Protist, Vol. ▮, ▮▮▮—▮▮▮, ▮▮ ▮▮▮▮
http://www.elsevier.de/protis
Published online date 12 October 2007

Protist

ORIGINAL PAPER

A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*

Sue Vaughan[2], Linda Kohl[2,3], Ian Ngai, Richard J. Wheeler, and Keith Gull[1]

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Submitted May 24, 2007; Accepted August 18, 2007
Monitoring Editor: Janine Beisson

The flagellum is attached along the length of the cell body in the protozoan parasite *Trypanosoma brucei* and is a defining morphological feature of this parasite. The flagellum attachment zone (FAZ) is a complex structure and has been characterised morphologically as comprising a FAZ filament structure and the specialised microtubule quartet (MtQ) plus the specialised areas of flagellum: plasma membrane attachment. Unfortunately, we have no information as to the molecular identity of the FAZ filament components. Here, by screening an expression library with the monoclonal antibody L3B2 which identifies the FAZ filament we identify a novel repeat containing protein FAZ1. It is kinetoplastid-specific and provides the first molecular component of the FAZ filament. Knockdown of FAZ1 by RNA interference (RNAi) results in the assembly of a compromised FAZ and defects in flagellum attachment and cytokinesis in procyclic trypanosomes. The complexity of FAZ structure and assembly is revealed by the use of other monoclonal antibody markers illustrating that FAZ1 is only one protein of a complex structure. The cytokinesis defects provide further evidence for the role of an attached flagellum in cellular morphogenesis in these trypanosomes.

Key words: flagellum; flagellum attachment zone filament; FAZ; cytokinesis; cytoskeleton; *Trypanosoma brucei*.

Introduction

The protozoan parasite *Trypanosoma brucei* causes African sleeping sickness in humans and Nagana in cattle. The parasite is widespread in sub-Saharan Africa, accounting for ~50,000 human deaths annually and there is a high economic cost with the loss of domestic cattle. The parasite undergoes a complex life cycle; alternating between insect vector (the tsetse fly) and mammalian host. A distinguishing morphological feature of this parasite is the single flagellum which is attached along the length of the cell body with a small overhang that protrudes from the anterior end of the cell (Fig. 1A) (for review see Gull 1999). This feature is unusual as the flagella/cilia of most eukaryotic cells extend out freely into the surrounding medium. The importance of the flagellum in many aspects of this parasite's cellular morphogenesis, organelle positioning and pathogenicity, in addition to motility have become clear from recent studies (Baron et al.

e-mail keith.gull@path.ox.ac.uk (K. Gull).
[1]Corresponding author; fax +44 1865 285691
[2]These authors contributed equally to this work.
[3]Present address: Muséum National d'Histoire Naturelle, 61 rue Buffon, 75231 Paris cedex 05, France.

doi:10.1016/j.protis.2007.08.005

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

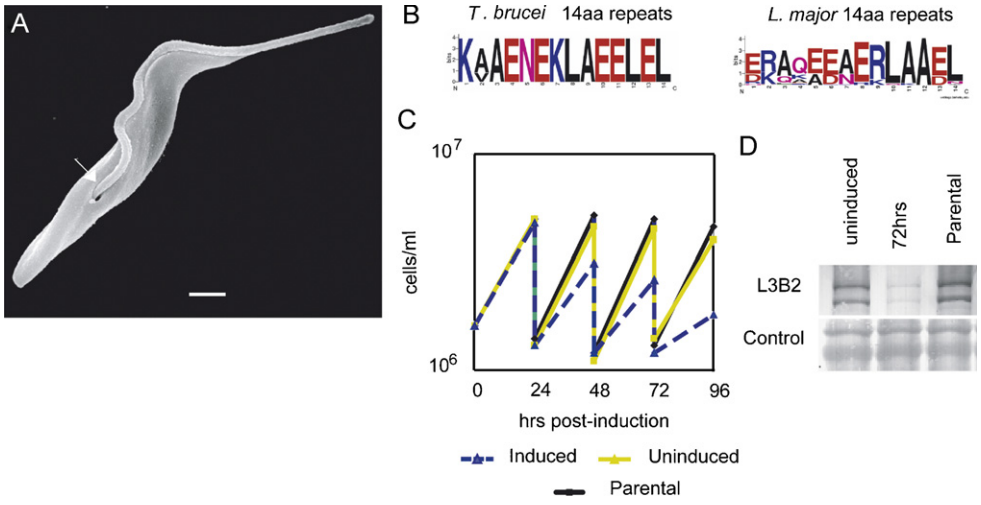

Figure 1. RNAi of FAZ1 affects growth in procyclic *T. brucei*; **A**: Scanning electron micrograph of a procyclic *T. brucei* cell. The single flagellum exits the flagellar pocket (arrow) at the posterior end of the cell and is attached along the length of the cell body (scale bar = 1 μm); **B**: Sequence logos illustrate the similarity of the 14aa repeats within the FAZ1 protein in *T. brucei* and *L. major*; **C**: Knockdown of FAZ1 by RNAi results in a growth effect after 24 h induction compared to the uninduced cells or the parental cell line; **D**: Western blotting with L3B2 antibody detects two bands >200 kDa in parental and non-induced, but at 72 h post-induction the level of protein detected is greatly reduced.

