1997). In addition to cell motility, the *T. brucei* flagellum is intimately linked to cell morphogenesis (Kohl et al., 2003). Genome comparison revealed that most genes involved in construction and function of the axoneme are conserved in trypanosomes (Berriman et al., 2005; Briggs et al., 2004; Kohl and Bastin, 2005). In contrast to many flagellated cell types, trypanosomes can easily be grown and transformed in culture, making them an attractive model for functional analysis of flagellum.

In this article, we investigated the importance of the axoneme in flagellum and cell motility of *T. brucei* by comparing control cells with RNAi mutants of various axoneme components. We describe the unusual beating of the flagellum that produces two waveforms: a principal one, which propagates from tip to base, and a reverse one, which propagates from base to tip and allows the cell to change direction. Ultra-structural analysis revealed that the central pair bears multiple projections and keeps a fixed position in wild-type flagella whereas it becomes highly variable in mutants of the central pair or of outer dynein arms. The contribution of the PFR was re-examined and, finally, this study demonstrates the role of flagellum motility in completing cell separation in both insect and bloodstream stages.

Results

Structural arrangement of the trypanosome flagellum

Most typical sub-structures present in classic 9+2 axonemes are visible in cross-sections of the *T. brucei* flagellum: a central pair and associated projections, radial spokes, nexin links, inner and outer dynein arms (Fig. 1A-C) (Sherwin and Gull, 1989; Vickerman, 1962; Vickerman, 1976). To obtain a clearer view of axoneme sub-structures, especially central-pair

projections, cells were treated with detergent and peripheral and central-pair microtubules were numbered as described previously (Sherwin and Gull, 1989). Compared with the canonical view of the *Chlamydomonas* axoneme, we noticed several differences (Fig. 1A-C). First, nine outer dynein arms were visible in all cross-sections (instead of eight in green alga flagella). Second, radial-spoke heads seemed to be larger. Third, organisation of central-pair projections appeared different (Fig. 1A, insert). At least three projections were visible on each microtubule but they were not symmetric. The C1 microtubule carries three electron-dense short projections (5-10 nm). The C2 microtubule exhibits two long (15-20 nm) projections (termed 2a and 2b) and a short projection. A bridge appears to connect both central microtubules; a kind of sheath was also detected in some sections. Longitudinal sections revealed several projections (Fig. 1D) but, as in other species (Mitchell, 2003b), close proximity of radial-spoke heads complicated the interpretation.

The PFR is associated to the axoneme via a thick connection on microtubule B of doublet 7, and via thinner filaments connecting to doublets 6 to 4 (Fig. 1A-C) (Hemphill et al., 1991). Double immunofluorescence analysis with antibodies against PFR proteins and tubulin suggested that the PFR is found between the axoneme and the cell body (Woods et al., 1989). Electron microscopy indicated that filaments appear to link the proximal zone of the PFR (initiating in the vicinity of doublet 7 of the axoneme) and the FAZ (flagellum attachment zone) filament that runs along the side of the cell body following the flagellum path (Sherwin and Gull, 1989). The axoneme is often seen in close proximity to the region of the cell body where the four specialised sub-pellicular microtubules associated to the FAZ filament are visible (Fig. 1B). We wondered how such an edifice could behave during

Fig. 1. Structural and molecular aspects of the trypanosome flagellum. (A) Drawing of cross-section through the *T. brucei* flagellum, indicating predicted location of proteins studied in this work. Microtubules are shown in black, nexin links in green, radial spokes in magenta, dynein arms in orange and central-pair projections in yellow. The PFR is shown in blue, with its proximal, intermediate and distal domain (relative to the axoneme). The inset shows a magnified central pair and its projections. To measure orientation of the central pair relative to the PFR, lines were drawn on the axis passing through the two microtubules or through the proximal domains of the PFR (dashed grey lines) and angles measured. (B) Cross-sections of wild-type flagellum from whole cells. (Left) The flagellum appears to lie on its side, with the PFR in proximity of the FAZ filament (dark spot between subpellicular microtubules) and the axoneme closer to the four specialised microtubules associated to the smooth endoplasmic reticulum (left side of the image). (Right) The PFR is positioned between the cell body and the axoneme. (C) Cross-section of demembrated flagellum where dynein arms, radial spokes and central-pair projections are more visible. (D) Longitudinal section of the flagellum showing the axoneme (Axo) with the central pair (CP) microtubules and some projections and the paraflagellar rod (PFR) confined between the axoneme and the cell body. (E) Representation of proteins analysed in this study with their main particular domains (from Pfam-domain searches). (F) Percentage of identity between flagellum proteins from *T. brucei* and two other kinetoplastids, *T. cruzi* and *L. major*, and between human, mouse and *Chlamydomonas reinhardtii*. Alignment was performed using the Jotun-Hein method, except for TbDNAH where Clustal W was used.

flagellum bending. We therefore, analysed cross-sections of wild-type flagella and observed that the most common disposition (~66% of sections, $n=40$) was the flagellum lying on its side, with both PFR and axoneme apposed to the region of the cell body, with the FAZ filament and the four specialised microtubules respectively (Fig. 1B, left panel). We defined this

state as the ‘side arrangement’. Half as frequent were profiles where only the PFR appeared in close contact with the cell body (‘PFR arrangement’) (Fig. 1B, right panel). Only in rare instances (one out of 40 sections) was the axoneme in closer proximity to the cell body than the PFR. Finally, a few profiles were seen with the flagellum alone, without it being in contact

