# A Trypanosome Structure Involved in Transmitting Cytoplasmic Information During Cell Division

Flávia F. Moreira-Leite, Trevor Sherwin,* Linda Kohl,† Keith Gull‡

**African trypanosomes are protozoan parasites that cause sleeping sickness in humans through a tsetse fly vector. The procyclic form of *Trypanosoma brucei* has a single, attached flagellum that describes a helical path along the cell from posterior to anterior. During division, a specific flagellum-flagellum connection is elaborated between the new and old flagellum. This connector was present only during cell duplication and was found to be involved in the replication of the helical cell pattern and polarity. This finding implicates the concept of cytotaxis in cell morphogenesis in trypanosomes.**

A procyclic (tsetse form) trypanosome has a single flagellum that emerges from an invagination of the plasma membrane termed the "flagellar pocket," close to the posterior end of the cell. The flagellar pocket is the sole site for membrane traffic to and from the cell surface. The flagellum is attached to the cell body throughout most of its length and follows a left-handed helical path toward the anterior end. Along the region of attachment, known as the flagellum attachment zone (FAZ) (*1, 2*), the membranes of the flagellum and cell body are intimately apposed. Inside the cell, this region of apposed membranes is associated with two internal cytoskeletal structures—the FAZ filament and a subset of four specialized microtubules—both of which run from the basal body to the anterior end of the cell, underlying the flagellum. The flagellar cytoskeleton is cross-linked to the cell body cytoskeleton through the FAZ, and the arrangement of these structures confers the typical polarity and helicity of a trypanosome cell. During cell duplication, unique mechanisms such as the interaction between the flagellum and the mitochondrial DNA are involved in the rigorous replication of this cell pattern and polarity (*3*).

The emergence of a short new flagellum (Fig. 1A) from the flagellar pocket is one of the earliest events in cell duplication of *T. brucei*. Later, separation of the basal bodies (which subtend the proximal ends of the flagella) produces a second flagellar pocket for the new flagellum (Fig. 1B) and mediates the segregation of the replicated mitochondrial DNA in the kinetoplasts (*3*). The new flagellum emerges closer to the posterior end of the cell and is always on the left side of the old flagellum (Fig. 1B) when the cell is viewed from its posterior end. Positioning of the new flagellum and FAZ defines a pattern that influences the helical arrangement of the cytoskeleton, the axis and polarity in cell division and cell shape, the position of membranous organelles, and the direction of motility of daughter cells (*2, 4*).

Examination of the cell cycle of *T. brucei* by scanning electron microscopy (SEM) (*5*) revealed that, although the proximal ends of the flagella moved apart during division, the distal tip of the new flagellum remained associated with the old flagellum throughout cell duplication (Fig. 1, B and C). The distal tip of the new flagellum always contacted the lateral aspect of the old flagellum, and always from the left when the cell was viewed from the posterior end.

We were prompted to investigate this phenomenon further because it indicated the existence of a specifically localized flagellum-flagellum association during duplication. This would implicate the position and orientation of the old flagellum in directing the morphogenesis of the new flagellum and, consequently, of the internal cytoskeleton.

When we observed negatively stained cytoskeletons (detergent-extracted cells) of dividing trypanosomes by transmission electron microscopy (TEM) (*5*), we noted the presence of a characteristic structure at the distal tip of the new flagellum (Fig. 2). We termed this structure the "flagella connector" (FC) because it appeared to link the two flagella. The FC had a roughly triangular profile (Fig. 2, B to E) and was present only at the distal tip of the new flagellum. This corresponded exactly to the position where, as determined by SEM, the new flagellum was associated with the old (Fig. 1). Thus, it could be that the FC mediates the physical attachment of flagella and that this attachment is detergent-resistant. The FC has a lamellar core (Fig. 2, C to E), which may represent segments of membrane rendered detergent-resistant by proteins responsible for