2007; Broadhead et al. 2006; Davidge et al. 2006; Kohl et al. 2003; Moreira-Leite et al. 2001; Robinson et al. 1995).

In contrast to other kinetoplastids, the trypanosomatids are characterised by their possession of this attached flagellum. The flagellum of *T. brucei* is composed of a classical 9+2 axoneme plus the paraflagellar rod (PFR), which is attached to the axoneme and is essential for motility of the parasite (Bastin et al. 1998). The flagellum attachment zone (FAZ) defines a complex structure that connects the flagellum to the long axis of the cell body via the FAZ filament and the microtubule quartet (MtQ) with associated membranous elements. The FAZ filament is located in a gap between sub-pellicular microtubules of the microtubule corset which underlies the plasma membrane. The MtQ is located immediately to the left of the FAZ filament when viewed from the posterior end of the cell and is associated with smooth endoplasmic reticulum (Sherwin and Gull 1989a). The MtQ is nucleated close to the basal body (at the proximal end of the flagellum) within the cytoplasm and has distinct biochemical characteristics such as resistance to salt (Robinson et al. 1995). A similar ultrastructure has also been well characterised in *T. cruzi* (Rocha et al. 2006) and, although epimastigote flagella are only partially attached, FAZ filament-like structures have been reported in the flagellar pocket areas of *Leishmania mexicana* (Weise et al. 2000) and *Crithidia fasciculata* (Brooks 1978).

The long slender cell shape of the parasite is maintained by the corset of sub-pellicular microtubules underlying the plasma membrane. Importantly, the MtQ is nucleated close to the basal bodies and therefore these microtubules are antiparallel (plus end at the anterior of the cell) to the main set of cortical microtubules (minus end at the anterior end of the cell). This sub-pellicular corset remains intact during the cell division cycle necessitating a very precise coordination between its duplication and the duplication and segregation of the many single copy organelles (Sherwin and Gull 1989b). The flagellar attachment zone is central to these processes. As the new flagellum extends out of the flagellar pocket at the posterior end of the cell, FAZ filament assembly lags slightly behind, so attaching the newly synthesised flagellum to the cell body as it follows a left-handed helical path (Kohl et al. 1999), guided by the flagella connector (Moreira-Leite et al. 2001). The flagella connector extends to a distance of ~0.6 of the old flagellum length and whilst the connector is effectively stationary, the new flagellum continues to extend in length and separation of the basal bodies (with attached mitochondrial DNA, termed the kinetoplast) of the old and new

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

flagella occurs. Mitosis initiates during basal body and kinetoplast segregation and cytokinesis initiates from the anterior end of the cell, separating both the old and new flagella and the precisely separated kinetoplasts and nuclei (Davidge et al. 2006). Due to the position of the FAZ between the two flagella in dividing cells we have developed arguments and evidence that the FAZ defines the position and polarity of the cleavage plane (Davidge et al. 2006; Robinson et al. 1995).

Despite the importance of an attached flagellum in cell morphogenesis, to date no molecular characterisation has been made of the FAZ filament components. Our previous studies in *T. brucei* and other studies in both *T. brucei* and *T. cruzi* have identified antibodies that recognise the FAZ and detect high molecular weight proteins by Western blotting (Kohl and Gull 1998; Ruiz-Moreno et al. 1995; Souto-Padron et al. 1989; Woods et al. 1989; Woodward et al. 1995). One of our monoclonal antibodies L3B2 recognises the FAZ filament on the cell body side by immunogold labelling of detergent-extracted whole mount cytoskeletons (Kohl and Gull 1998) and in this present study we have screened an expression library with the L3B2 antibody and identified a large repeat-containing protein named FAZ1. Following knock down of FAZ1 expression using an inducible RNAi approach we find that flagellum attachment is compromised following induction with an accumulation of cells exhibiting partially or fully detached flagella. Assembly and probably progression of FAZ assembly is compromised, but intriguingly, knockdown of FAZ1 does not prevent localisation of certain other components of the FAZ. However, cytokinesis defects are also evident when FAZ1 is depleted demonstrating the importance of flagellum attachment and the FAZ filament in cell morphogenesis.

