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Abstract

Flagella and cilia are elaborate cytoskeletal structures conserved from protists to mammals, where they fulfil functions related to motility or sensitivity. Here we demonstrate novel roles for the flagellum in the control of cell size, shape, polarity and division of the protozoan *Trypanosoma brucei.* To investigate flagellum functions, its formation was perturbed by inducible RNA interference silencing of components required for intraflagellar transport, a dynamic process necessary for flagellum assembly. First, we show that down-regulation of intraflagellar transport leads to assembly of a shorter flagellum. Strikingly, cells with a shorter flagellum are smaller, with a direct correlation between flagellum length and cell size. Detailed morphogenetic analysis reveals that the tip of the new flagellum defines the point where cytokinesis is initiated. Second, when new flagellum formation is completely blocked, non-flagellated cells are very short, lose their normal shape and polarity and fail to undergo cytokinesis. We show that flagellum elongation controls formation of cytoskeletal structures present in the cell body that act as molecular organisers of the cell.

Keywords: cytokinesis/flagellum/morphogenesis/polarity/trypanosome

Introduction

Flagella and cilia are eukaryotic organelles whose core structure (a set of microtubules and associated proteins called the axoneme) shows remarkable conservation from protists to mammals (Silflow and Lefebvre, 2001). Despite their highly conserved structural architecture, flagella and cilia are involved in widely different functions: motility (of the cell or of material in its vicinity), reproduction and sensitivity (Bastin *et al*., 2000b; Silflow and Lefebvre, 2001; Tabin and Vogan, 2003).

Trypanosomes are flagellated protozoa belonging to the order of Kinetoplastida and are responsible for tropical diseases such as sleeping sickness. They possess a single flagellum, with a classic axoneme and an additional lattice-like structure called the paraflagellar rod (PFR) (Gull, 1999). In recent years, trypanosomes have turned out to be a useful model to study flagellum assembly and targeting (Bastin *et al*., 1999a; Godsel and Engman, 1999; Ersfeld and Gull, 2001). The flagellum emerges from a flagellar pocket and is attached along the length of the cell body with the exception of its distal tip. Within the cytoplasm adjacent to the flagellum, a defined set of cytoskeletal structures (called flagellum attachment zone or FAZ) lies underneath the plasma membrane and follows the path of the flagellum. The FAZ is composed of two individual structures: (1) a set of 4 microtubules, of distinct biochemical characteristics, connected to the smooth endoplasmic reticulum and (2) an electron-dense filament (Sherwin and Gull, 1989a). Both structures are initiated from the basal body area and run towards the anterior of the cell right up to its end. FAZ function is unknown but its positioning suggests a potential role in cellular organisation (Robinson *et al*., 1995). During cell replication, the old flagellum

is maintained, and a new one is assembled from a new basal body complex, always localised at the posterior end of the cell. FAZ structures are also replicated and associated to the new flagellum (Kohl *et al*., 1999). As the new flagellum elongates, its distal tip remains in constant contact with the old flagellum. A discrete cytoskeletal structure, termed the flagellum connector (FC), has been identified at this point, and is only present during cell duplication (Moreira-Leite *et al*., 2001). It has been postulated that the FC would anchor the tip of the new flagellum to the old flagellum. As a result, new flagellum assembly would take place following the path of the existing one and act as a guide to cell formation, hence replicating the existing organisation (Moreira-Leite *et al*., 2001; Beisson and Wright, 2003). Cytokinesis is initiated at the anterior end of the cell and proceeds on a helical manner, cutting the cell in two following the new flagellum/FAZ as axis (Vaughan and Gull, 2003).

The trypanosome flagellum is highly motile and the involvement of both the paraflagellar rod and the axoneme has been demonstrated (Bastin *et al*., 1998; Bastin *et al*., 1999b; Bastin *et al*., 2000a; Kohl *et al*., 2001; Durand-Dubief *et al*., 2003; McKean *et al*., 2003). However, trypanosomes with reduced motility remain viable in culture (Bastin *et al*., 1998). Judging from the omnipresence of the flagellum during the trypanosome life and cell cycle, we postulated that this structure could fulfil other functions than just motility and could be involved in the trypanosome cell cycle. To evaluate this possibility, we considered the possibility of perturbing flagellum construction by interfering with intraflagellar transport (IFT). IFT is defined as the transport of protein particles (“rafts”) between the outer doublet microtubules and the membrane of flagella or cilia (Rosenbaum and Witman, 2002). Transport towards the distal tip of the flagellum is powered by heterotrimeric kinesin II

(Kozminski *et al*., 1995) whereas particles are brought back towards the basal body by a dynein motor (Pazour *et al*., 1999; Porter, 1999). Inhibition of IFT prevents flagella or cilia assembly in most eukaryotes examined to date: *Chlamydomonas*, *Tetrahymena*, *Caenorhabditis elegans* and *Mus musculus* (Rosenbaum and Witman, 2002). It has been suggested that IFT is used to transport axoneme precursors to the distal tip of the flagellum, which is the site of assembly of the organelle (Rosenbaum and Witman, 2002).

Here, we report the identification of *T. brucei* homologues of several IFT proteins and the consequences of knocking down their expression by inducible RNA interference (RNAi). Firstly, trypanosomes with shorter flagella were obtained, demonstrating the importance of IFT for flagellum length control. Strikingly, cells bearing shorter flagella were smaller, with a linear relationship between flagellum length and cell size. These short cells retain normal organisation and could divide, with the anterior tip of their flagellum apparently defining the point of initiation of cytokinesis. Secondly, cells without a flagellum were even shorter, lost both their shape and polarity and could not divide. These results demonstrate novel roles for the flagellum in cell morphogenesis and division.

Results

IFT is required for flagellum formation in trypanosomes

By mining the *T. brucei* genome sequence data bases, we identified several trypanosome genes coding for homologues of proteins involved in IFT: either motor proteins or proteins present in cargoes but whose exact function is unknown. All the corresponding trypanosome proteins show a high degree of conservation, suggesting that IFT could be functional in trypanosomes. For example, TbDHC1b exhibits all specific signatures of dynein heavy chains involved in IFT (Figure 1A). One gene from each category was selected for this work: *TbDHC1b* (motor) and *TbIFT88* (cargo) respectively.

To address the question of a potential involvement of the flagellum in the trypanosome cell cycle (Robinson *et al*., 1995; Moreira-Leite *et al*., 2001), we decided to perturb flagellum formation by silencing *TbDHC1b* or *TbIFT88* via RNA interference (RNAi) (Fire *et al*., 1998; Ngô *et al*., 1998; Bastin *et al*., 2000a; Wang and Englund, 2001; Morris *et al*., 2002). Trypanosomes were transformed with plasmids able to express *TbDHC1b* or *TbIFT88* dsRNA under the control of an inducible promoter (Wang *et al*., 2000). After transfection and selection, recombinant cell lines were induced to express *TbDHC1b* or *TbIFT88* dsRNA by growth in the presence of tetracycline for 48 hours. In both cases, RT-PCR demonstrated a specific and efficient reduction in the amount of targeted RNA (Figure 1B). Cells from non-induced (TbDHC1b)RNAi or (TbIFT88)RNAi mutants behaved as normal trypanosomes. During the course of induction, cells from (TbDHC1b) RNAi and (TbIFT88) RNAi mutants were examined by light microscopy and the flagellum was

identified by immunostaining with an anti-paraflagellar rod antibody (Kohl *et al*., 1999). In both cases, non-flagellated cells were detected 48 hours after induction of RNAi (Figure 1C,D). Phenotypes looked very similar for both mutants except that kinetics appeared faster for *TbDHC1b* (Figure 1E). The proportion of non-flagellated cells increased rapidly to reach a maximum 70-80% of the population after 3 days (Figure 1E). This demonstrates the essential role of TbDHC1b (a motor protein) and of TbIFT88 (an unrelated protein present in IFT particles) in flagellum formation. Together with the presence of apparently *bona fide* trypanosome homologues of all proteins known to be involved in IFT in other organisms, these results strongly suggest the existence of IFT in trypanosomes and its functional conservation in flagellum assembly. A progressive reduction in both (TbDHC1b) and (TbIFT88) RNAi mutants growth rates was noticed during the course of induction of RNAi (Figure 1F), suggesting an important role for flagellum formation in trypanosome development.