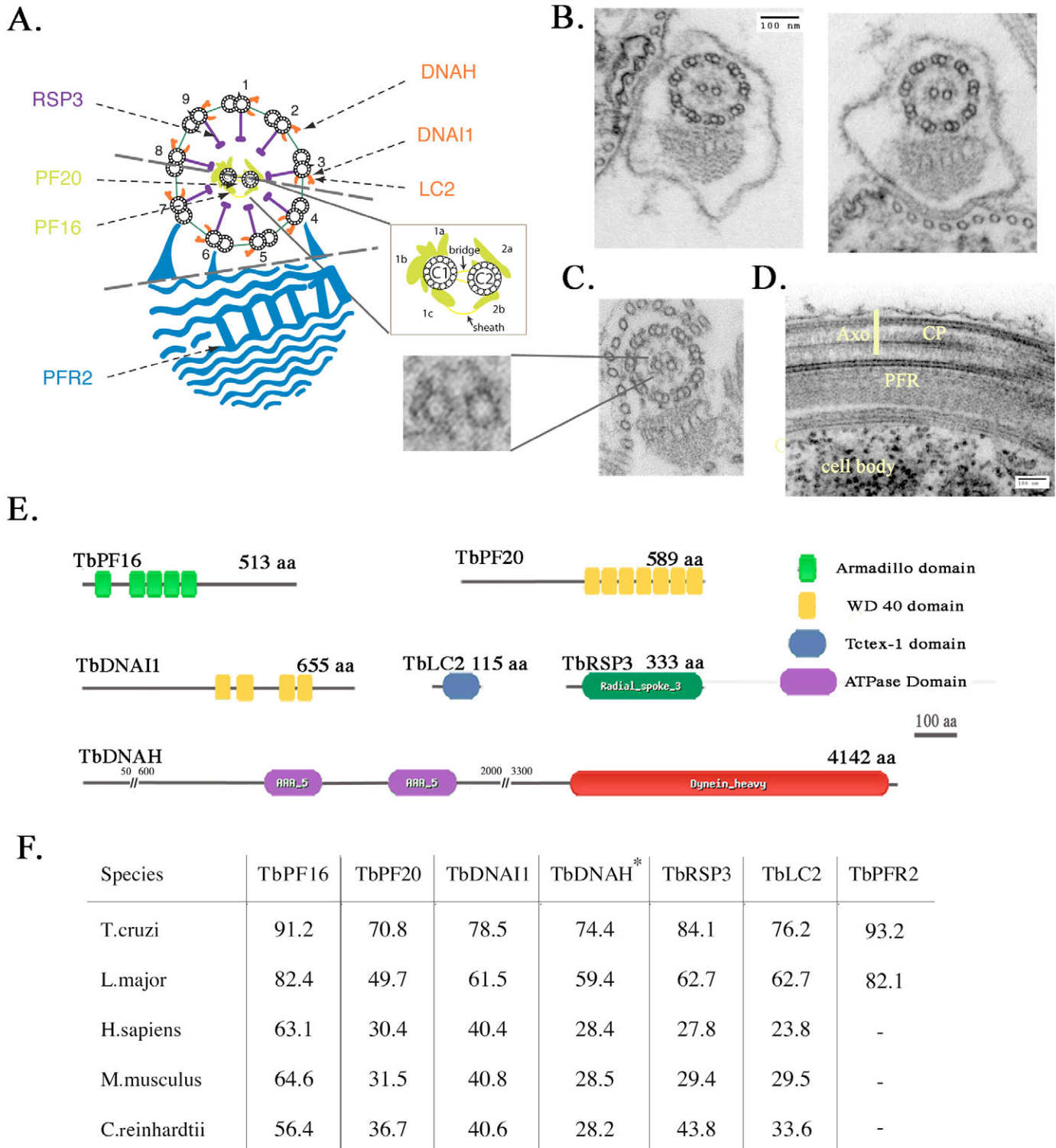


Fig. 1. See previous page for legend.

to a cell body, probably corresponding to the free distal end of the flagellum.

To determine the orientation of the central pair relative to peripheral microtubules, we used PFR positioning as an unambiguous landmark. Indeed, the PFR is firmly anchored to the axoneme (as described above) and these connections can only be disrupted by trypsin treatment (Russell et al., 1983). We measured the angle between the axis of the central pair and that of the proximal domain of the PFR in cross-section of wild-type flagella (Fig. 1A). This revealed that the orientation of the central pair is almost invariant in all axoneme cross-sections analysed at an angle of $-7.0^\circ \pm 6.5$ ($n=36$). This orientation was maintained in samples fixed after membrane removal following detergent extraction (angle of $-10.3^\circ \pm 5.3$, $n=42$). Similar determined orientation has also been observed in the related parasite *Leishmania mexicana* where the central pair and the PFR appear almost parallel in 80 out of 80 cross-sections examined (M. Wiese, Bernhard Nocht Institute, Hamburg, Germany, personal communication). In contrast to species with planar flagellum wave forms (Mitchell, 2003a), the arrangement of the trypanosome flagellum around the cell body severely restricted the possibility of examining central-pair orientation relative to flagellum bending. Nevertheless, longitudinal sections suggested that the central pair is twisted exactly in the same way as peripheral microtubules, following the arrangement of the trypanosome flagellum around the cell body (data not shown). These data suggest that the central pair of the trypanosome axoneme adopts a fixed orientation relative to the peripheral microtubules – as observed in metazoa.

Identification and RNAi silencing of trypanosome axoneme genes

To establish the importance of the axoneme in flagellum motility of trypanosomes, we first searched for representative genes encoding axoneme proteins (Fig. 1E). We selected the central-pair protein PF16, an armadillo-containing polypeptide, found on the C1 microtubule and essential for flagellum motility in *Chlamydomonas* (Smith and Lefebvre, 1996) and in mouse (Sapiro et al., 2002). As central-pair projections appear slightly different in trypanosomes, we selected another representative of this complex, the PF20 protein, rich in WD40 repeats and present on the bridge between the two singlet microtubules (Smith and Lefebvre, 1997). RSP3 was chosen as specific marker of the radial spoke because its inactivation leads to complete spoke disruption (Williams et al., 1989). Dynein arms are multi-subunit structures made of one to three heavy chains that have motor activity, and a variable number of intermediate and light chains. The latter ones are required for regulation and assembly of the dynein complex but have no intrinsic motor activity (reviewed in DiBella and King, 2001; Kamiya, 2002). The intermediate chain protein DNAI1 (IC78) is an essential component of the outer dynein arm (Wilkerson et al., 1995) and was selected as it is the first gene where mutations associated to human ciliary disorders have been identified (Pennarun et al., 1999). For the light chain, we selected LC2 (TcTex2), whose mutations are responsible for genetic distortion in mouse (Pazou et al., 1999). We searched the genome databases of *T. brucei* and of the related parasites *Leishmania major* and *Trypanosoma cruzi* and found orthologues of each axoneme gene in all three species. In *T. brucei*, only one corresponding gene of each was

found with the exception of dynein heavy chains where a total of 12 members were identified. One gene encodes the conventional cytoplasmic dynein and two separate ones present the signature of dynein heavy chains involved in retrograde intraflagellar transport (Kohl and Bastin, 2005). Both latter genes are essential for flagellum assembly (Kohl et al., 2003) (S. Absalon and P.B., unpublished data). At the time this project was launched, only one axonemal dynein-heavy-chain gene was fully sequenced and this was selected for further studies. Sequence alignments and analysis suggests that this protein would belong to the inner dynein-arm group, with closest homologues being the human dynein heavy chain 1 or the sea urchin heavy chain 6.

Alignments of *T. brucei* predicted-axoneme proteins with orthologues of *Chlamydomonas*, mouse and human show a significant conservation degree (Fig. 1E). Identity was 30-40% between human and trypanosome orthologues, with the exception of PF16, which was more than 60% identical. These values are in the same range as between *Chlamydomonas* and human flagellar genes (Pennarun et al., 1999; Sapiro et al., 2000). By contrast, the PFR genes, initially identified in *T. brucei* (Deflorin et al., 1994; Schlaeppli et al., 1989), are exclusively present in trypanosomatid and euglenid genomes so far with no counterpart in other species (Kohl and Bastin, 2005).

To determine the role of the axoneme in flagellum motility of *T. brucei*, we silenced expression of the above genes by inducible RNAi. A fragment of each gene was amplified by PCR from genomic DNA and cloned in an expression vector allowing inducible expression of double-stranded RNA (dsRNA) (Wang et al., 2000). These plasmids were stably transformed and RNAi was triggered by addition of tetracycline that switches on expression of dsRNA. The extent of RNAi silencing was evaluated by RT-PCR (Durand-Dubief et al., 2003) using mRNA for aldolase as a control (supplementary material Fig. S1). This confirmed silencing at the RNA level for all cell lines after 2 or 3 days of induction with the exception of (TbDNAH) where mRNA amounts were not or only slightly affected. Upon RNAi silencing, examination by light microscopy revealed variable reduction in motility 24 hours after addition of tetracycline in the (TbPF16)RNAi cell line and 24 hours later for the other mutants. An exception was the (TbDNAH)RNAi cell line, which showed no difference to controls, as expected from the RT-PCR data. An important parameter needs to be taken into account for correct interpretation: RNAi targets RNA and not protein. Hence, proteins present in flagella that were assembled before the onset of RNAi remain present and only disappear according to their rate of turnover. Typically, in the case of silencing of flagellar genes, a protein can be missing in the new flagellum but still be present in the old flagellum (Bastin et al., 2000; Kohl et al., 2003; McKean et al., 2003).