Results

FAZ1 is a Novel Repeat-Containing Protein

Monoclonal antibody L3B2 labels the FAZ filament structure in the cell body side by immunogold labelling (Kohl et al. 1999). A procyclic *T. brucei* expression library was probed (Birkett et al. 1992) with the L3B2 antibody and three clones were identified and sequenced. Database mining of the *T. brucei* genome project (Hertz-Fowler et al. 2004) with these sequences identified one open reading frame (ORF) Tb927.4.3740 on chromosome IV. We have named this protein FAZ1. This ORF is predicted to be 5079 bp long with a predicted mass of 192.5 kDa and pI 4.3. The ORF is conserved in *T. cruzi* (contigs Tc00.1047053506201.140, .170) and, intriguingly, in *L. major* there are two orthologues next to each other on chromosome 34 (LmjF34.0690 and LmjF34.0680).

FAZ1 protein is characterised by a short variable N-terminus domain, a conserved second domain, then a large repeat-containing domain and a conserved C-terminus domain. The repeat domain consists of 36×14aa repeats in *T. brucei* and *T.* cruzi, which are identical and in the *L. major* LmjF34.0690 orthologue there are 70×14aa repeats that are similar to the repeats in the *T. brucei* and *T. cruzi* orthologues. Sequence logos illustrate the similarity between them (Fig. 1B). However, these 70 repeats are degenerate, such that there is other sequence between some of the repeats. The second orthologue LmjF34.0680 contains 3×222nt repeats, but dot blot comparison between *L. major* sequences suggests some homology with the 14aa sequence repeats (data not shown). FAZ1 is not predicted to contain a transmembrane domain and searching motif databases did not reveal any further information.

New Flagellum Growth Continues under FAZ1 Ablation

In order to address the function of FAZ1 we made an inducible RNAi cell line. Induction of RNAi led to a growth effect after 24 h induction (Fig. 1C) and knockdown of protein expression was confirmed by Western blotting with L3B2 at 72 h post-induction (Fig. 1D). Depletion of FAZ1 at earlier time points was analysed by labelling cells with L3B2. Observing the position and number of kinetoplasts, nuclei and flagella in *T. brucei* cells is a well-established method to identify the position in the cell division cycle. Early in the cell cycle cells possess a single kinetoplast, nucleus and flagellum (termed 1K1N). As the cell cycle progresses the new flagellum grows posterior to the old flagellum and the kinetoplast segregates to produce a 2K1N cell. Following mitosis the resulting 2K2N cell has two full length flagella. Cytokinesis occurs to cleave the cell between the two flagella resulting in two 1K1N siblings. 1K1N cells were scored for the presence, absence or reduction of labelling of L3B2 following induction as these cells are either the start or end of the cell division cycle (Fig. 2A). The cell cycle of procyclic *T. brucei* cells is ~8.5 h and even by 15 h