Flagellum is required for cell shape and polarity

In both (TbDHC1)RNAi and (TbIFT88)RNAi mutants, non-flagellated cells exhibited a striking modification in cell shape. Normal trypanosomes are long and slender cells (Figure 2A), whose shape is defined by a corset of subpellicular microtubules (Sherwin and Gull, 1989a). They swim with the distal end of the flagellum leading, whilst cell shape is defined by a narrow *anterior* end and a wider *posterior* end, which is the site of microtubule elongation (Sherwin and Gull, 1989b). The nucleus is in a central position and the mitochondrial genome is localised at the posterior end of the cell. Trypanosomes possess a single mitochondrion containing a

concatenated DNA network called the kinetoplast that is tightly linked to the basal body of the flagellum (Robinson and Gull, 1991). This cellular organisation was severely perturbed in the non-flagellated cells: the distinct shape was lost and the kinetoplast appeared extremely close to the nucleus (Figure 2B). No posterior / anterior end could be recognised, neither for the whole cell, nor for its microtubular cytoskeleton (Figures 2B-C), dramatically demonstrating that the flagellum is required for definition of cell shape.

We then asked whether such cells were still polarised. As a polarity marker, we used an anti-clathrin antibody (Morgan *et al*., 2001). In trypanosomes, endocytosis takes place *via* a restricted area of the cell surface, called the flagellar pocket, situated at the base of the flagellum. In non-induced trypanosomes (Figure 2D), clathrin localised almost exclusively at the posterior end of the cell, between the kinetoplast and the nucleus (Morgan *et al*., 2001). In non-flagellated cells from (TbDHC1b) (data not shown) and (TbIFT88) (Figure 2E) RNAi mutants induced for 72 hours, clathrin was no longer localised at its defined position. Instead, it was found dispersed throughout the cytoplasm, suggesting severe defects in cellular organisation and polarity.

Polarised growth is another feature of the trypanosome cell, proceeding *via* extension and insertion of microtubules at the posterior end of the cell. This is illustrated by the mean of a specific monoclonal antibody that recognises only tyrosinated α-tubulin, a marker of tubulin recently incorporated in microtubules (Robinson *et al*., 1995 ; Sherwin and Gull, 1989b). In replicating normal cells (identified by the presence of two basal body complexes), this antibody stained the basal bodies, the posterior end of the cell and the distal tip of the new flagellum

(Figure 2F). In contrast, in (TbIFT88)RNAi mutants induced for 72 hours, 60.5 % (n=162) of the non-flagellated cells having duplicated their basal bodies did not show the typical polar incorporation of tyrosinated tubulin. Instead, relatively weak non-distinct staining was observed throughout the cytoskeleton (Figure 2G). In addition, 19.1 % of replicating non-flagellated cells exhibited incorporation of tyrosinated tubulin at both ends of their cytoskeleton (Figure 2H). These trypanosomes often display a nut-like shape. These results demonstrate that non-flagellated cells have lost cues for correct replication of their cytoskeleton.

Flagellum length determines cell size and cytokinesis

During the course of RNAi knock-down targeting *TbDHC1b* or *TbIFT88* RNA, we noticed the presence of a large number of cells with a flagellum shorter than usual (Figure 3A-B). The associated FAZ filament (red staining on figure) also looked shorter. Curiously, these cells looked smaller. To determine whether both phenomena were related, the total cell body size of 100 uniflagellated trypanosomes was measured in (TbDHC1b) (Figure 3C) and (TbIFT88) (data not shown) RNAi cell lines either non-induced, or induced for 24 or 48 hours and plotted versus the length of the flagellum. In both cases, there was a striking and almost linear correlation (r^2=0.834) between the length of the flagellum and the size of the cell. In other words, as trypanosomes grew shorter and shorter flagella due to the progressive inhibition of synthesis of IFT proteins, their cell body size was also smaller. To evaluate whether this reduction was affecting equally all cell parts or not, we measured distances from the anterior tip of the cell to the nucleus, from the nucleus to the kinetoplast and from the kinetoplast to the posterior end in the same cells as above (Figure 3D). These measurements showed that the reduction in size was

mostly caused by a reduction in the distance between the anterior end of the cell and the kinetoplast, i.e., the zone along which the flagellum is attached. In contrast, the distance between the kinetoplast and the posterior end of the cell was not modified. This amazing correlation suggests that flagellum elongation could control cell size.

In order to understand how the length of the flagellum could be involved in this process, we turned our attention to the “mother” cell from which shorter trypanosomes are derived. Trypanosomes divide by binary fission after having replicated basal bodies and kinetoplasts, FAZ and flagella and nuclear mitosis (Figure 3E, see below for more details). One simple explanation for the smaller size observed in cells with a short flagellum would be to postulate that elongation of the new flagellum is requested for normal cell growth at the posterior end (Figure 3E, top). In these conditions, the posterior end of the mother cell would be too short and the progeny inheriting the new flagellum would be too small. To evaluate this possibility, we measured cell body size and the length of the new flagellum in trypanosomes with 2 nuclei from (TbDHC1b)RNAi (Figure 3F) and (TbIFT88) RNAi (data not shown) cell lines either non-induced, or induced for 24 or 48 hours. These data showed that despite the production of shorter and shorter new flagella (and finally no new flagellum at all), cell elongation proceeded normally to reach ~25 µm. Therefore, cell elongation at the posterior end is not modified in induced (TbDHC1b) and (TbIFT88) (data not shown) RNAi cell lines and could not account for the observed results.

A second possibility to explain the smaller size of the cell with the short flagellum is that the tip of the new flagellum defines the position of cell cleavage (Figure 3E, bottom). In these conditions, cell cleavage would be “too posterior”,

producing a smaller daughter cell with the short new flagellum, and, since cell elongation proceeded normally, a larger daughter cell inheriting the old flagellum (of normal length). To evaluate this possibility we examined again measurements of cell size and flagellum length of the 100 uniflagellated cells from (TbDHC1b) (Figure 3C) and (TbIFT88) (data not shown) RNAi cell lines either non-induced, or induced for 24 or 48 hours described above. Strikingly, the size of the cell inheriting the old flagellum (that of normal length) progressively increased during the course of induction (notice the shift of red squares on Figure 3C). This strongly favours the hypothesis that the tip of the new flagellum defines the initiation of cytokinesis (Figure 3E, bottom).

In these conditions, it was surprising to find that non-flagellated cells could be produced at all as the new flagellum appears critical for cell division. To understand how these non-flagellated cells were born, we first examined the normal trypanosome cell cycle (Figures 4A-C). Cells in the early phase of their cell cycle possess a mature basal body (subtending the flagellum) and a pro-basal body, a single flagellum and the associated FAZ, a single kinetoplast and a single nucleus (Figure 4A). Basal bodies duplicate first, followed by mitochondrial genome replication and segregation (Sherwin and Gull, 1989a). The new basal body and the associated kinetoplast migrate towards the posterior end of the cell, which is increasing in length. A new FAZ is assembled, presumably nucleated from the basal body area, prior to the formation of the new flagellum (Kohl *et al*., 1999). The mature posterior basal body subtends the new flagellum that elongates towards the anterior end of the cell (Figure 4B). FAZ is also elongated at that stage, following the path of the new flagellum. Mitosis then occurs and the most posterior nucleus migrates

between the 2 kinetoplasts (Figure 4C). Cytokinesis is initiated from the anterior tip and proceeds in a helical manner towards the posterior end to generate two siblings, each inheriting a single nucleus, kinetoplast/basal body and flagellum/FAZ.