Motility analysis

Motility phenotypes were further characterised at the individual cell level by video-microscopy (supplementary material, Movies 1-4) and at the population level by sedimentation assays (Fig. 2). The flagellum of trypanosomes from wild-type, 29-13, non-induced RNAi cell lines or a control-induced RNAi cell line silencing a gene not involved in motility (called F6) beats normally and cells swim with their

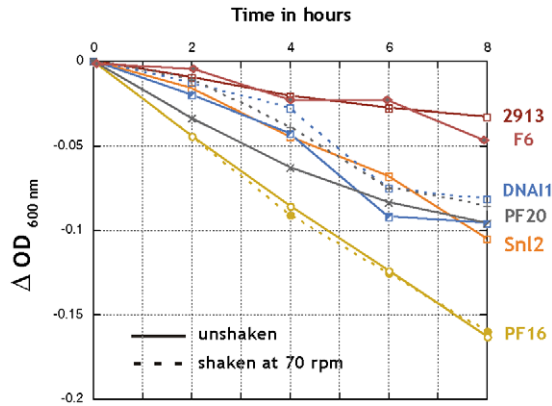


Fig. 2. Sedimentation shows reduction of motility. Trypanosomes were incubated in cuvettes and optical density was measured before and after mixing. The recipient cell line 29-13 was used as a negative control and the PFR2 RNAi-mutant *snl-2* as positive control. RNAi mutant cell lines were induced for 2 days and grown with (full lines) or without (dotted lines) shaking. Reduction in cell motility was severe in both conditions.

flagellum leading (supplementary material Movie 1). The principal flagellar wave travels from tip to base and is responsible for forward cell propulsion. The distal end of the flagellum beats at a frequency of 12–14 Hz ($n=83$, measured on three separate non-induced samples), with no significant differences between cells in motion and cells attached to the slide. The amplitude of the wave is constant as it is transmitted along the free part of the flagellum and the anterior end of the cell but is reduced progressively as it nears the base of the organelle which is embedded in the cell body. This intimate association with the cell body probably contributes to weaken both wave amplitude and frequency, and also prevented precise measurements of these parameters at the proximal end. At variable time intervals, the flagellum switches for short periods to a base-to-tip wave propagation, whose beat frequency is 3–5 Hz (supplementary material Movie 1). The amplitude was wider compared with what was observed for the principal wave. This switch leads to profound re-orientation of the anterior end and then of the whole cell without obvious backward motility.

(TbPF16)RNAi cells induced for 2 days appeared paralysed and sedimented at the bottom of the flask (Fig. 2, supplementary material Movie 2). The flagellum appeared to be partially detached from the main cell body in ~30% of the cells ($n=322$). The second cell shown on the movie possesses two flagella. Whereas the old one is still motile, the new one is paralysed and partially detached from the cell body. The amplitude and the frequency of the propulsive wave were strongly reduced and often abolished. The reverse wave occurred less frequently but was occasionally visible. The other central-pair mutant, (TbPF20)RNAi displayed a similar phenotype, although less intense (Fig. 2 and data not shown). (TbRSP3)RNAi mutants also presented significant alterations in motility (data not shown). As this mutant is being studied intensively elsewhere (Ralston et al., 2006), we have not investigated this any further. (TbDNAH)RNAi did not show significant modifications of swimming pattern and behaved like wild-type or non-induced cells (data not shown). By

contrast, (TbDNAI1)RNAi cells stop swimming and their flagella exhibited interesting features (Fig. 2, supplementary material Movie 3). The propulsive wave was absent, however, the reverse wave was still active and maintained continuously the 3–5 Hz beat frequency ($n=38$). This indicates a central role for TbDNAI1 in the tip-to-base flagellum movement. Finally, we re-examined motility of a trypanosome mutant deprived of most of the PFR structure after *PFR2* RNAi silencing (Fig. 2, supplementary material Movie 4). The sedimentation assay reproduced previous experiments (Bastin et al., 1999b; Durand-Dubief et al., 2003) as expected. However, video-microscopy highlighted an intriguing characteristic: instead of the classic twisted shape of the cell body, >90% of the cells seemed bent at approximately right angle (supplementary material Movie 4). Interestingly, the distal tip of the flagellum is still motile, sometimes displaying a normal beat amplitude at the free distal end where movements appear mostly planar. This was only poorly or not transmitted at all to the cell body, which appeared rigid. In addition, the flagellum rapidly oscillated between principal and reverse waves, resulting in the absence of cell propulsion.

Ultra-structural defects of the axoneme

We next examined axoneme ultra-structure in the various mutants by transmission-electron microscopy 3 days after induction of RNAi (Fig. 3, supplementary material Fig. S2). First, we looked at the disposition of the flagellum relative to the cell body. In both central-pair mutants, a drop in the proportion of cells with the ‘side arrangement’ was observed: only 35% of cross-sections in (TbPF16)RNAi mutants ($n=87$) and 30% in (TbPF20)RNAi mutants ($n=21$) instead of 66% in controls. In the case of (TbPF20)RNAi cells, this was accompanied by an increase in the proportion of ‘PFR arrangements’ (60% profiles) and a slight increase in flagella without cell body (~10%). For (TbPF16)RNAi cells, 35.6% of cross-sections through flagella were seen without a cell body, in good agreement with the frequency of cells with a detached flagellum (30%). These data suggest that the ‘side arrangement’ of the flagellum is linked to flagellum motility.

The central-pair microtubules are destabilised in sperm flagella of PF16 mouse knock-out mutants (Zhang et al., 2002). About 20% of flagella profiles of (TbPF16)RNAi and (TbPF20)RNAi mutants induced for 3 days present only one central microtubule, with a few without central pair at all (Fig. 3A, Table 1). It should be noted that the majority (12 out of 15) of these sections are likely to be close to the distal tip of the flagellum as they were seen near the narrow anterior end of the cell, or without the cell body at proximity. Both central-pair microtubules were visible in all other mutants examined. To gain more precise insights on structural defects of the

Table 1. Presence of central-pair microtubule in various cell lines

Cell type	Number of microtubules in the central pair (%)		
	2	1	0
WT ($n=40$)	97.5	2.5	0
(TbPF16)RNAi ($n=83$)	78.3	18	3.7
(TbPF20)RNAi ($n=69$)	79.8	20.2	0
(TbDNAI1)RNAi ($n=23$)	100	0	0

Table 2. Presence of outer dynein arms in various cell lines

Cell type	Total images [‡]	Number of missing outer-dynein-arms		
		None	1-3	>3
WT*	40	40	0	0
WT [†]	30	30	0	0
(TbPF16)RNAi*	34	8	25	1
(TbPF16)RNAi [†]	20	11	9	0
(TbPF20)RNAi*	11	7	4	0
(TbPF20)RNAi [†]	32	16	16	0
(TbDNAI1)RNAi [†]	43	1	5	37

*, whole cells; [†], detergent-treated.

[‡]Only images in which outer dynein arms could unambiguously be recognised were selected for analysis, hence the reduced number of samples.

axoneme, the flagellum membrane was removed by treatment with Nonidet P-40. The buffer used did not contain sodium chloride and stability of central-pair microtubules was not modified compared with non-extracted samples, as observed for *Chlamydomonas* (Mitchell and Sale, 1999). However, several projections were frequently missing, indicating defects in the organisation of central-pair microtubules. Another unexpected modification was apparent: at least one outer dynein arm was absent, usually on doublet 5 in the case of (TbPF20)RNAi, and on doublets 5 and 6 for (TbPF16)RNAi (Table 2). This was not a consequence of detergent extraction because a similar proportion of flagella that was missing at least one outer dynein arm was found on images of fixed intact cells (Table 2). This defect was never observed on sections of wild-type cells ($n=70$). By contrast, inner dynein arms did not appear modified.