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

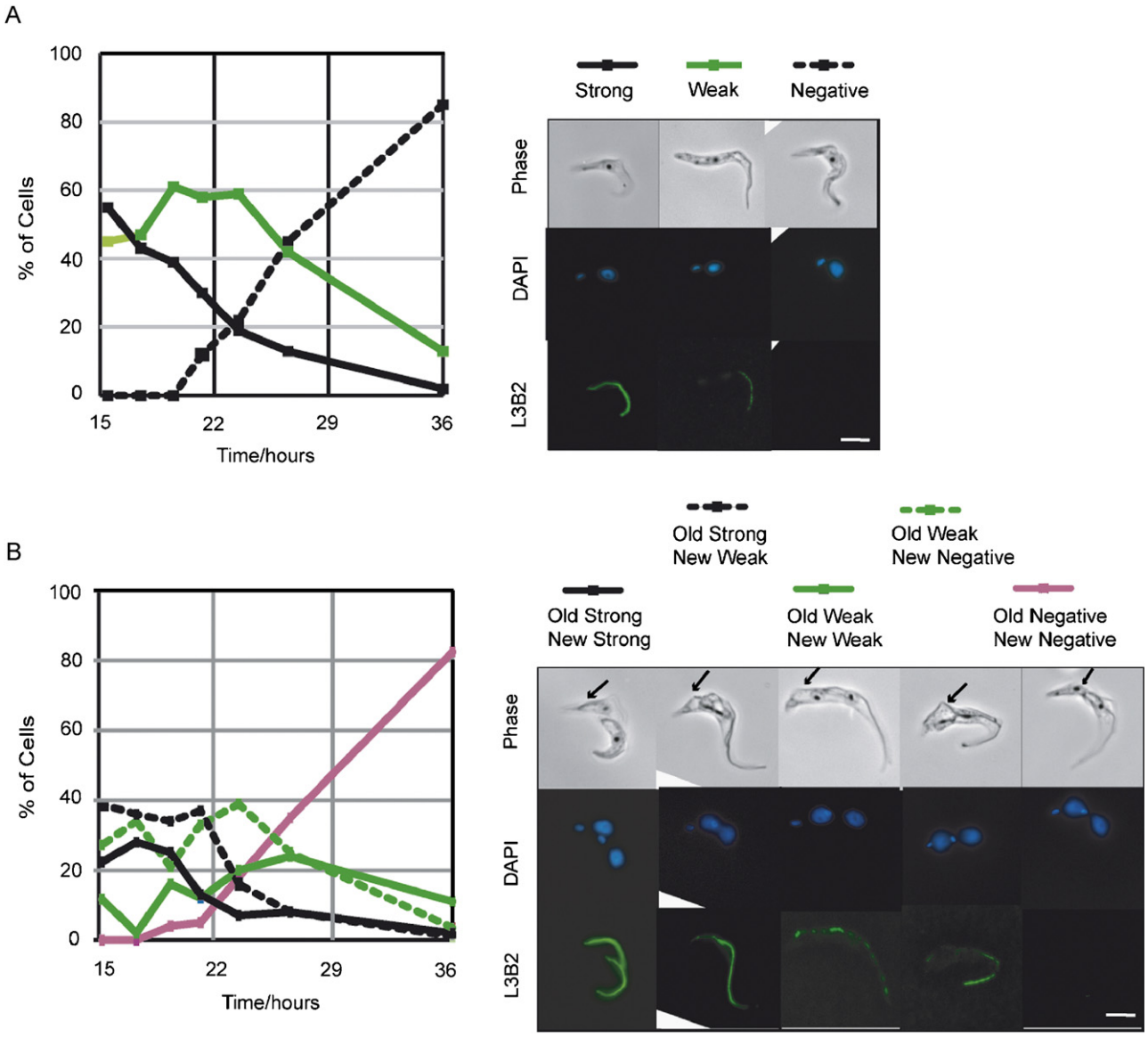

Figure 2. Presence of a flagellum in the absence of FAZ1; **A**: Reduction in the labelling of L3B2 in 1K1N cells between 15 and 36 h post-induction. Cells were scored as strong, weak or negative for L3B2 labelling. Even by 15 h post-induction ~40% of cells have weak labelling and by 36 h >80% of cells are negative for L3B2 ($n = 100$ cells/time point); **B**: In 2K2N cells there is an old and new flagellum with accompanying FAZ in normal cells. Cells were scored as strong, weak or negative for L3B2 labelling of the old and new FAZ filament post-induction (arrows mark the new flagellum). By 36 h post-induction >80% of the old and new FAZ are negative for L3B2 labelling ($n = 100$ cells/time point). Scale bar = 5 μm.

post-induction many 1K1N cells have reduced L3B2 labelling, demonstrating that knockdown of FAZ1 by RNAi is fast acting. By 36 h post-induction more than 80% of 1K1N cells were negative for L3B2 (Fig. 2A). Significantly, all of these cells possessed a flagellum.

The old flagellum remains intact and a new flagellum grows posterior to the old flagellum during the cell division cycle, which allows us to make comparisons between them and check if a new flagellum is able to be built in the induced cells. At the start of induction both the old and new flagella could be labelled with L3B2 (Fig. 2B), but as induction progressed labelling of the new FAZ filament of the new flagellum was found to be reduced or absent, but a new flagellum was still

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

assembled. By 36 h post-induction more than 80% of these cells possessed two flagella without FAZ1 labelling (Fig. 2B), confirming that FAZ1 is not essential for new flagellum growth.

An Aberrant FAZ is Assembled in the Absence of FAZ1

Although growth of a new flagellum is not affected by knockdown of FAZ1, an obvious question is whether FAZ1 is essential for flagellum attachment. Previous studies have shown that flagellar detachment is lethal in procyclic *T. brucei* (Moreira-Leite et al. 2001; Nozaki et al. 1996). 1K1N cells with a single flagellum were analysed and attachment errors were present at 24 h post-induction and by 48 h post-induction ~30% of cells have attachment errors, which correlates with the reduction in growth rate (Fig. 3A). The most common form of attachment errors (~50%) are cells with a partially detached flagellum that have a 'loop' of detachment usually in the central portion of the cell, but the flagellum is attached either side of the loop. A further 33% are fully detached and 17% are detached at the anterior end of the cell body. Furthermore, an increase in new flagella with detachment phenotypes in 2K2N cells was found at 72 h post-induction (Fig. 3B), demonstrating that defects in flagellum attachment occur during assembly.