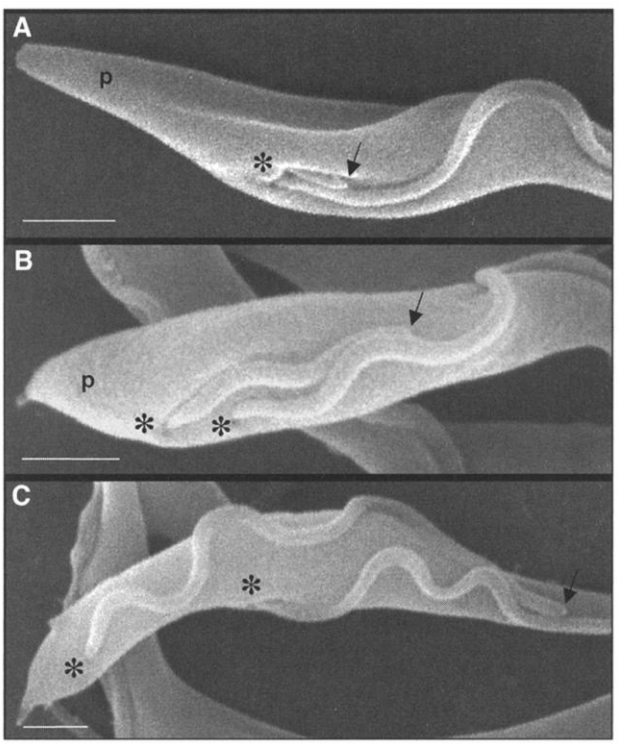


**Fig. 1.** The distal tip of the new flagellum remains associated with the old flagellum during cell duplication of *T. brucei*, even as the proximal ends of both flagella move apart. (**A** to **C**) SEM micrographs of trypanosomes in different stages of the cell cycle with new flagella of different lengths: late $G_1$/S (A), $G_2$ (B), and postmitotic (C) phases. Arrows point to the tip of the new flagellum; asterisks mark the region of the flagellar pocket and the basal body (p, posterior end of the cell). Scale bars, 1 μm.

School of Biological Sciences, 2.205 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, UK.

*Present address: Discipline of Ophthalmology, Faculty of Medicine and Health Sciences, University of Auckland, Auckland, New Zealand.

†Present address: Muséum National d'Histoire Naturelle, Laboratoire de Biophysique, 43 rue Cuvier, 75231 Paris Cedex 05, France.

‡To whom correspondence should be addressed. E-mail: k.gull@man.ac.uk

the attachment. The *T. brucei* flagellum contains, in addition to the axoneme, a massive paracrystalline structure—the paraflagellar rod (PFR) (*6*). Examination of cytoskeletons showed that the FC connected the distal tip of the axoneme of the new flagellum to the lateral aspect of the axoneme, but not the PFR, of the old flagellum (Figs. 2 and 3).

We used two approaches to test whether the FC was indeed performing the flagellum-flagellum attachment independent of other internal cell body components. First, we treated whole-mount cytoskeletons with 60 mM $Ca^{2+}$ (*5*), which depolymerizes the subpellicular microtubules, leaving the axonemal microtubules and the PFR intact (*7*). The FC not only survived such treatment, but its substructure became even more clearly distinguishable (Fig. 3A). The FC appeared to have three main subcomponents: a "fuzzy" component at the extreme distal tip of the new flagellum, a short link structure, and a plate-like structure with a lamellar core that "sits" directly on the microtubules of the axoneme of the old flagellum (Fig. 3A).

The morphology of the connector, as seen in whole cytoskeletons and $Ca^{2+}$-flagella preparations, supports the idea of a junctional complex between two membrane domains, linked (directly or indirectly) to the internal axonemal microtubules. Because the FC changed position in the old flagellum as the new flagellum extended, the junctional complex must be moving along the old axoneme toward its distal tip, a motility that could be powered by its association with kinesin-type motor proteins.

The second approach used to test the localized "tip-to-side" flagellum-flagellum attachment hypothesis was to produce trypanosomes where flagellum–cell body attachment was disrupted. Trypanosomes lacking flagellum–cell body attachment were made by inducible double-stranded RNA interference (RNAi) (*8–10*) of the gene encoding Fla1 (*11*), a surface membrane glycoprotein vital for flagellum–cell body attachment in *T. brucei* (*12*) and the related human parasite *T. cruzi* (*13*). Inducible RNAi of Fla1 was produced by inserting a 1-kb segment of its coding sequence in the pZJM vector (*14*) and stably transfecting procyclic trypanosomes (*15, 16*).