Observation of (TbDNAI1)RNAi flagella cross-section (Fig. 3B, Table 2) demonstrated the importance of DNAI1 for the assembly of the outer dynein arm: out of 46 profiles analysed, 32 missed all nine dynein arm complexes and 13 missed at least one of them. However, neither inner dynein arms nor the central-pair microtubules displayed structural problems. These data demonstrate that DNAI1 is essential for outer-dynein-arm assembly in trypanosomes as it is the case in *Chlamydomonas* and humans.

The orientation of the central pair was examined in (TbPF16)RNAi, (TbPF20)RNAi and (TbDNAI1)RNAi mutants. Whereas the central pair was at a slightly negative angle relative to the PFR in wild-type flagella, its orientation appeared highly variable in all three mutants, with a wide dispersion over at least 180° (Fig. 3D). Whereas only 10% of sections through wild-type flagella ($n=82$) showed an angle of more than $\pm 15^\circ$, this value raised to 65% for (TbPF20)RNAi ($n=65$), 46% for (TbPF16)RNAi ($n=116$) and 62% for (TbDNAI1)RNAi ($n=44$). It was difficult to unambiguously distinguish the C1 microtubule from the C2 microtubule in these images, especially in central-pair mutants where projections were modified, so these values are probably underestimates because variations of up to 360° cannot be ruled out. This dispersion was also found in detergent-extracted cells for all three mutant cell lines (Fig. 3C,D). Nevertheless, about half of the sections in flagella of mutant cell lines still presented an almost parallel orientation to the PFR. These could represent completely twisted central pairs ($\pm 180^\circ$) or correspond to cells where RNAi silencing is not complete. Ultra-structural analysis of the (TbDNAI1)RNAi mutant

revealed that ~85% of cells lacked most of the dynein arms, an indication that the remaining 15% could correspond to cells behaving like controls. Owing to the short size of profiles seen in longitudinal section, it was not possible to determine whether the central pair was fixed at a particular angle along the length of a given flagellum or whether it was rotating within the same axoneme. A few longitudinal sections in the (TbPF16)RNAi-mutant strain suggested that the central pair maintains a fixed orientation (parallel to the bend plane) during flagellum bending (Fig. 3C). By contrast, analysis of flagella of paralysed PFR mutants showed that orientation of central-pair microtubules remains similar to wild-type (Bastin et al., 1998) (data not shown). These data show that defects of central-pair microtubules or outer dynein arms result in loss of the fixed central-pair-microtubule orientation.

Flagellum motility is required for septation

Upon induction of RNAi silencing targeting TbPF16 or TbPF20, or – to a lesser extent – TbDNAI1, we noticed a reduction in the growth rate (Fig. 4) and an accumulation of large cellular aggregates in the culture flasks, suggesting cell-separation defects. Examination of dividing wild-type or non-induced trypanosomes revealed that daughter cells are only joined at their posterior end, with their flagella beating in opposite directions. Owing to the flagellum arrangement on the cell body, both cells tend to twist in opposite direction, which could contribute to tension on the mid-body and participate to cell separation (Fig. 5A, supplementary material Movie 5). Examination of (TbPF16)RNAi cells at early stages of induction (24-48 hours) revealed that numerous bi-flagellated cells possess a motile and an immotile flagellum (the old and the new flagellum, respectively). Strikingly, when cells are about to divide, the progeny carrying the old flagellum is motile and drags its immotile counterpart behind it, indicating that motility could indeed contribute to cell separation (Fig. 5B, supplementary material Movie 6). At later stages of induction (48-72 hours), both flagella are paralysed and progression through cytokinesis appears much slower (Fig. 5C,

Fig. 3. Central-pair-presence and -orientation in normal and mutant cell lines. (A) Cross-sections of flagella from (TbPF16)RNAi and (TbPF20)RNAi cells induced for 3 days. The central pair can be positioned at very different angles relative to the PFR axis. In several sections, one or both microtubules of the central pair can be absent. (B) Loss of some outer dynein arms in (TbPF20)RNAi- and (TbPF16)RNAi mutants, and of most of them (eight) in (TbDNAI1)RNAi mutants. Yellow arrows indicate the position of the missing arm. (C) Longitudinal section of a (TbPF16)RNAi mutant where the central pair is visible in a bent region, in this case it appears parallel to the bend plane. (D) Central-pair orientation relative to the PFR proximal axis. A negative angle indicates that lines drawn from the axis of the central pair and of the PFR would cross in direction of doublet 4 of the axoneme (right side of images in B). Sections were classified as belonging to any 15° interval. Owing to the difficulty in unambiguously distinguishing the C1 from the C2 microtubule, angles were limited to 180° but could be more important. As no significant differences could be seen between sections of whole cells or de-membrated samples, data from both sets were pooled. From top to bottom: wild-type ($n=82$), (TbPF20)RNAi ($n=65$), (TbPF16)RNAi ($n=116$) and (TbDNAI1)RNAi ($n=44$). Whereas a determined orientation is found in wild-type cells, it appeared highly variable in axoneme mutants.