Examination of the ultrastructure of the flagellum and FAZ by transmission electron microscopy (TEM) highlighted a number of abnormalities. Cross sections of uninduced appeared as wild-type ($n = 93$) (Fig. 4A) with the FAZ filament being of appropriate size and position next to the MtQ. However, in 35% of cross sections ($n = 98$) at 72 h post-induction the gap where the FAZ filament is situated between two sub-pellicular microtubules was found to be unusually wide (compare Fig. 4A arrow with Fig. 4B brackets) with more electron dense material juxtaposed to the cell body membrane (Fig. 4B). In the remaining 65% of cross sections, 17% have abnormalities in positioning or absence of the endomembrane

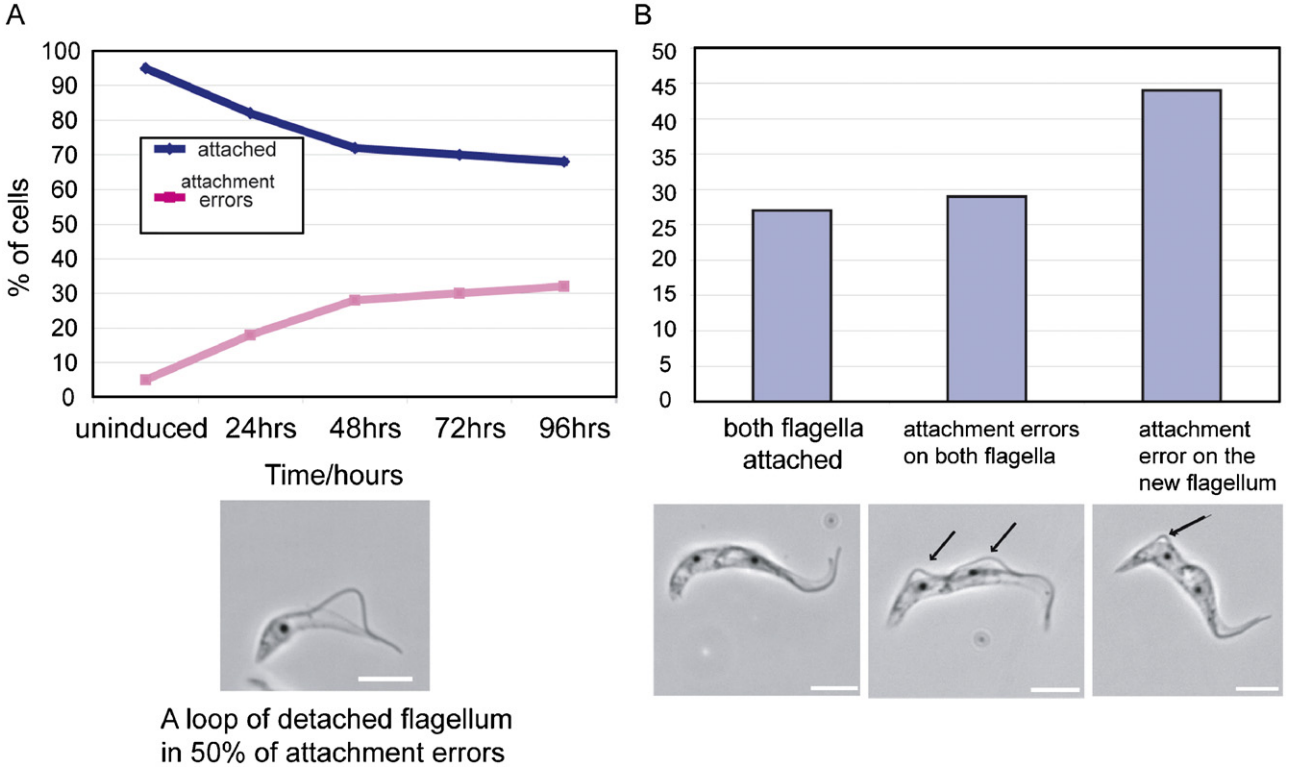

Figure 3. Compromised flagellar attachment in FAZ1 RNAi cell line. **A**: Counts were made of the number of 1K1N cells with an attached or unattached flagellum. By 48 h post-induction ~30% of cells had a partially or fully detached flagellum ($n = 200$ cells/time point); **B**: The old and new flagella of 2K2N cells were scored for flagella attachment or detachment at 72 h post-induction. The new flagellum is always positioned posterior to the old flagellum in these cells ($n = 200$ cells) (arrows highlight detached flagella). Scale bar = 5 μm.