After 48 hours of induction, many (TbDHC1b) (Figures 4D-F) RNAi or (TbIFT88) (Figures 4G-I) RNAi mutant cells in duplication still possessed an old flagellum but no new flagellum was visible, neither by direct Nomarski or phase contrast observation nor by immunolabelling of flagellar components. Surprisingly, despite the absence of a new flagellum, these trypanosomes were able to assemble a short FAZ, initiated from the expected localisation at the basal body area and extending towards the anterior end where its tip seemed in contact with the old FAZ. However, this FAZ extended to roughly only one third of its normal length. In addition, its shape looked different: instead of the harmonious, undulating aspect observed in flagellated non-induced trypanosomes, it looked straight, almost stretched. This structure was immunolabelled by monoclonal antibody markers of both the FAZ filament (Kohl *et al*., 1999) (Figure 4 D-I) and of the 4 specialised microtubules associated to the FAZ (Gallo *et al*., 1988) (data not shown), suggesting that formation of these 2 structures is linked.

These cells underwent apparently normal nuclear mitosis, but the posterior nucleus appeared beyond the posterior kinetoplast/basal body (Figures 4E and 4H) instead of being between the 2 kinetoplasts/basal bodies (compare with Figure 4C). Cell measurements showed that nuclear positioning relative the posterior end was correct but kinetoplasts/basal bodies segregation was often incomplete. The interkinetoplast distance in binucleated cells without a new flagellum was 2.67 ± 0.46 µm for induced Tb(DHC1b)RNAi (n=40) or 3.60 ± 0.94 µm in the case of

(Tb(IFT88)RNAi (n=54) instead of 5.33 ± 0.47 µm (n= 101) in normal non-induced binucleated cells. As shown previously for cells assembling a short flagellum, cell body elongation at the posterior end proceeded normally (Figure 3F, cells with no new flagellum or flagellum length = 0). Such cells divided to generate two types: a short, non-flagellated sibling, with a limited FAZ filament (Figure 4J), and a longer, apparently normal flagellated sibling (see below). Interestingly, the anterior tip of the new, short, FAZ was always positioned at the initiation of the cleavage furrow (indicated by arrowheads on Figures 4F and I). These results show that even in the absence of a new flagellum, FAZ formation can be initiated but its elongation cannot proceed fully. However, these cells can divide, suggesting that the tip of the FAZ would be the critical determinant for initiation of cell division.

Flagellum connector in flagellated and non-flagellated trypanosomes

Recently, Moreira-Leite and co-workers demonstrated the existence of a particular cytoskeletal structure connecting old and new flagella during cell duplication (Moreira-Leite *et al*., 2001). They postulated that this structure could play an essential role in the cell cycle by directing the replication of the existing helical pattern typical of trypanosomes. Therefore, we looked whether the FC could still be assembled during the production of non-flagellated trypanosomes. First, we used a rabbit antiserum as a marker of the FC in immunofluorescence assay. In wild-type as in non-induced duplicating (TbDHC1b)RNAi or (TbIFT88)RNAi cells, this antibody stained the tip of the new flagellum and did not produce any significant signal in uniflagellated cells (Figures 5A-D). The tip of the old flagellum was not stained either. The signal was detected as soon as the new flagellum exited the flagellar

pocket (Figure 5B) and present throughout flagellum elongation (Figures 5C-D), as expected from a marker of the FC (Moreira-Leite *et al*., 2001). In (TbDHC1b) or (TbIFT88) RNAi mutant cells induced for 24-48 hours assembling a shorter new flagellum than usual, the FC was always detected by immunofluorescence analysis with the anti-FC marker antibody (Figure 5E). Surprisingly, about one third of cells without a new flagellum still possessed a FC signal on the old flagellum (Figure 5F), suggesting that at least some components of that structure could still be present. Unfortunately, the signal produced by the anti-FC antiserum was too weak to be used in double immunofluorescence with the anti-FAZ antibodies. Finally, no FC signal at all could be visualised in non-flagellated cells.

Electron microscopy analysis of negatively stained cytoskeletons (detergent-extracted cells) of biflagellated cells from non-induced (TbDHC1b)RNAi cell line confirmed the typical lamellar structure of the FC (Moreira-Leite *et al*., 2001), sitting on the axoneme (slightly bent at this point) from the old flagellum, with some fuzzy extensions towards the tip of the axoneme from the new flagellum (Figure 5G). In contrast, we could not identify a FC by electron microscopy examination of trypanosomes with an old but without a new flagellum (Figure 5H). This differs from the immunofluorescence analysis with the marker antibody. This result may be due to differences in the methods used for sample preparation (cells need to be detergent-extracted for electron microscopy analysis), or in the assay employed. Immunofluorescence could identify components present at the FC position, but these might not be assembled in a stable structure visible by electron microscopy. These images also revealed the total absence of axonemal microtubules from the new basal body in induced (TbDHC1b)RNAi cell (Fig. 5H).

Formation of FAZ structures is required for basal body segregation and cytokinesis

We next asked whether non-flagellated, non-polar trypanosomes with only a short FAZ could re-enter the cell cycle and duplicate and divide. In agreement with the role of the flagellum in the control of cell size, non-flagellated cells were tiny, showing a two-fold reduction of their cell body compared to normal trypanosomes (9.8 ± 2.2 µm, n= 121, instead of 19.4 ± 2.5 µm, n= 106). Non-flagellated cells could duplicate their basal bodies but no new FAZ filament was assembled (Figures 4K and 4L). Segregation of these duplicated basal bodies and kinetoplasts was seriously reduced in binucleated, non-flagellated *(TbIFT88)* RNAi mutants induced for 72 hrs. Only 28.4 % of them showed clear segregation of both basal bodies and kinetoplasts (Figure 4L) whereas 57.4 % segregated their basal bodies but these failed to migrate apart and had a single large kinetoplast stuck between them (Figure 4K), and the remaining 14.2 % did not segregate neither their basal bodies nor their kinetoplasts. These data can be correlated with the poor basal bodies/kinetoplasts segregation observed during the generation of non-flagellated cells and strongly suggest that FAZ/flagellum elongation is crucial for this segregation process. However, these cells could complete nuclear mitosis, but they failed to divide, looked arrested in their cell cycle, and presumably died. These data demonstrate that the presence of the old flagellum is required for formation of a new FAZ (cells with an old flagellum could produce a short FAZ, see Figure 4D-I) and reveal that in the absence of a new FAZ, cytokinesis cannot occur.

The importance of the FAZ for cell division was further demonstrated in a particular cell type: the flagellated siblings derived from cells like the ones shown on Figures 4F and 4I. These cells could reiterate once or twice the same cycle but then encountered some amazing difficulties. They still retained the old flagellum and could duplicate their basal bodies and kinetoplast, however, segregation and migration apart was highly inefficient and no new FAZ filament, neither FAZ-associated microtubules were assembled. Instead, FAZ proteins accumulated in patches of material dispersed around the basal bodies area whereas FAZ proteins associated to the filament along the old flagellum remained in place (Figure 4M). These cells did not undergo cytokinesis, yet nuclear mitosis still proceeded, producing multinucleated cells (Figures 4M,N). Taken together, these data show that formation of a new FAZ is required for cytokinesis. Mitosis without cytokinesis has been observed previously and is probably explained by the absence of that particular cell cycle checkpoint in trypanosomes (Robinson *et al*., 1995; Grellier *et al*., 1999; Ploubidou *et al*., 1999).