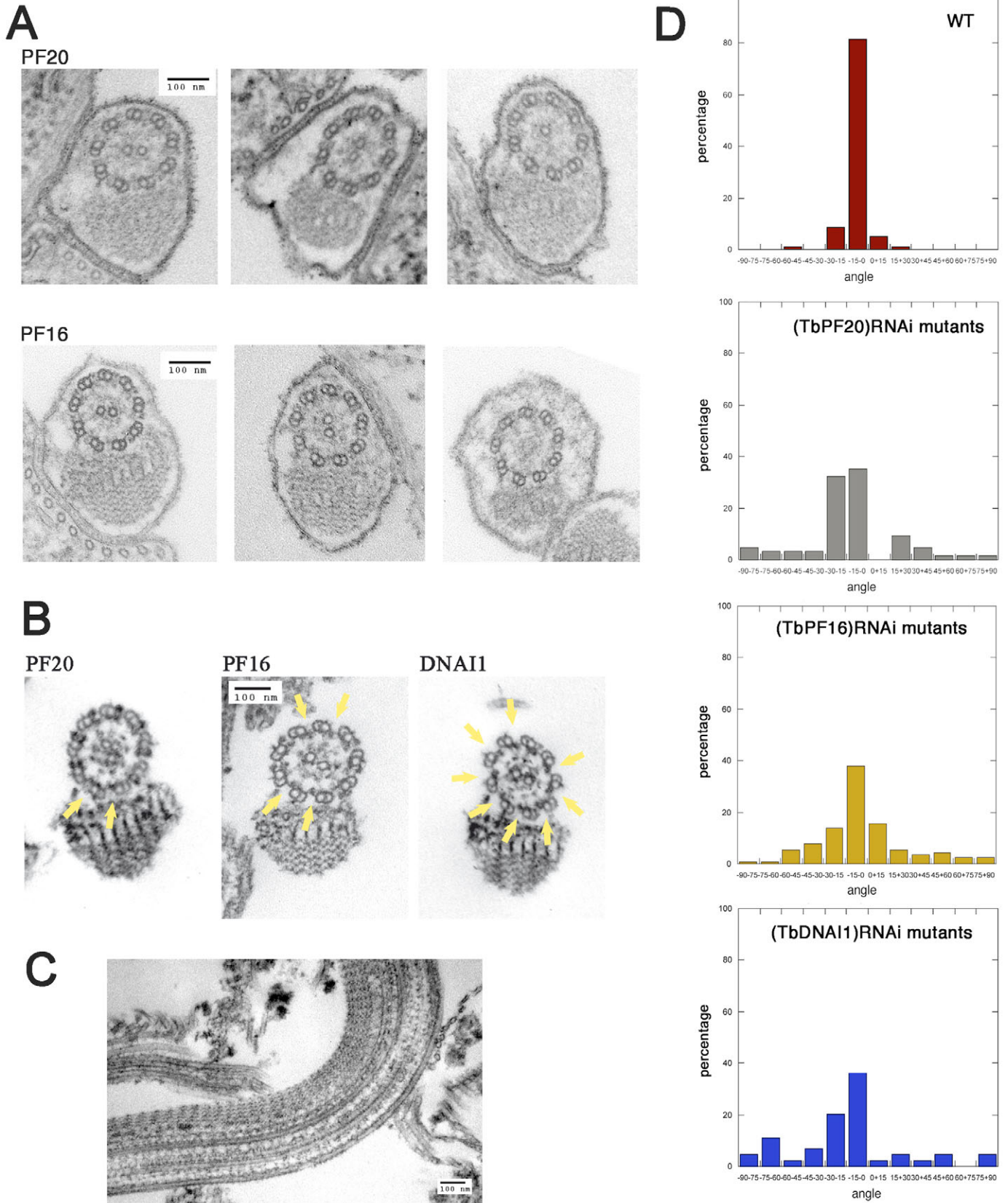


Fig. 3. See previous page for legend.

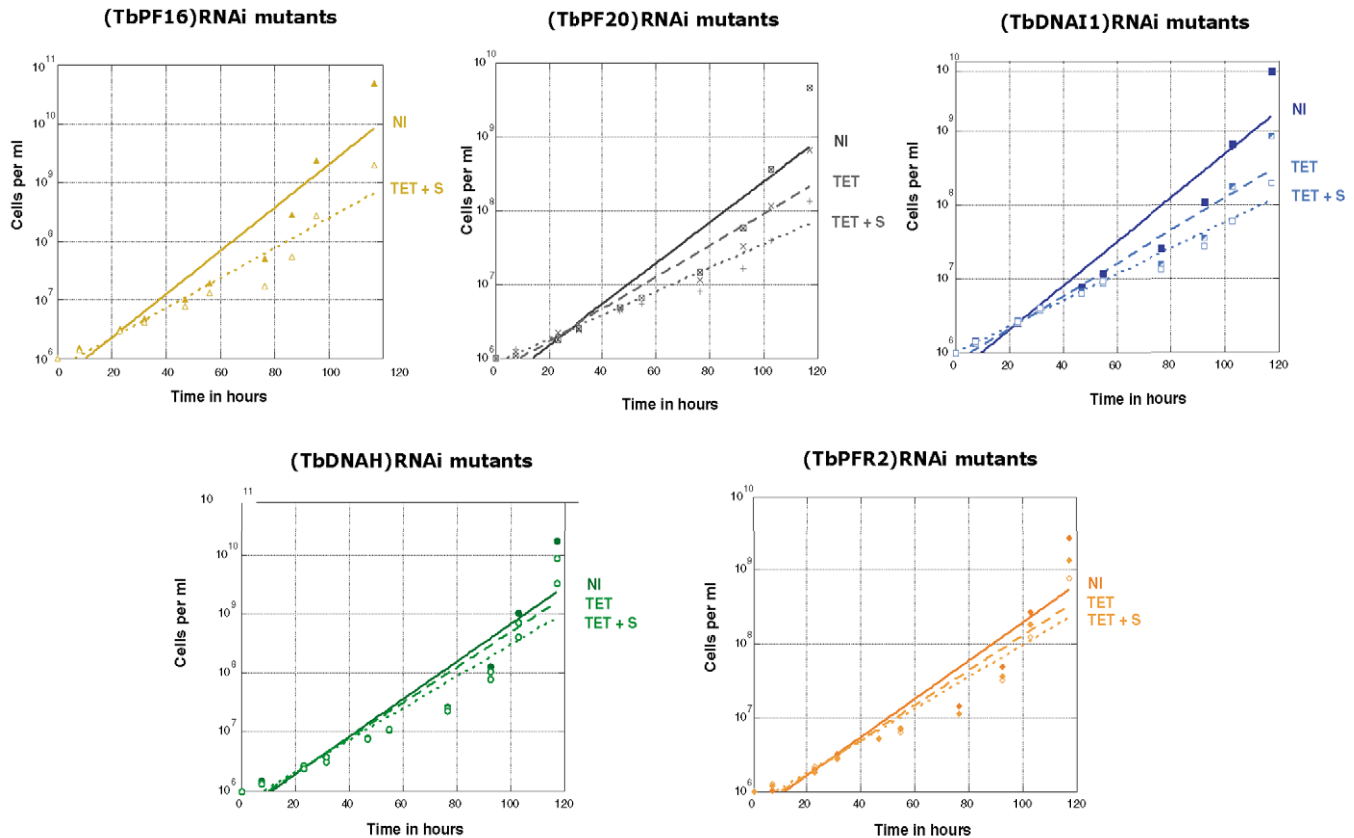


Fig. 4. Cumulative growth curves of the axoneme or PFR mutants under non-induced conditions (full line), induced conditions (dashed line) and induced conditions while shaking the culture (dotted line).

supplementary material Movie 7), which could explain the slow-growth phenotype we observed (Fig. 4). Similar results were observed for (TbPF20)RNAi and, to a lesser extent, for (TbDNAI1)RNAi, whereas induced cell lines with weaker-motility phenotypes, such as (TbLC2)RNAi (which displayed jerky but active movements, data not shown) and (TbDNAH)RNAi (which behaved essentially like wild-type) (Fig. 4). Despite showing significant reduction in motility, *snl-2* or (PFR2)RNAi mutants showed little cytokinesis defects and grew almost as fast as wild type and non-induced controls (Fig. 4) (Bastin et al., 1999b; Bastin et al., 1998). Observation of dividing-induced *snl-2* trypanosomes indicated that daughter cells are still able to twist while attached (supplementary material Movie 8), a feature not seen in induced (TbPF16)RNAi or (TbPF20)RNAi. This motility might be sufficient to sever the mid-body and allow cytokinesis completion. If this were exclusively dependent on mechanical shearing force, we reasoned that shaking the culture could compensate this defect. We therefore cultivated non-induced and induced cell lines with or without agitation, and compared their growth rate (Fig. 4, Fig. 5D). Shaking the culture lead to a spectacular reduction in the number of cell aggregates for all cell lines examined, confirming that mechanic force can compensate for the cell division defect (Fig. 5D). However, this failed to restore normal cell growth, indicating that other parameters need to be altered. Detailed cell biology revealed that these cells had difficulties in segregating their basal bodies, a key stage of the trypanosome cell cycle (our unpublished

data). An alternative explanation is that cells cannot feed properly because of defects in endocytosis, which solely takes place at the level of the flagellar pocket. This is clearly not the case because the endocytic network of cells from unshaken cultures of (TbPF16)RNAi mutants is not modified compared with controls (Fig. S2).