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

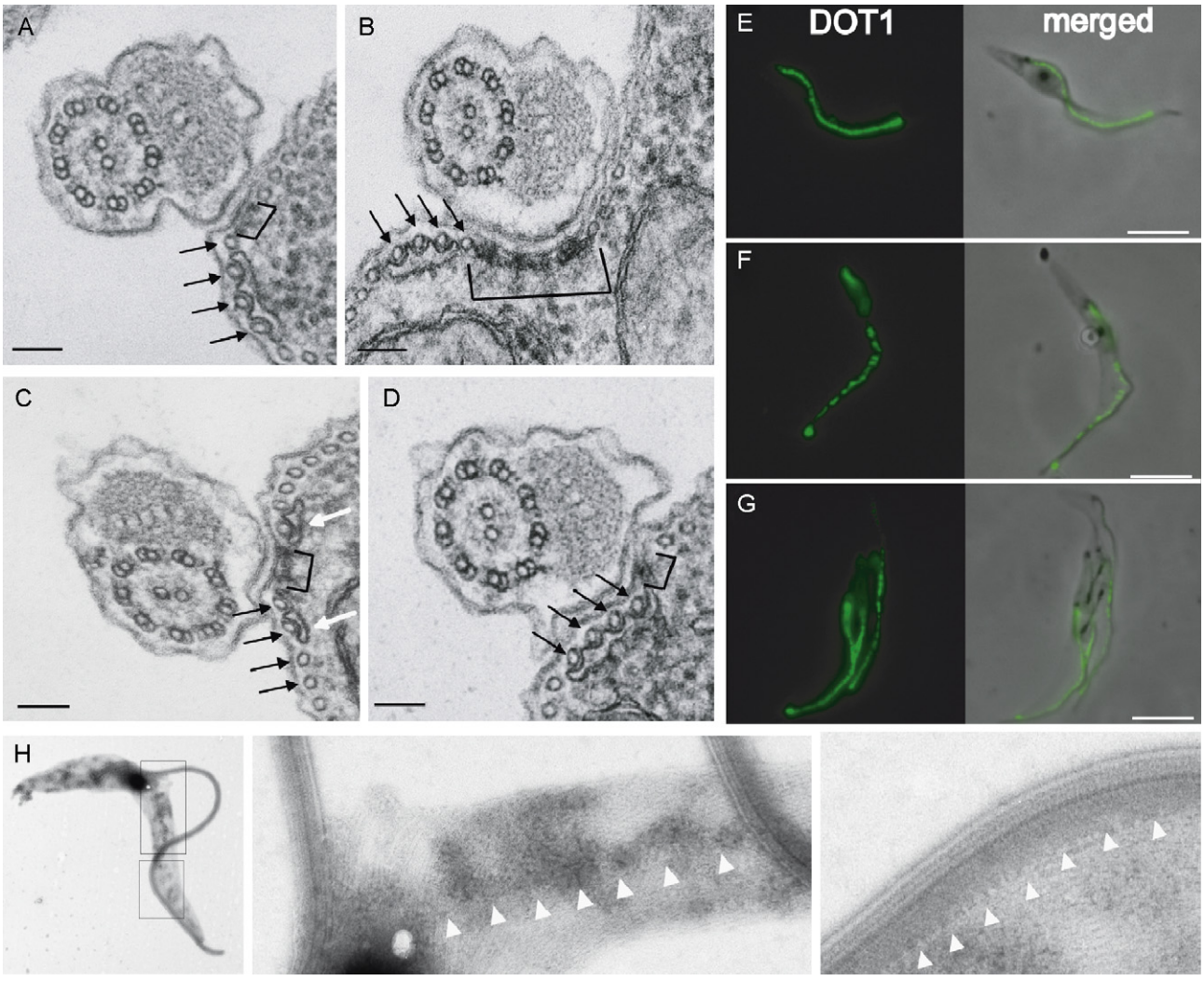

Figure 4. Aberrant FAZ assembly following knockdown of FAZ1; **A**: TEM cross section of the flagellum and FAZ area in uninduced cells are as wild-type. MtQ is marked with arrows. FAZ filament area is marked with a bracket; **B**: TEM cross section of FAZ1 RNAi 72 h post-induction. The FAZ filament area (bracket) is unusually wide compared to uninduced cross sections and there is considerably more electron dense material; **C**: TEM cross section of FAZ1 RNAi 72 h post-induction showing errors in the positioning of endomembrane on either side of the gap (white arrows) where the FAZ filament is situated. The endomembrane is usually located across the MtQ (compare with A, B and D); **D**: TEM cross section of FAZ1 RNAi 72 h post-induction with a normal FAZ structure; **E**: DOT1 in non-induced cells labels the FAZ filament; **F** and **G**: Labelling of DOT1 is patchy in induced cells by 72 h post-induction following knockdown of FAZ1. G is a multinucleated cell; **H**: A detergent-extracted whole mount cytoskeleton at 72 h post-induction with a partially detached flagellum. Ultrastructure resembling the FAZ can be seen in the cell body in the area of detachment, but further along the flagellum attachment appears normal (A—D scale bars = 100 nm; E—G scale bars = 5 μm).

associated with the MtQ (Fig. 4C) with the remainder of the cross sections as per non-induced (Fig. 4D).