Discussion

IFT is required for flagellum formation

In this report, we have shown that silencing of two different proteins presumably associated with the IFT machinery lead to the inability of trypanosomes to form a new flagellum. A new basal body was formed, but it was not able to subtend a new flagellum (Figure 5F). Two different proteins were separately targeted: TbDHC1b, a motor protein and TbIFT88, a protein present in the IFT particles. Together with the presence of trypanosome orthologues of all IFT genes identified so far in other organisms, these data strongly suggest that IFT is functional in trypanosomes and is involved in flagellum formation. We could not directly identify movement of IFT rafts as elegantly demonstrated in *Chlamydomonas* (Kozminski *et al*., 1993) but structures morphologically resembling IFT rafts have been identified in cross-section of wild-type trypanosome flagella (Sherwin and Gull, 1989a; Bastin *et al*., 2000b). As in *Chlamydomonas* flagella, these rafts also localise between the B tubule of the peripheric doublets of the axoneme and the plasma membrane.

The requirement for the IFT machinery in cilia or flagella formation has been demonstrated in several very different flagellated organisms : protists such as the green algae *Chlamydomonas* (Kozminski *et al*., 1995) and the ciliate *Tetrahymena* (Brown *et al*., 1999), invertebrates such as sea urchin (Morris and Scholey, 1997) and nematodes (Signor *et al*., 1999) and vertebrates such as mouse (Nonaka *et al*., 1998). To date, trypanosomes would represent the oldest eukaryotes where the role of IFT proteins in flagella formation has been demonstrated.

The old flagellum appears unaffected by RNAi knock-down of either TbDHC1b or TbIFT88. This may look surprising knowing that in *Chlamydomonas*, IFT is

required for both formation and maintenance of flagella (Kozminski *et al*., 1995). In trypanosomes, maintenance of the old flagellum is probably explained by the fact that RNAi targets only RNA for degradation, hence proteins existing before expression of dsRNA are not affected (Bastin *et al*., 2000a; Moreira-Leite *et al*., 2001). This means that TbDHC1b and TbIFT88 proteins associated with the old flagellum should remain present. If IFT is also involved in maintenance of the old flagellum in trypanosomes (retrograde movement of non-assembled PFR proteins has been observed previously in fully grown flagella, Bastin *et al*., 1999b), then turn-over of trypanosome IFT proteins in an assembled flagellum appears remarkably slow. Indeed, old flagella are still present up to 7 days after silencing of *TbDHC1b* or *TbIFT88*.

IFT controls flagellum length and cell size

Strikingly, during the course of induction of RNAi silencing of *TbDHC1b* or *TbIFT88*, many cells assembled shorter flagella than normal. These flagella appeared functional and looked normal except for their length. In *Chlamydomonas*, IFT is required to control flagellum length by continuously balancing microtubule turn-over at the distal end of the axoneme (Marshall and Rosenbaum, 2001). This is illustrated by the temperature-sensitive mutant *FLA10*, that fails to assemble or to maintain its flagella when grown at the restrictive temperature. However, incubation of these cells at intermediate temperatures between restrictive and permissive leads to flagella of intermediate lengths (Marshall and Rosenbaum, 2001). These results suggest that alterations in IFT could lead to changes in flagellum length. In *T. brucei* RNAi mutants, inducible expression of dsRNA leads to destruction of RNA and inhibition of new protein synthesis, but existing protein synthesised before are not

affected and only disappear according to their turn-over rate (Bastin *et al*., 2000a). As a result, when RNAi gets into action, the amount of proteins coded by the targeted RNA is progressively reduced before being absent. In the case of our *T. brucei* IFT mutants, one could imagine that as the pool of TbDHC1b or TbIFT88 protein is reduced, it is still sufficient for initiating flagellum assembly but not enough for supporting the construction of a full-length flagellum. This result suggests that regulating the amount of available IFT proteins could act as a way to regulate flagellum length.

The link between flagellum length and cell size was even more spectacular. As the cells grew shorter flagella, cell body size appeared smaller (Figure 3). The correlation was almost linear and is explained by a shorter distance between the anterior tip and the kinetoplast, i.e. the part of the cell body flanked by the flagellum. Our measurements have shown that this was not due to a failure in cell body elongation but rather to mis-positioning of the cleavage furrow (Figure 3), suggesting that the tip of the flagellum (or of the associated FAZ) could define the point of initiation of division.

Trypanosomes are parasitic organisms with a complex life cycle where they alternate between an insect vector and a mammalian host. Due to the changes in environment, trypanosomes need to adapt to each condition and react by activating various biochemical pathways and by changing their surface coat (Matthews, 1999). These changes are accompanied by extensive modulation of cell size and shape (Vickerman, 1985) and interestingly the flagellum follows these changes tightly. The “procyclic” trypanosome stage that was used for this study normally colonise the midgut of a tsetse fly, and its size varies between 20 and 25 µm. In the next part of

the life cycle, trypanosomes become much longer (up to 40 µm), and this increase in cell size is correlated with an increase in flagellum length (Van Den Abbeele *et al*., 1999). When trypanosomes are present in the bloodstream of their mammalian host, two stages can be discriminated: the long slender, proliferating form, and the short stumpy, non-proliferating but differentiating form (from bloodstream to insect stage). The slender to stumpy differentiation program incorporates a round of cell division. Interestingly, when such dividing cells were examined just prior to cytokinesis, the average length of the new flagellum was shorter than the one measured from simply replication slender cells at the same stage (21 µm instead of 25 µm) (Tyler *et al*., 2001). It is tempting to speculate that regulating the amount of functional IFT particles act as a way to control both flagellum length and cell size.

Finally, it should be noted that the “amastigote” form of *Trypanosoma cruzi*, a related trypanosome species, possesses a very short flagellum not extending beyond the flagellar pocket and its cell body size and shape resembles that of non-flagellated *T. brucei* mutants described here (de Souza, 1984).

FAZ as a molecular organiser of the cell

As discussed above, the tip of the new flagellum seems to dictate where cytokinesis will initiate. Surprisingly, binucleated cells without a new flagellum but with an old one are still able to divide and to produce a flagellated and a non-flagellated progeny (Figure 4D-I). This means that presence of the new flagellum alone is not responsible for cleavage initiation. We propose that this function is actually played by the FAZ. In all cells without a new flagellum but with an old one, a new FAZ was still made, as shown by staining with markers for the FAZ filament or

for the 4 microtubules, but it was much shorter than normal. The extremity of that structure looked always in contact with the old FAZ (Figures 4D,E,G,I). When such cells were observed during early stages of cytokinesis, the anterior end of the FAZ was always positioned at the point where cleavage seemed to have been initiated (Figure 4F&I). In agreement with a role for FAZ in cytokinesis, cells that are not able to produce a new FAZ fail to undergo cytokinesis. This is the case of all non-flagellated daughter cells (Figure 4K), but also of the progeny that inherited the old flagellum. Although this flagellated daughter can reiterate once or twice the same cycle as its mother, it then fails to assemble a new FAZ and also fails to undergo cytokinesis (Figures 4M-N). In addition, when the new FAZ is absent, segregation of the basal body/kinetoplast complex is severely reduced when not completely abolished (Figure 4K,M), suggesting that FAZ could also be involved in basal body segregation. This is further supported by the incomplete segregation of basal bodies in cells producing only a short new FAZ without a new flagellum (interkinetoplast distance reduced to 2-3 µm instead of 5 µm in non-induced cells).