We also investigated the consequences of reduced motility in bloodstream form trypanosomes. Cells were transfected with double-stranded RNA corresponding to genes encoding PFR2 or GFP as control. Consequences of RNAi silencing were monitored by immunofluorescence with a specific anti-PFR2 monoclonal antibody (Kohl et al., 1999). At early time points (5–8 hours), numerous bi-flagellated cells were seen with reduced PFR2 staining at the distal tip of the new flagellum, without apparent modification of the old flagellum (Fig. 5E). This is explained by the fact that PFR is assembled by the addition of new subunits at the distal end of the new flagellum and that turn-over in the old flagellum is slow (Bastin et al., 1999a). In two separate experiments, we observed 53% and 57% of bi-flagellated cells where PFR2 abundance was clearly reduced in the new flagellum compared with the old one. After 15 hours, these cells failed to complete the late step of cytokinesis, with two progenies stuck to each other at their posterior end (Fig. 5F). Nevertheless, these cells were still able to duplicate their basal bodies and to assemble a new flagellum (PFR is not required for flagellum assembly), but the new flagellum did not stain with the anti-PFR2 antibody. These cells enter mitosis but again failed in cytokinesis and, in contrast to

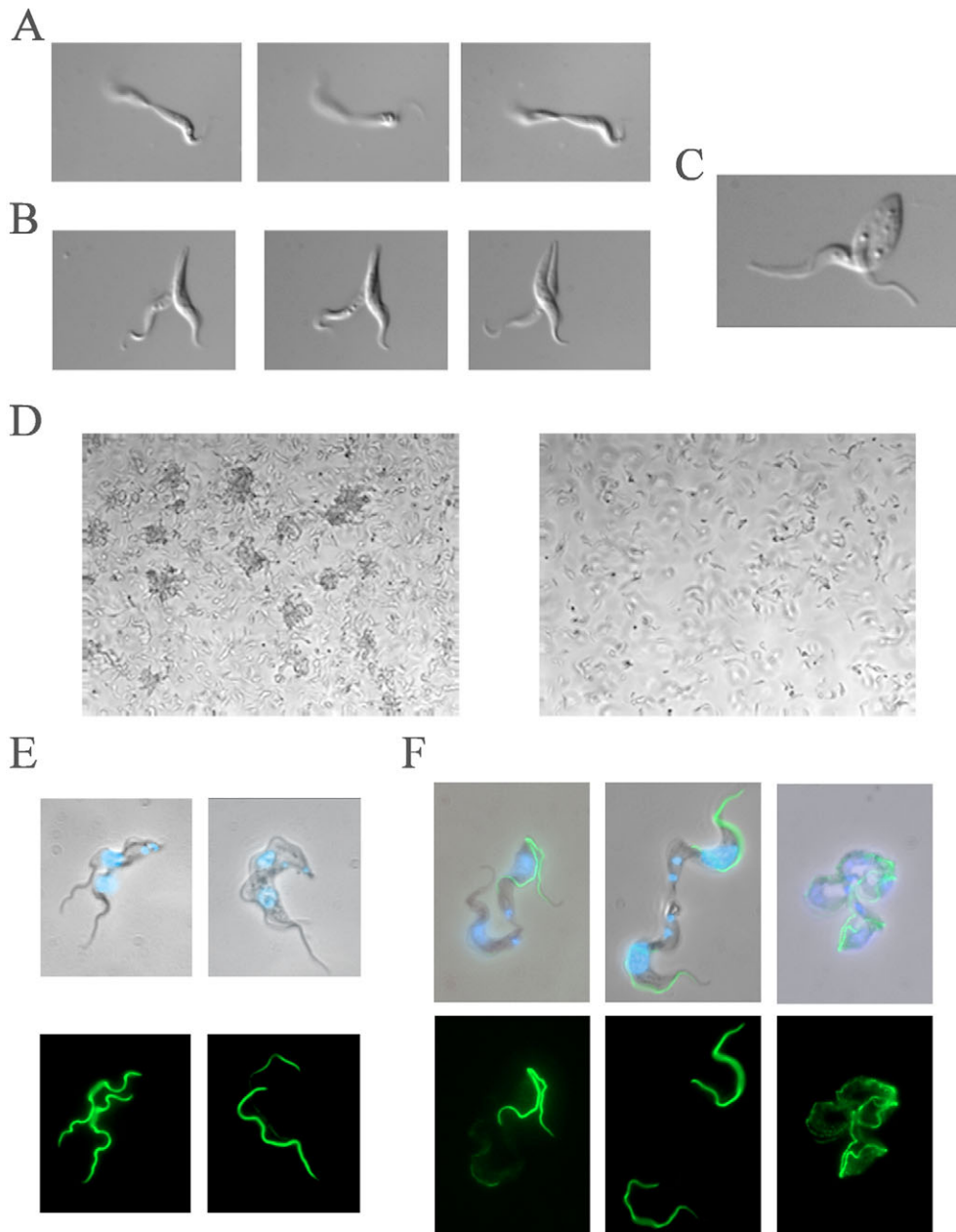


Fig. 5. Cell-body separation is impaired when axoneme motility is strongly affected. (A-C) Still images of movies of (TbPF16)RNAi mutants (A) non-induced or induced for (B) 48 hours or (C) 72 hours. (A) Both flagella are motile and the cell is actively moving and twisting, but no net movement is observed. (B) The left flagellum is motile but the right one is not. The active flagellum drags the couple towards its anterior end. (C) Both flagella are paralysed and division is slowed down. (D) Field of (TbPF20)RNAi-mutant cells induced for 72 hours, grown (left) without shaking or (right) with shaking. Large cell aggregates are visible when cultures are not shaken; most of them disappear upon shaking. (E) Bloodstream form trypanosomes electroporated with (left) *GFP* or (right) *PFR2* dsRNA. After 5 hours, flagella were labelled with the anti-PFR2 antibody (green, bottom images) and DNA-stained with DAPI (blue); superimposed phase-contrast image (top images). Cells with two flagella that received *GFP* dsRNA showed complete PFR2 staining in both flagella, whereas those with *PFR2* dsRNA did not stain for PFR2 at the distal part of the flagellum, the normal site of PFR construction. (F) After 18 hours cells that had failed to divide (left images) were abundant only in the *PFR2*-transfected samples. The cells could re-enter the cell cycle (middle images) but rapidly degenerated (right images).

procyclic stage trypanosomes, appear to degenerate (Fig. 5F). This was confirmed by the reduced growth rate of cells transformed with *PFR2* dsRNA compared with those electroporated with *GFP* dsRNA (data not shown). These results indicate that flagellum motility is also essential to complete cell separation in bloodstream form trypanosomes.

Discussion

The attachment and arrangement of the trypanosome flagellum associated with the presence of the PFR, whose integrity is required for correct cell motility (Bastin et al., 1998), represent unusual features that render investigation of movement challenging. Observation of live wild-type trypanosomes revealed the complex flagellum beating pattern, with a principal wave responsible for propulsion, propagating from tip to base. The flagellum frequently switched to a reverse wave

with high amplitude and low frequency. This allowed the cell to re-orient and then to change direction. This alternating direction of wave propagation has been described for *Crithidia* (Holwill and McGregor, 1976) but not for *Leishmania* where only the tip-to-base wave is observed (Santrich et al., 1997).