After growing a nonsynchronous culture of stably transfected cells for 24 hours in the presence of tetracycline (to induce RNAi of Fla1), we specifically observed the subpopulation of cells in late $G_2$ or mitosis, because in these cells it was easy to discern both old and new flagella within the same cell (*17*). Our previous work (where cells in this stage of the cell cycle were particularly informative) showed that inducible RNAi in trypanosomes can act to ablate a flagellum protein in the new flagellum while leaving the old flagellum unaffected (*10*). Induction of RNAi in Fla1 produced trypanosomes with a new flagellum forming a loop detached from the cell body for most of its length, but remaining tethered to the old flagellum by the tip FC (Fig. 3B). In these cells, the old flagellum remained attached to the cell body and the ultrastructure of the FC appeared unaltered (Fig. 3B). An indication of the strength of FC attachment came from the observation of live induced cells by differential interference contrast microscopy. Films of these cells (*18, 19*) show that the loop of the new flagellum beats vigorously and freely while the distal tip

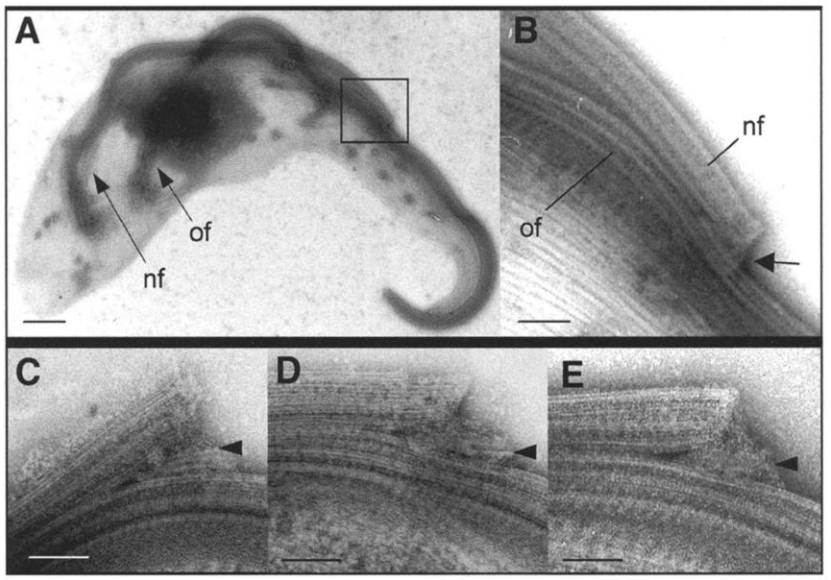


**Fig. 2.** A characteristic detergent-resistant structure, the flagella connector (FC), is present at the tip of the new flagellum and contacts the old flagellum. (**A**) Negatively stained cytoskeleton of a trypanosome undergoing cell duplication (nf, new flagellum; of, old flagellum). (**B**) Higher magnification of the boxed area in (A), showing the FC at the tip of the new flagellum, linking the tip of the axoneme of this flagellum with that of the old flagellum. (**C** to **E**) Images of the FC in different cells, showing the central lamellar core (arrowheads), possibly representing membrane remnants. Scale bars, 1 μm (A), 200 nm (B to E).