A model for the role of IFT, flagellum and FAZ in trypanosome cell cycle and division

The contribution of the flagellum and its associated structures (FAZ and FC) to cell morphogenesis and cell cycle can be summarised in the following working model. First, basal body duplicates (Sherwin and Gull, 1989a) and a new FAZ is assembled, prior to flagellum exit from the flagellar pocket (Kohl *et al*., 1999). These steps are independent from the formation of the new flagellum as they still take place in (TbDHC1b) and (TbIFT88) RNAi mutant cells with an old flagellum but without a

new one (Figure 4D-I). Next, flagellum elongates and somehow drives FAZ elongation. From that point in the cell cycle, FAZ elongation is controlled by flagellum growth as production of flagellum that do not reach wild-type length also leads to incomplete FAZ. As FAZ elongates, it could participate to basal bodies segregation. In the absence of a new flagellum, FAZ is much shorter and basal bodies segregation is less efficient.

During the whole process, cytoskeleton elongation occurs normally at the posterior end (Sherwin and Gull, 1989b), a process apparently independent of flagellum presence. Once flagellum growth is terminated, FAZ elongation also finishes, the FC is disassembled and the cell initiates cleavage at the anterior end of the FAZ, following the helical path of that structure. In the absence of a new flagellum, FAZ does not elongate properly but cell growth continues unabated, nuclear mitosis takes place, and the posterior nucleus migrates in a normal position relative to the posterior end, suggesting that these processes are independent of flagellum formation. Because of the reduced kinetoplast/basal body separation, the 2 basal body/kinetoplast complexes are now sandwiched between the 2 nuclei. The presence of the short FAZ seems sufficient to initiate division. When it cleaves, it produces a shorter non-flagellated progeny, with its basal body/kinetoplast complex very close to the nucleus, and a longer, flagellated, progeny, with an excessively long posterior end. FAZ could also be involved in cell polarity as non-flagellated cells shows rapid loss of polarity and organisation. The non-flagellated sibling can duplicate its basal body/kinetoplast complex but these organelles are poorly or not segregated and the cell fails to divide. No new FAZ is assembled in these cells, suggesting that the presence of the old flagellum is required for formation of FAZ.

The flagellated progeny can reiterate once or twice the cycle described above but then also fails to assemble a new FAZ and to undergo cytokinesis. It should be noted that this flagellated cell retains its elongated shape and polarity.

Conclusion

Our results demonstrate a crucial role for the flagellum in definition of cell shape, polarity and size, as well as in initiation of cytokinesis and basal bodies segregation. Modulation of flagellum length via IFT could represent an original way to control cell size and shape, as suggested by the variations of these parameters during the trypanosome life cycle. Flagellum control of cell morphogenesis is probably mediated by the formation of the FAZ structure (FAZ filament and the four associated microtubules). Understanding how these cytoskeletal elements are assembled and controlled promises to reveal more intriguing cell biology features.

Materials and Methods

Identification of *TbDHC1b* and *TbIFT88* genes and generation of RNAi mutants

The TIGR (Institute for Genomic Research) and Sanger Centre *T. brucei* data bases (TIGR genome project www.tigr.org/tdb/mdb/tbdb, Sanger genome project http://www.sanger.ac.uk/Projects/T_brucei/) were screened by BLAST search using the full-length sequence of the *Chlamydomonas DHC1b* and *IFT88* genes. Different homologous sequences were identified and the genes were re-constructed based on alignments with available gene sequences. Sequences have been submitted to GenBank data base as BK000491 (TbDHC1b) and AF521959 (TbIFT88). For RNAi, a segment of the *TbDHC1b* or of the *TbIFT88* gene was amplified by PCR from genomic DNA and cloned between 2 facing regulatable T7 promoters in the pZJM vector allowing tetracycline-inducible expression of double-stranded (ds) RNA (Wang *et al*., 2000). Primers designed to amplify an 832 bp internal region of *TbDHC1b* (position 399-1230 of the *T. brucei* nucleic acid sequence, corresponding to amino acids 1801-2076 of the *Chlamydomonas* DHC1b sequence, accession number CAB56748 (Porter *et al.*, 1999)) were 5'-CGATGAATTCCTCGAGTCAAAATAGATCAGCTTTCAG-3' and 5'-CGATCGAAGCTTCCCAAAAGCTGCTGTCGGTG-3'. This region is only conserved in DHC1b proteins and not in other dyneins. Highest overall identity of that region with other dyneins reaches 53-57 % with no regions >14 bp of total identity (data not shown). Such an identity is not high enough to generate cross RNAi in the system we used (Durand-Dubief *et al*., 2003). For *TbIFT88*, a 941 bp internal region (positions 301-1241 of the *T. brucei* nucleic acid sequence, corresponding to amino acids 353-668 of the *Chlamydomonas* IFT88 sequence, accession number AF298884 (Pazour

et al., 2000)) was amplified using primers 5' CGATGAATTCCTCGAGGATTAAGGAGGAAC GTACAC-3' and 5'CGATCGAAGCTTAGCGCCTC GTAGAGTCGCTTG-3'. No related sequence could be identified in the *T. brucei.* Amplified fragments were ligated in the pZJM vector and control-sequenced. Plasmids were linearised with *Not* I and electroporated in the 29-13 procyclic trypanosome cell line that expresses T7 RNA polymerase and tet-repressor (Wirtz *et al*., 1999). Transfected cells were plated and selected by addition of 2 µg phleomycin per ml of medium. For screening (Bastin *et al*., 1999a), resistant cells were grown with (induced) or without (non-induced) 2 µg tetracycline per ml for 48 hours, fixed and processed by immunofluorescence using the anti-PFRA monoclonal antibody L8C4 as a marker of the flagellum (Kohl *et al*., 1999). For longer induction experiments, 1 µg of fresh tetracycline was added daily.

Immunofluorescence and phenotype analysis

Cells were spread on poly-L-lysine coated slides and fixed in methanol at –20°C before rehydration and processing for immunofluorescence as described (Sherwin *et al*., 1987). The following antibodies were used: L8C4 (Kohl *et al*., 1999) (marker of the flagellum), L3B2, L6B3 (Kohl *et al*., 1999) and DOT-1 (Woods *et al*., 1989) (markers of the FAZ filament), 1B41 (Gallo *et al*., 1988) (marker of the 4 specialised microtubules), BBA4 (Woods *et al*., 1989) (markers of the basal body), YL1/2 (Kilmartin *et al*., 1982) (anti-tyrosinated α-antibody, marker of recently assembled tubulin) and anti-clathrin heavy chain (Morgan *et al*., 2001). Slides were viewed using a DMR Leica microscope and images were captured with a Cool Snap HQ

camera (Roper Scientific). Images were analysed and cells parameters were measured using the IPLab Spectrum software (Scanalytics).

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Legends to figures

Figure 1. TbDHC1b and TbIFT88 are required for flagellum assembly. (A) Partial alignment of protein sequences of DHC1b central domain from *T. brucei* (Tb), *C. reinhardtii* (Cr), *C. elegans* (Ce)*, D. melanogaster* (Dm), *H. sapiens* (Hs) and the cytoplasmic dynein from *S. cerevisae* (Sc). Conserved residues are shown in blue, green and red blocks indicate dynein and DHC1b signatures respectively. (B) RT-PCR analysis demonstrates specific reduction of *TbDHC1b* or *TbIFT88* RNA amounts in induced samples. Total RNA extracted from non-induced and 48 hrs-induced (TbDHC1b)RNAi or (TbIFT88)RNAi mutants was used as templates for RT-PCR using specific primers for *TbDHC1b*, *TbIFT88* or an unrelated RNA as a control. Knock-down of*TbDHC1b* (C) or of *TbIFT88* (D) lead to production of non-flagellated cells over time. Non-induced (left), 48 hrs (middle) and 72 hrs (right) induced cells were stained with the anti-PFRA antibody L8C4 as a marker of the flagellum (green) and with DAPI (blue), showing nucleus and kinetoplast (mitochondrial genome) staining. Pictures were merged with the phase contrast image. (E) RNAi was induced in (TbDHC1b)RNAi or (TbIFT88)RNAi cell lines, cells were fixed at indicated times and processed for immunofluorescence with the anti-PFRA. Cells possessing at least one flagellum were scored as positive. Arrows indicate when nonflagellated cells start to be detected. (F) During the same experiment, cell growth was monitored in the presence (+, induced) or absence (-, non-induced) of tetracycline.