RNAi that targets distinct representatives of each axoneme structure (central pair, radial spokes, dynein arms) resulted in significant modifications of flagellum- and cell-motility, thus demonstrating that the axoneme is indeed functional in trypanosomes and that its constituents appear to behave in a related manner as to what was observed in *Chlamydomonas* and mammals. Analysis of *Chlamydomonas* inner and outer dynein-arm mutants suggested different contributions in the control of beat frequency and waveform (Brokaw and Kamiya, 1987; Yagi et al., 2005). In *T. brucei*, absence of the outer dynein arm in the (TbDNAI1)RNAi mutant is accompanied by

absence of propulsive wave and loss of forward motility. Nevertheless, the reverse wave is still operating at an apparently normal beat frequency, indicating that inner dynein arms are the main motor for this type of flagellar motility. Outer dynein arms are essential for reverse motility in *Chlamydomonas*, an organism that also swims with the flagella leading (Wilkenson et al., 1995; Pazour et al., 1999). Cooperation of various dynein motors has been reported previously in *Chlamydomonas* and is likely to also play an important role in the complex motility displayed by *T. brucei*. The presence of nine axonemal dynein heavy chains indicates a sophistication equivalent to what had been observed in other organisms with complex beating pattern (Omoto et al., 1996; Porter, 1996). Silencing the *T. brucei* LC2 leads to a clear modification of swimming pattern that turned out to be jerky. This pattern is reminiscent to what had been observed in *Chlamydomonas* (Pazour et al., 1999), re-asserting the conserved function of the various dynein components in flagellum beating across species. Nevertheless, this did not prevent cells from moving forward and growth rate was not modified. The difference in behaviour compared with *TbDNAI1*-knockdown mutants can possibly be linked to a slightly less efficient silencing of the LC2 RNA, as suggested by RT-PCR (supplementary material Fig. S1).

The presence of a central pair of singlet microtubules with associated projections is a characteristic of motile flagella (Mitchell, 2004), albeit several types of motile cilia or flagella naturally devoid of central pair have been reported (Nonaka et al., 1998; Schrevel and Besse, 1975). Several proteins are specifically associated to the central pair and their inactivation usually drastically reduced flagellum motility in *Chlamydomonas* (Rupp et al., 2001; Smith and Lefebvre, 1996; Smith and Lefebvre, 1997; Yokoyama et al., 2004) and mice (Sapiro et al., 2002). In addition, patients with ultra-structural defects of the central pair displayed the most severe forms of primary ciliary dyskinesia (Tamalet et al., 2001). Our study revealed that the trypanosome central-pair structure is asymmetric, with specific projections, is essential for flagellum motility and does not rotate relative to peripheral doublets. This latter characteristic is reminiscent to all metazoa examined to date but differs from what is encountered in *Chlamydomonas* (reviewed in Omoto et al., 1999). Our data revealed that the orientation seems slightly variable (Fig. 3D), a feature previously reported in *Euglena gracilis*, which – like trypanosomes – belong to the euglenoid group and possess a PFR (Melkonian et al., 1982). The fixed orientation is preserved after detergent extraction, suggesting the existence of a specific system to maintain it. Although the orientation of the central pair relative to the axis of the PFR appears invariant, the positioning of the whole edifice (axoneme + PFR) on the cell body was more variable, probably as a consequence of motility. This indicates torsion of this dual complex, with some defined constraints because the PFR always appeared closer to the FAZ filament, and the axoneme closer to the adjacent smooth-endoplasmic-reticulum-associated microtubules. By contrast, flagellum arrangement along the cell body was frequently modified in axoneme mutants and numerous paralysed flagella are partially or, sometimes, completely detached from the cell body (with the exception of anchoring via the basal body-flagellar pocket), suggesting that this physical constraint is modified.

Intriguingly, the orientation of the central pair is highly variable in (*TbPF16*)RNAi, (*TbPF20*)RNAi and (*TbDNAI1*)RNAi mutants. In *Chlamydomonas*, the central pair twists during flagellum bending as to adopt a specific orientation with the C1 microtubule towards the active dynein doublet (Mitchell, 2003a; Wargo and Smith, 2003). However, in vitro reactivation of isolated axonemes showed that this orientation is lost in radial-spoke mutants and outer-dynein-arm mutants (the latter case only at a high Ca^{2+} concentration). These results suggest interactions between the central-pair complex and the outer dynein arms and are consistent between trypanosomes and green algae, despite the different behaviour of the central pair. The loss of specific outer dynein arms in both central-pair mutants in trypanosomes also underlines the likelihood of such interactions. Comparison of axonemes from *T. brucei* and *Chlamydomonas* revealed several differences, including in central-pair projections and radial-spoke heads. Identifying the differences between flagella of organisms with a rotating or a fixed central pair will be challenging. In any case, these data underline once more the complexity of the central-pair apparatus (Mitchell, 2004) and highlight the need for detailed examination of cilia and flagella from different origins.

Overall, our results demonstrate that the trypanosome axoneme possesses typical ultra-structural and molecular features of conventional 9+2 axonemes and that the central pair maintains a fixed orientation. Combined to the availability of the complete genome sequence and the amenability of reverse genetics, this makes the trypanosome an attractive model to study genes involved in human genetic disorders linked to flagellum motility defects. Central-pair defects are amongst the most severe cases of primary ciliary dyskinesia (Tamalet et al., 2001) and modifications of central-pair orientation have been reported (Rutland and de Jongh, 1990). In addition, *DNAI1* was the first gene whose importance in human genetic disorders has been revealed (Pennarun et al., 1999), and the similarity between ultra-structural defects seen in patients and the RNAi phenotype reported here opens the opportunity for functional studies of human genes in trypanosomes. Functional complementation of RNAi mutants by expression of heterologous genes from *T. cruzi* has been successful without cross-RNAi (Rusconi et al., 2005), suggesting it can be attempted for human genes.

These results prompted us to re-examine the compartment of PFR mutants that present a normal axoneme at the ultra-structural level but cannot swim (Bastin et al., 1998). Our data show that the distal tip is still motile in many flagella, displaying an almost normal wave amplitude, but that the waves are not properly propagating towards the base. The flagellum alternates rapidly between main (direct) and reverse wave without effective motility. This could be linked to the striking change in shape because these trypanosomes do not exhibit the usual twisted aspect with the flagellum around the cell body. Instead, PFR-mutant cells appeared bent at right angle with an almost straight posterior end. These data support the hypothesis that the PFR participates to cell motility by contributing to flagellum strengthening during wave propagation (Santrich et al., 1997). In the related parasite *Leishmania*, the flagellum is only tethered along a short region of the cell body where it exits from the flagellar pocket. FAZ filament and four microtubules are present at this site (Wiese

et al., 2003) and trypanosome anti-FAZ antibodies stain that area distinctly (our unpublished data). Dark-field microscopy with stroboscopic illumination showed that wave propagation is also reduced in proximity to the cell body, suggesting a related function in *Leishmania* (Santrich et al., 1997). *PFR2*-knockout mutants of *Leishmania* retained the ability to produce waves, although of a lower amplitude, and waves were drastically reduced as the wave progressed in the direction of the cell body. Finally, flagella movements also appeared planar in *PFR2*-knockout cells (Santrich et al., 1997), suggesting that the PFR contributes to the complex three-dimensional swimming pattern of trypanosomes and *Leishmania*.

In trypanosomes, like in many groups of protists, the new flagellum is constructed while the old one remains present (Brugerolle, 1992; Noel et al., 2003; Sherwin and Gull, 1989). Both flagella are beating throughout this process and, during the progression of cytokinesis, the daughter cells re-orientate in such a way that their flagella are pointing in opposite directions. As a result, movement of these two flagella, in addition to the twisted motility imposed by the flagellum disposition around the cell body (Hill, 2003), exerts tension on the mid-body and contributes to completion of cell separation. Other modes of movement participate to this process in the ciliate *Tetrahymena* and in the slime mould *Dictyostelium*, where the shearing force is provided by complex cilia beating (Brown et al., 1999) and amoeboid movement (Tuxworth et al., 1997), respectively. In all three cases, cells can re-enter the normal cell cycle, progress through mitosis and, therefore, end up multi-nucleated – indicated by the absence of a checkpoint on that final stage of cell division. Separation defects were rare in PFR mutants, an observation that may be related to the fact that these cells can still move and exhibit twisting movements that could be sufficient to allow cytokinesis. Similarly, no striking cytokinesis defects were noticed in (TbDNAI1)RNAi mutants or trypanin mutants (Hutchings et al., 2002), which both can beat but without net forward motility.