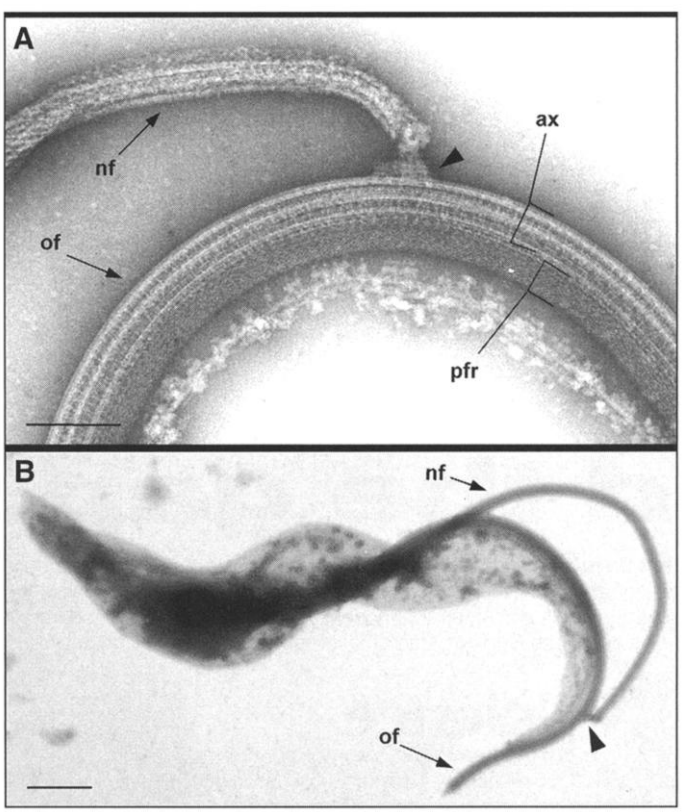


**Fig. 3.** Evidence for the existence of flagellum-flagellum attachment at the FC. (**A**) $CaCl_2$ treatment removes the subpellicular microtubules, but does not affect the axoneme (ax) or the paraflagellar rod (pfr) (other labels as Fig. 2). Attachment at the FC also resists $CaCl_2$ treatment (arrowhead). (**B**) Induction of RNAi of Fla1 for 24 hours produces a phenotype where the new flagellum (but not the old) describes a loop, being detached from the cell body but still attached to the old flagellum by its tip via the FC (arrowhead). Scale bars, 200 nm (A), 1 μm (B).

remains firmly attached to the old flagellum. Thus, FC attachment must have been of considerable strength because it was not disrupted by the vigorous movement of the new flagellum loop. The Fla1-RNAi phenotype showed that the nature of FC attachment is likely to be different from that of lateral flagellum–cell body attachment. The FC must disappear before the completion of cytokinesis, and preliminary data indicate that the connection between the old and new flagella disappears at, or soon after, the start of cytokinesis.

The FC is a unique structure that, during cell duplication, physically links a forming cell component with the preexisting version. It is unlikely that the old flagellum is providing a template for the formation of the new flagellar axoneme, because this has not been observed in other flagellates. Rather, a direct consequence of the "tip-tethering" is that the old flagellum restricts and directs the positioning and orientation of the new flagellum in relation to the topography of the cell body. The exact configuration of the attachment we have described guides and maintains the relative posterior position, circumferential position, and helical track of the new flagellum. This process is likely to be essential for defining the arrangement of internal cytoskeletal structures that are linked to the attached flagellum, such as the FAZ and the subpellicular microtubules.

There is evidence that the arrangement of these structures is intimately associated with the definition of cell shape and division plane (*2, 4, 20*). We have performed immunofluorescence microscopy on Fla1-RNAi cells to detect the internal FAZ organization. These experiments showed that when the flagellum was detached, the internal FAZ structures were not formed properly. It appears, therefore, that the cytotactic cues of the FC are subsequently translated to define the lateral attachment process, which in turn influences the positioning and polarity of the internal FAZ structures so important for cell morphogenesis and division. The use of the attached flagellum in providing information for the morphogenesis of trypanosomatid parasites seems to be at its most extreme in the trypomastigote form (*21, 22*).

This reproduction of the existing helical pattern in such a distinctive manner implicates the concept of cytotaxis, defined by Sonneborn (*23*) as "ordering and arranging of new cell structure under the influence of preexisting cell structure" in cell morphogenesis in trypanosomes. Since the early illustration of cytotaxis in *Paramecium* by Beisson and Sonneborn (*24*), a few other examples have come from other ciliates (*25*), and more recently the phenomenon has been implicated in maintaining the bipolar pattern of budding in yeast (*26*). Our results not only show a clear example of Sonneborn's concept of cytotaxis, but also provide, in the form of the FC, a definition of the structure and mechanism that executes the phenomenon in this instance. Future knowledge of the composition of the FC will enable the molecular mechanisms involved to be elucidated.