Figure 2. Non-flagellated trypanosomes lose cell shape and polarity. (A,B) Cells were stained with the anti-PFRA antibody as a marker of the flagellum (green) and with

DAPI (blue). (A) Non-induced (TbDHC1b)RNAi cell showing the typical trypanosome polarity, asterisk indicates the posterior end of the cell. (B) (TbDHC1b)RNAi trypanosome induced for 48 hours without a flagellum did not exhibit normal cell shape. (C) Electron micrograph of a (TbDHC1b)RNAi non-flagellated cell (48 hrs induction) revealing absence of polarity and partial microtubule disorganisation. (D,E) Phase contrast images of trypanosomes stained with DAPI (blue, left panels) and with anti-clathrin antibodies (red, right panels). Non-induced (TbIFT88) RNAi cells (D) showed a defined signal at the posterior end, between the kinetoplast and the nucleus, whereas non-flagellated (TbIFT88) RNAi cells induced for 72 hours (E) showed dispersion of clathrin vesicles throughout their cytoplasm. (F-H) Non-flagellated trypanosomes exhibit abnormal cell growth. Phase contrast images of (TbIFT88) RNAi trypanosomes stained with DAPI (blue, left panels) and with the anti-tyrosinated tubulin antibody YL1/2 (yellow, right panels). (F) Non-induced trypanosome, showing normal polarised cell growth that takes place by microtubule elongation at the posterior end (arrows). The arrowheads indicate the position of the new flagellum. (G,H) (TbIFT88)RNAi cells induced for 72 hours: incorporation of tyrosinated tubulin was seen throughout the cytoskeleton (G) or at 2 opposite ends (H).

Figure 3. Flagellum length controls cell size. (A,B) Field of (TbDHC1b)RNAi trypanosomes induced for 48 hours. Flagellum was labelled with monoclonal antibody L8C4 (green), FAZ filament with L6B3 (red lines) and basal body with BBA4 (red spots) and cells were counterstained with DAPI (blue). (A) DIC image, (B) merged fluorescence. Cells with a shorter flagellum appeared smaller (arrows) than

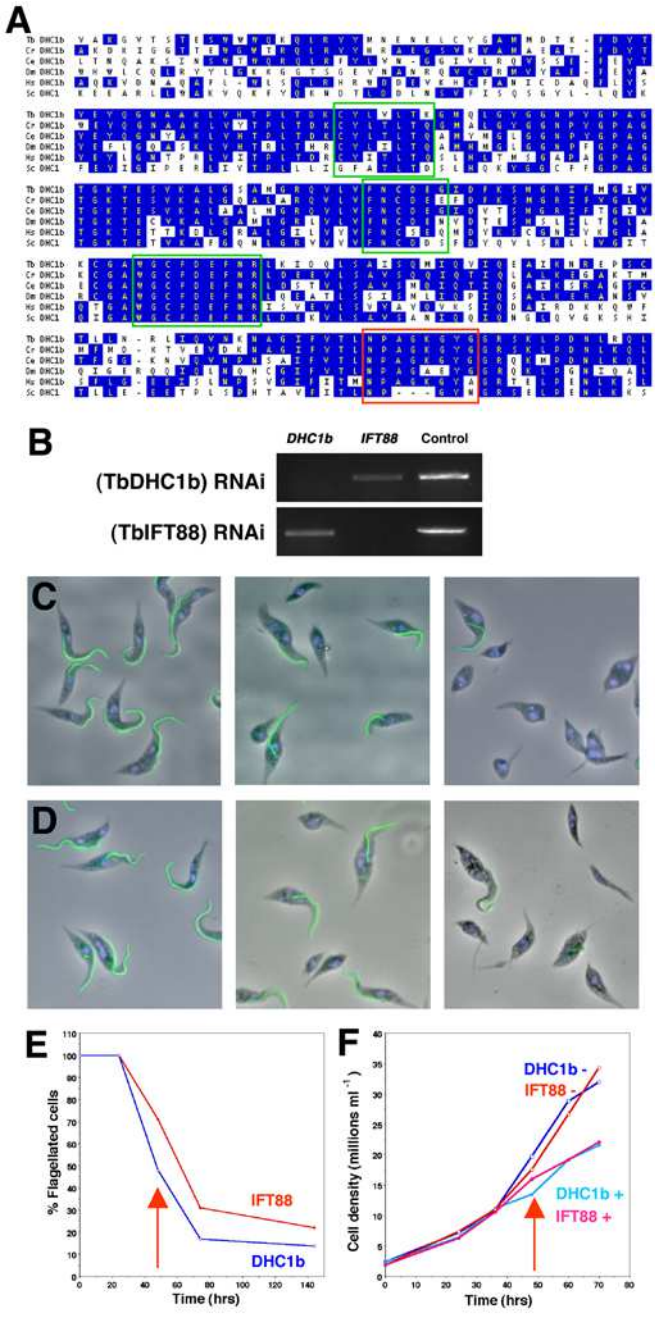
cells with a normal flagellum (arrowheads). (C-D,F) (TbDHC1b)RNAi trypanosomes were either non-induced (black squares), or induced for 24 (blue squares) or 48 hours (red squares). (C) Uniflagellated cells. Cell body size was measured and plotted versus the measured flagellum length. Each point represents an individual cell. (D) In the same uniflagellated trypanosomes, the distance between the anterior tip of the cell and the kinetoplast was measured and plotted versus flagellum length. Non-flagellated cells were not included as recognition of posterior/anterior end is virtually impossible (see text and Figure 2). (E) Two models for the generation of small cells with short flagella. Nucleus is shown in blue, flagellum in green, FAZ in red and basal body/kinetoplast (shown as a single circle for simplicity) in black. Normal cell size of a binucleated (“2K2N”) or of a uninucleated (“1K1N”) trypanosome is delimited by the dashed lines. Point of cleavage initiation is indicated by the thick arrow and probable cleavage progression by the dashed arrow (see text for details). (F) Biflagellated cells. Total cell size was measured and plotted versus the measured new flagellum length. Although the assembled new flagellum gets shorter or is even absent, total cell body size remains as in non-induced trypanosomes.

Figure 4. New and old flagellum are required for cell morphogenesis. (A-C) Cell cycle of normal, non-induced, (TbDHC1b)RNAi trypanosomes. Flagellum was labelled with monoclonal antibody L8C4 (green), FAZ filament with L6B3 (red lines) and basal body with BBA4 (red spots) and cells were counterstained with DAPI (blue). Top panels, DIC merged with flagellum staining (green), bottom image, merged fluorescence. Yellow arrows indicate kinetoplast position. After 48 hours of induction, (TbDHC1b)RNAi (D-F) and (TbIFT88)RNAi (G-I) trypanosomes cannot

assemble a new flagellum but nevertheless formation of a short new FAZ is observed. Panels are as (A-C) except that (TbIFT88)RNAi cells were shown by phase contrast (as for the whole figure). (J-K) Non-flagellated (TbIFT88)RNAi cells (induced for 72 hrs) stained with the anti-basal body BBA4 and anti-FAZ L6B3 (both in red) and counterstained with DAPI (blue). Kinetoplasts segregation failed, even after nuclear mitosis was completed (K). (L) Non-flagellated (TbDHC1b)RNAi cells (induced for 72 hrs) stained with the anti-basal body BBA4 and anti-FAZ L6B3 (both in red) and counterstained with DAPI (blue). Although basal body duplication and segregation occurred, no new FAZ formation was detected. Similar observation was made for the 4 FAZ-associated microtubules, identified by immunolabelling with antibody 1B41 (not shown). (M,N) After 72 hrs induction, flagellated cells have been immunolabelled by antibody 1B41 (red) and stained with DAPI (blue), showing lack of assembly of FAZ-associated microtubules, accumulation of patches of material around the basal bodies area and absence of cytokinesis.