Materials and Methods

Trypanosome cell lines and culture

T. brucei cells at the procyclic stage (corresponding to parasites developing in the midgut of the tsetse fly) were cultured at 27°C in semi-defined medium 79 containing 10% foetal calf serum and hemin. Wild-type (strain 427), 29-13 (Wirtz et al., 1999) and derivatives, snl-2 (Bastin et al., 2000) and PFRaI (Durand-Dubief et al., 2003) cell lines were used. For induction of RNAi, cells were grown in the presence of 1 µg of fresh tetracycline per ml of culture medium. Culture density was maintained between 1 and 10 millions cells per ml by regular dilution and growth curves were presented as cumulative data, allowing global view for long-term experiments. In the case of bloodstream trypanosomes, the cell line 90-13 (Wirtz et al., 1999) was grown in HMI-9 medium with 10% foetal calf serum as described (Hirumi and Hirumi, 1989).

Identification of axoneme genes

The *T. brucei* GeneDB database (<http://www.genedb.org/genedb/trypp/blast.jsp>) was screened by BLAST search using known axoneme genes. Genes encoding a putative dynein heavy chain (Tb10.70.1720), the dynein light chain LC2 (Tb11.02.5620), the central-pair proteins PF16 (Tb927.1.2670) and PF20 (Tb10.61.2920), and the radial-spoke protein RSP3 (Tb11.47.0034) were identified using *Chlamydomonas* sequences. The *TbDNAI1* (Tb11.02.2640) gene was identified by BLAST search using the human gene (NP036276). Protein sequences were aligned using MegAlign package from DNASTAR software (Lasergene).

Plasmid construction and RNAi-mutant cell line generation

Primers were designed to PCR-amplify fragments of genes from wild-type *T. brucei* genomic DNA. Forward and reverse primer sequences were: for PF16, 5'-CGAT-GAATTCCTCGAGTTGATGCCTTGCAGCAGGCC-3' and 5'-CGATCGAAGCT-TAGACAATGACAGCGAGAGCG-3'; for PF20, 5'-GAGACTCGAGTCATCGA-GCCAACCATAACC-3' and 5'-AGAGAAGCTTCTGACATCACAAAGTTCGCC-

3'; for RSP3, 5'-CGACCGAAGCTTATGCAAGGGCAAACCAAGC-3' and 5'-CGACCAGATCTCTACTTGGCGAATGAGATC-3'; for LC2, 5'-CGATGAAT-TCCCTCGAGATGGTGAACGAGTACCATG-3' and 5'-CGATCGAAGCTTCTAC-TCCCAGTACAGCGC-3'; for DNAI1, 5'-GAGACTCGAGACAAGGTGAA-ACATCGTGCC-3' and 5'-AGAGAAGCTTCAGGATCGAAGACACATGCG-3'; for DNAH, 5'-GAGACTCGAGCCACGGATGGAAGTGATGAG-3' and 5'-AGA-GAAGCTTGGAGGAGTTTCGGCGTATTC-3'. PCR products were purified and cloned in the pZJM vector (Wang et al., 2000). After electroporation, cells that had integrated the plasmid were selected for 3 weeks in culture medium containing 20% foetal calf serum and the appropriate antibiotics (15 µg/ml G418, 20 µg/ml hygromycin, 2 µg/ml phleomycin). For analysis, five to 20 transformed cell lines were induced and characterised, and one was selected for sub-cloning by limiting dilution (Bastin et al., 1999a).

Transient transfection of double-stranded RNA (dsRNA) (Ngô et al., 1998) in bloodstream trypanosomes was performed with in vitro synthesised *PFR2* or *GFP* dsRNA (Durand-Dubief and Bastin, 2003). Transfected cells were returned to culture, incubated for 5-20 hours in normal culture medium and analysed by video microscopy or fixed and processed for immunofluorescence with the anti-PFR2 monoclonal antibody L8C4.

Electron microscopy

Cells were washed in 0.1 M cacodylate buffer (pH 7.2) and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, 0.5% tannic acid solution for 2 hours at room temperature. Fixed samples were washed three times in 0.1 M cacodylate buffer (pH 7.2) and post fixed in 1% osmium-cacodylate solution (0.1 M, pH 7.2) for two hours in the dark under agitation. Samples were stained en bloc with a 2% aqueous uranyl solution at 4°C overnight, were gradually dehydrated in ethanol and finally embedded in Spurr resin followed by polymerisation for 3 days at 70°C.

For detergent-extraction of flagella (which facilitates observation of axoneme ultra-structure) (Witman et al., 1978), cells were treated with PEM (100 mM PIPES, 2 mM EGTA, 1 mM MgSO₄) containing 1% Nonidet P40 solution for 10 min (Sherwin and Gull, 1989). Washed samples were fixed in 2.5% glutaraldehyde, 0.5% tannic acid solution for two hours at room temperature and processed as described above. 60 nm thick sections were cut with a ultracut E Reichert microtome and picked up on copper-rhodium-coated grids. They were viewed using a Hitachi 7100 TEM and images were captured with a Hamamatsu C4742-95 digital camera. Images were analysed with Image J software (NIH).

Antibodies and immunofluorescence

Cells were spread on poly-L-lysine-coated slides and fixed in methanol at -20°C before processing as described (Sherwin et al., 1987). Indirect immunofluorescence was performed with the anti-PFR2 specific monoclonal antibody L8C4 (Kohl et al., 1999). DNA was stained with DAPI. Slides were viewed using a DMR Leica microscope and images were captured with a Cool Snap HQ camera (Roper Scientific). Images were analysed using the IPLab Spectrum software (Scanalytics).

Motility analysis

After induction of RNAi for the indicated periods, cell motility was analysed by video-microscopy (individual cell analysis) using differential-interfering-contrast or dark-field-microscopy. Beat frequency of individual flagella was measured using stroboscopic illumination (details below). Beat frequency was evaluated on individual cells by adjustment of the flash frequency with the beat frequency of the distal part of the flagellum (terminal 5-10 µm of the flagellum). Cells were observed with an Olympus BH2 microscope equipped with dry lenses (Zeiss 25×) or oil-immersion lenses. In the latter case, the objective lenses used were either an Olympus 40× D-Apo UV-oil 1.30 with diaphragm or a Reichert 100× Plan-oil 1.25 with iris, combined with an Olympus dark-field oil condenser DWC 1.4-1.2. Records were obtained using stroboscopic illumination by a Strobex (Chadwick-Helmuth, El Monte, CA). Video images were recorded with a Panasonic WV-F 15 E S-VHS video camera (constant frame rate of the European standard, 50 Hz) connected to a Hamamatsu video image processor (DVS 3000) and to a Panasonic AG 7330 S-VHS video recorder synchronized to the stroboscopic illumination with a fiber optic 'video-sync' (module number 9630, Chadwick-Helmuth). The S-VHS video tapes were digitalised by use of a Formac DV digitaliser connected to an Apple iBook G4. Montage of the Quick Time movies was obtained with the iMovie software. Interfering with flagellum motility reduces cell mobility and as a consequence cells stop swimming and sediment at the bottom of the culture flask, a factor that can be monitored in sedimentation assays (Bastin et al., 1999b).

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