These results suggest that a FAZ structure (albeit aberrant) is formed in the induced cells, so we investigated the labelling pattern of two other monoclonal antibodies that recognise the FAZ filament — DOT1 and CD10 (whose antigens are unknown) (Woods et al. 1989). Interestingly, DOT1 labelling is present along the length of the cell body at 96 h post-induction, but unlike the even labelling of non-induced cells (Fig. 4E); the distribution of labelling is patchy (Figs 4F, G). Labelling with CD10 is severely reduced by 96 h

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

post-induction, but does not completely disappear in all cells (data not shown). These results suggest that at least some components of the FAZ filament structure are still localising relatively correctly following induction and the patchy DOT1 labelling likely represents compromised assembly. Notably, in cells with a partially detached flagellum, we did not find a direct correlation between flagellar detachment and absence of DOT1 and L3B2 labelling in the area where the flagellum is detached. This suggests a complex disorganisation of the FAZ filament at this point, rather than its wholesale absence. Ultrastructural features resembling the FAZ filament can be observed in areas of flagellar detachment of detergent-extracted whole mount cytoskeletons (Fig. 4H).

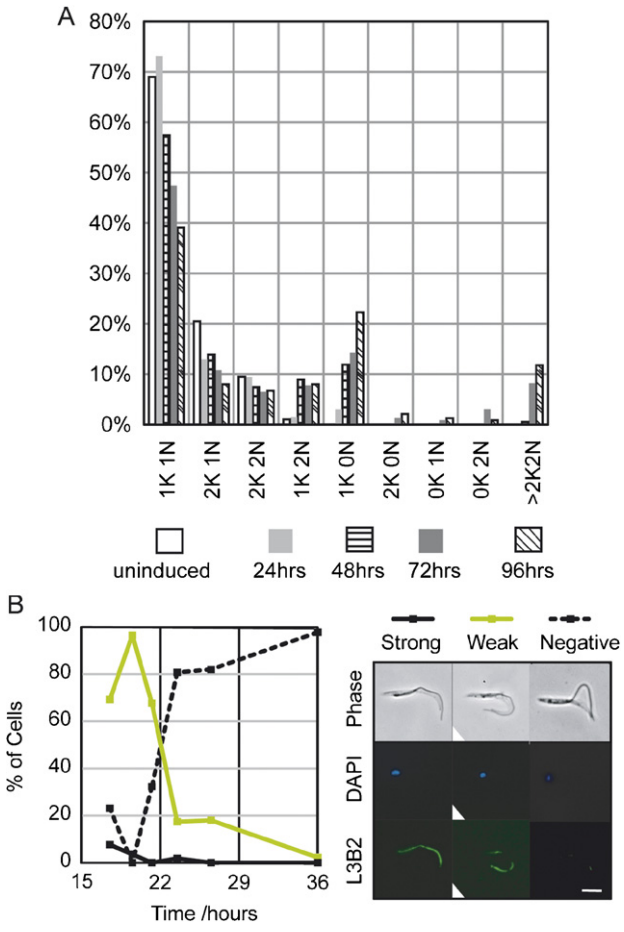

Figure 5. Cytokinesis defects in the FAZ1 RNAi cell line; **A**: The number of nuclei and kinetoplasts were counted for each cell in populations of uninduced, 24, 48, 72 and 96 h post-induction (500 cells/time point). The expected configurations are 1K1N, 2K1N and 2K2N cell types. In the induced population there is the appearance of 1K0N cells (zoids), 1K2N cells and cells >2K2N); **B**: 1K0N zoids were scored as strong, weak or negative for labelling of L3B2 antibody. By 36 h post-induction >80% of these cells were negative for L3B2, but still possess a flagellum. Scale bar = 5 μm.

FAZ1 Knockdown Results in Defects in Cytokinesis

Counts made of the number of kinetoplast and nuclei in cells following induction revealed an abundance of 1K0N cells (zoids) (Robinson et al. 1995) (Fig. 5A). This was clearly evident by 48 h post-induction with zoids representing 12% of the population, rising to 22% by 96 h post induction. There is a concomitant rise in 1K2N cells indicating that cells have completed nuclear S-phase and mitosis, but that the cleavage plane has not positioned correctly between the two nuclei resulting in a 1K0N and a 1K2N. The rise in 1K2N cells does not match the number of zoids, which could be explained by the increase in multinucleated cells (>2K2N) by 72 h post-induction suggesting that early cells can divide but this ability is reduced with time. The reduction in labelling of L3B2 of zoids (Fig. 5B) was also observed and all possessed a flagellum.

Discussion

In this paper we identify the first FAZ filament protein, called FAZ1. As outlined in the introduction, our previous monoclonal antibody studies have shown that the FAZ filament structure is complex and FAZ1 most likely represents just one protein within this structure. We previously showed that L3B2 is localised to the cytoplasmic side of the FAZ filament by immunogold labelling of detergent-extracted whole mount cytoskeletons (Kohl et al. 1999) and the FAZ1 sequence is not predicted to contain a transmembrane domain. Coupled with the knowledge that FAZ1 is a large repeat-containing protein, we can envisage FAZ1 as a cytoskeletal structural protein on the cell body side of the FAZ filament. We should add that two high molecular weight bands are recognised by Western blotting with L3B2 (Kohl et al. 1999) and this could represent allelic variation due to heterogeneity in the number of repeats or the two bands could represent different post-translational modifications of the protein.