Figure 5. The flagellum connector (FC) in wild-type and in induced (TbIFT88)RNAi cells. (A-D) Normal wild-type trypanosomes were stained with an anti-FC antiserum. Left panels, phase contrast image merged with DAPI staining (blue) and anti-FC immuno-staining (green). Central panels, FC staining only. Right panels: magnification of the area where the FC has been identified. Top, phase contrast image only. Bottom, phase contrast image merged with FC staining (green). Yellow star indicates the position of the old flagellum, yellow arrow indicates the tip of the new flagellum. (A) The left cell has got only one flagellum, its tip is not stained. The right cell has a new flagellum in the process of elongation and its distal tip shows

discrete and well defined staining. In contrast, the distal tip of the old flagellum is not stained. Similar observations were made as the new flagellum elongated (B-D). (E-F) (TbIFT88)RNAi trypanosomes were induced for 36 hours and stained with the anti-FC antiserum (green). (E) Binucleated cell whose new flagellum is too short (compare with D). FC is still present. (F). Binucleated cell without a new flagellum. FC is still present on the old flagellum of ~30% of these cells. (G) Electron micrograph of a negatively stained cytoskeleton of (TbDHC1b)RNAi non-induced trypanosome. This cell is biflagellated and the discrete pyramidal structure of the FC can be recognised in the enlarged box. (H). Electron micrograph of a negatively stained cytoskeleton of (TbDHC1b)RNAi trypanosome induced for 48 hours. The old basal body subtends the flagellum (enlarged left box) whereas no flagellum at all is visible on the new basal body (enlarged right box). In these conditions, the FC is not detected.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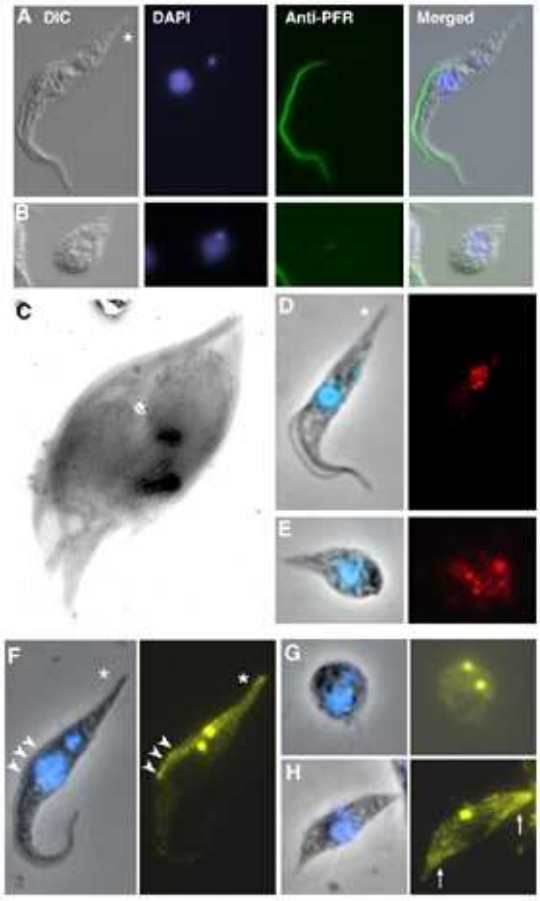
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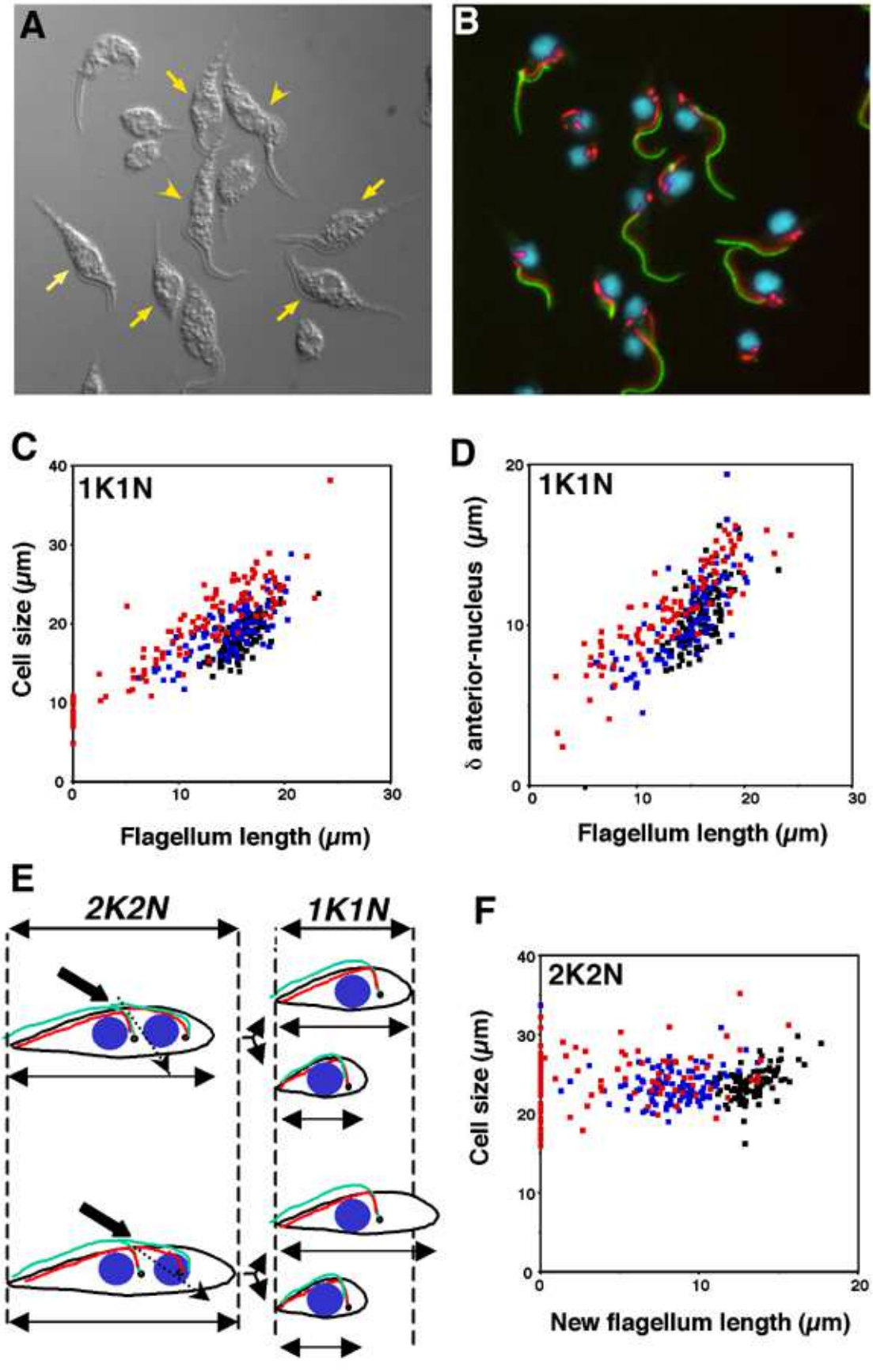
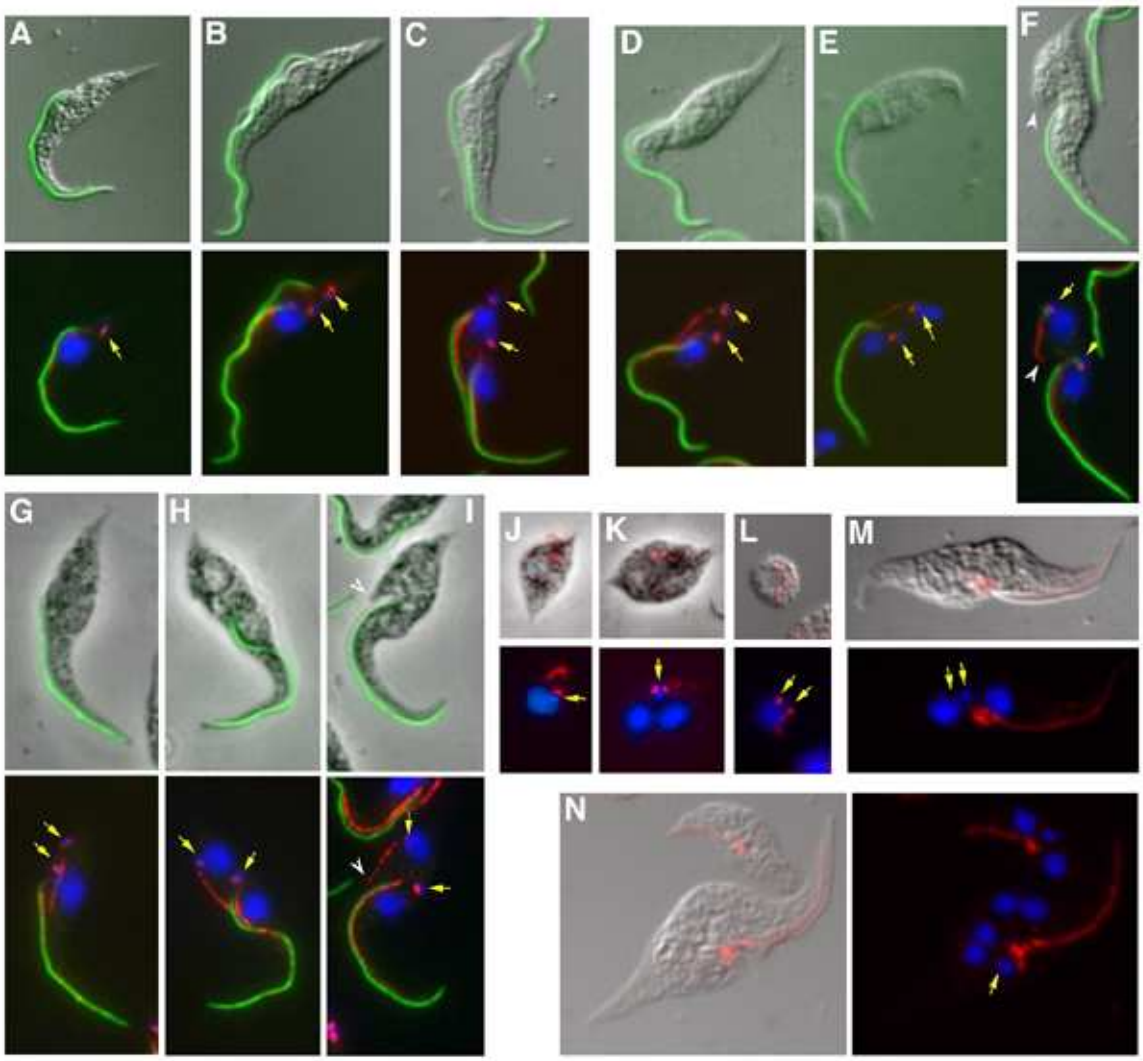
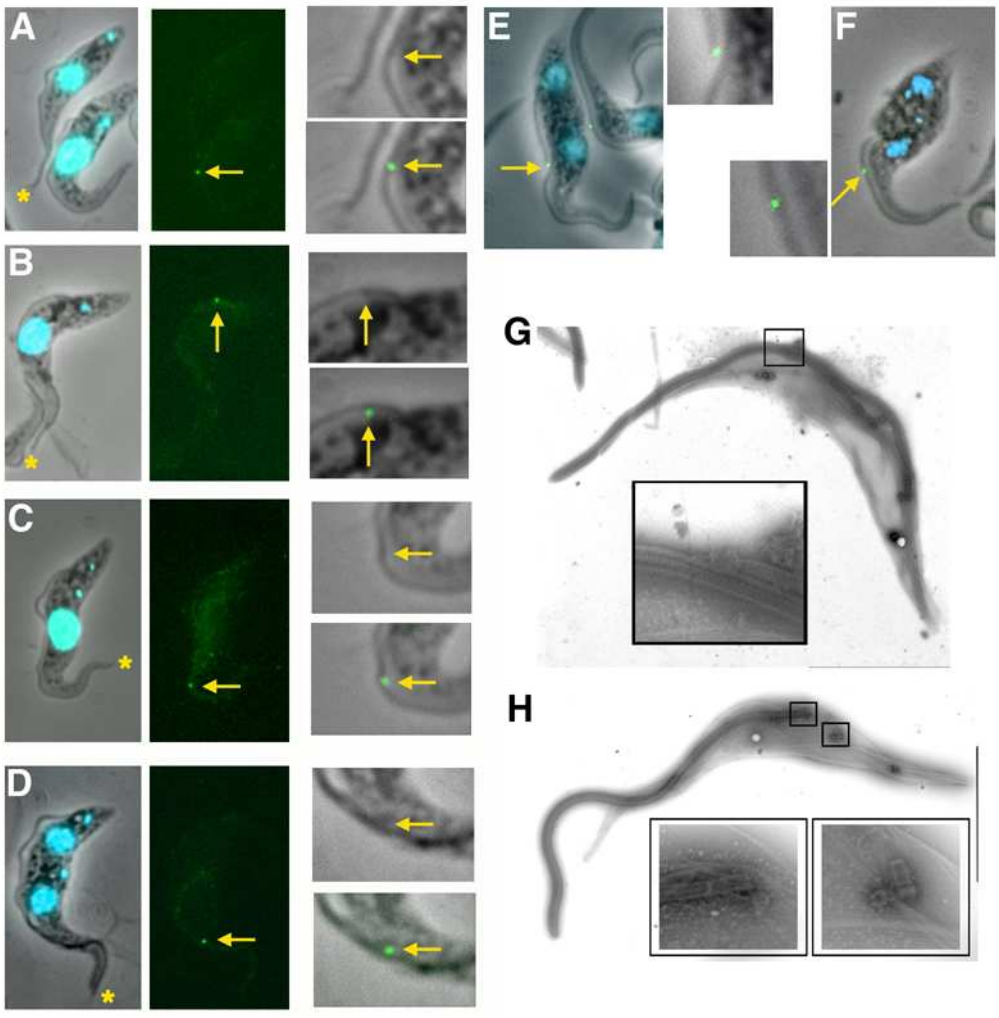
G
H


A
B
C
1K1N
Cell size (μm)
Flagellum length (μm)
D
1K1N
δ anterior-nucleus (μm)
Flagellum length (μm)
E
2K2N
1K1N
F
2K2N
Cell size (μm)
New flagellum length (μm)


A
B
C
D
E
F
G
H
I
J
K
L
M
N


A
B
C
D
E
F
G
H