### References and Notes


1. L. Kohl, T. Sherwin, K. Gull, *J. Eukaryot. Microbiol.* **46**, 105 (1999).
2. K. Gull, *Annu. Rev. Microbiol.* **53**, 629 (1999).
3. D. R. Robinson, K. Gull, *Nature* **352**, 731 (1991).
4. D. R. Robinson, T. Sherwin, A. Ploubidou, E. H. Byard, K. Gull, *J. Cell Biol.* **128**, 1163 (1995).
5. Procyclic *T. brucei brucei* (427 strain) cells were cultivated and prepared for SEM and TEM essentially as described (*22*). Subpellicular microtubules were removed by treatment of samples with 60 mM $CaCl_2$ for 1 min immediately after detergent extraction and before fixation. Preparations were observed in a Cambridge Stereoscan 360 at 15 kV for SEM and in a Philips EM400T for TEM.
6. P. Bastin, K. R. Matthews, K. Gull, *Parasitol. Today* **12**, 295 (1996).
7. M. T. Dolan, C. G. Reid, H. P. Voorheis, *J. Cell Sci.* **80**, 123 (1986).
8. A. Fire *et al.*, *Nature* **391**, 806 (1998).
9. H. Ngo, C. Tschudi, K. Gull, E. Ullu, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14687 (1998).
10. P. Bastin, K. Ellis, L. Kohl, K. Gull, *J. Cell Sci.* **113**, 3321 (2000).
11. T. Nozaki, P. A. Haynes, G. A. M. Cross, *Mol. Biochem. Parasitol.* **82**, 245 (1996).
12. D. J. LaCount, S. Bruse, K. L. Hill, J. E. Donelson, *Mol. Biochem. Parasitol.* **111**, 67 (2000).
13. R. Cooper, A. Ribeiro de Jesus, G. A. M. Cross, *J. Cell Biol.* **122**, 149 (1993).
14. Z. Wang, J. C. Morris, M. E. Drew, P. T. Englund, *J. Biol. Chem.* **275**, 40174 (2000).
15. Inducible RNA interference of Fla1 was produced using the pZJM vector system (*14*). About 1000 base pairs of the coding sequence of the *fla1* gene (nucleotides 601 to 1636) was amplified from genomic DNA using the polymerase chain reaction and the following primers: forward primer, 5′-CGAAGCTTGTTGGAGGTTTTC-3′ (Hind III site underlined); reverse primer, 5′-GCTCGAGGAACGCCGCCAACC-3′ (Xho I side underlined). The *fla1* fragment was initially cloned in pGEMT Easy vector (Promega) and subsequently cloned into the Hind III and Xho I sites of pZJM (*14*). 29-13 *T. brucei* cells (*16*), which harbor integrated genes for the T7 RNA polymerase and the tetracycline repressor, were transfected and selected using hygromycin (50 μg/ml), G418 (15 μg/ml), and phleomycin (5 μg/ml). Stably transfected cells were induced for up to 24 hours in the presence of tetracycline (1 μg/ml) to produce the described phenotype.
16. E. Wirtz, S. Leal, C. Ochatt, G. A. M. Cross, *Mol. Biochem. Parasitol.* **99**, 89 (1999).
17. Trypanosome cells in $G_1$ of the cell cycle have a single kinetoplast (mitochondrial DNA), nucleus, and flagellum. Kinetoplast (K), nucleus (N), and flagellum are all replicated and segregated at precise times within the cell cycle. Examining cells for the presence of 1K1N, 2K1N, and 2K2N configurations, and noting whether a cell has a new flagellum (and, if so, noting its length), allows individual cells within a population to be ascribed to a cell cycle position with some accuracy.
18. A movie showing the flagellum motion is available at *Science* Online (www.sciencemag.org/cgi/content/full/294/5542/610/DC1).
19. Cells were filmed in a Leitz DMRBE microscope (Leica) equipped with differential interference contrast optics and a COHU high-performance charge-coupled device camera. Films at 12 frames per second were made and processed using Scion Image 1.62a software.
20. A. Ploubidou, D. R. Robinson, R. C. Docherty, E. O. Ogbadoyi, K. Gull, *J. Cell Sci.* **112**, 4641 (1999).
21. Our examination of the proliferative epimastigote forms of *T. cruzi* and promastigote forms of *Leishmania* suggest that the FC is absent. Morphogenesis of the trypomastigote form of kinetoplastids (with its basal body/kinetoplast positioned at the posterior end of the cell most distant from the point of cytokinesis) appears to have a critical requirement for full flagellum attachment and positioning—hence the importance of having a flagella connector in the trypomastigote form.
22. T. Sherwin, K. Gull, *Philos. Trans. R. Soc. London Ser. B* **323**, 573 (1989).
23. T. M. Sonneborn, *Proc. Natl. Acad. Sci. U.S.A.* **51**, 915 (1964).
24. J. Beisson, T. M. Sonneborn, *Proc. Natl. Acad. Sci. U.S.A.* **53**, 275 (1965).
25. G. W. Grimes, *Am. Zool.* **22**, 35 (1982).
26. T. Chen *et al.*, *Science* **290**, 1975 (2000).


27. We thank all members of the Gull lab for useful comments and discussions. Supported by grants from the Wellcome Trust and the Biotechnology and Biological Sciences Research Council (K.G.). F.F.M.-L. is the recipient of an Overseas Research Student Award.

25 June 2001; accepted 17 August 2001