In this study we have demonstrated that growth of a new flagellum is independent of

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

FAZ1. This was also the case with *T. brucei* FLA1, which is a membrane glycoprotein that localises along the flagellum/cell body area by immunofluoresence. This protein is more likely to be a membrane protein associated with the cytoskeleton, rather than an actual component of the FAZ filament structure since it contains two predicted transmembrane domains (LaCount et al. 2002; Moreira-Leite et al. 2001; Nozaki et al. 1996). Interestingly, knockdown of FLA1 by RNAi caused complete flagellar detachment in *T. brucei* and raises some interesting points with regard to assembly of the FAZ filament and its connection to the flagellum. Ultrastructural material resembling the FAZ filament was observed in FLA1 RNAi cells (our unpublished observations), suggesting that FAZ filament assembly of the cell body side is not completely blocked when connections to the flagellum are affected.

Knockdown of FAZ1 by RNAi compromises flagellum attachment in ~30% of cells and whilst the level of knockdown by RNAi could certainly influence the level of the phenotype, the fact that not all cells have detached flagella most likely highlights the complexity of FAZ filament. We found a continued presence of FAZ filament structure in TEM cross sections as well as the presence of DOT1 labelling in RNAi-induced cells strongly suggesting that knockdown of FAZ1 does not prevent many FAZ filament proteins from localising. However, assembly does appear to be aberrant as in a third of TEM cross sections there were wide gaps where the FAZ filament is located with what looked like disorganised and multiple FAZ filament structures. This could represent multiple attempts at assembling the FAZ filament components at the same position due to faltering progression of FAZ assembly. The patchy DOT1 labelling in the induced population certainly supports the idea of intermittent assembly. Previously we showed that FAZ filament assembly closely follows flagellum assembly (Kohl et al. 1999) and faltering progression could lead to areas of detachment or a total lack of flagellum attachment if FAZ filament assembly falls too far behind flagellum assembly, thus explaining why the flagellum is only partially detached in many cells. Our model is that of an unstable FAZ filament following knockdown of FAZ1 whereby there is a failure to assemble all of the necessary components correctly, leading to a weak and unstable FAZ filament and faltering progression of assembly (Fig. 6). The assembly of a correct FAZ is clearly complex and must involve both the FAZ filament assembly (including FAZ1 and other unidentified filament proteins) followed by the membrane attached maculae adherens junctions at the plasma membrane and transmembrane proteins including FLA1.

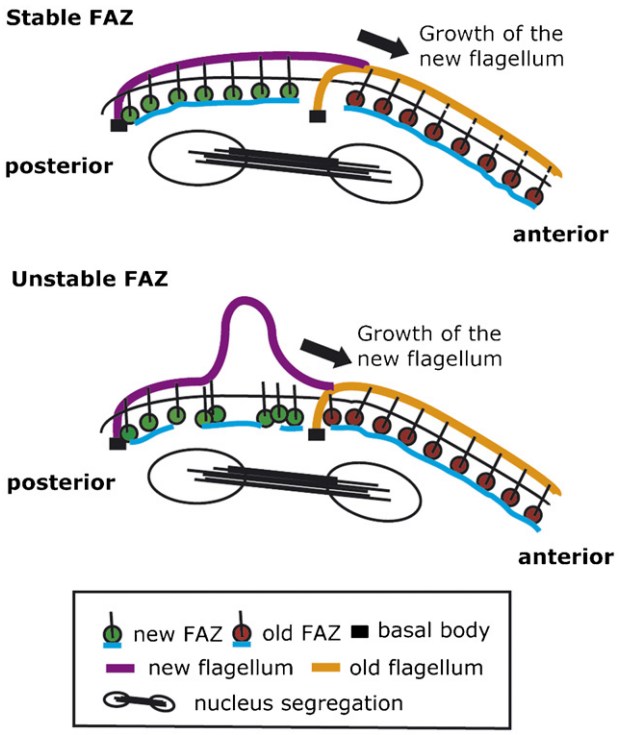

Figure 6. A model depicting a stable versus an unstable FAZ following knockdown of FAZ1. In the unstable FAZ model, assembly of the new FAZ filament structure is compromised resulting in intermittent FAZ assembly and detached flagella. Posterior and anterior ends of the cell are marked. The new flagellum is always positioned posterior to the old flagellum.

In *T. brucei* cytokinesis initiates at the anterior end of the cell and follows a helical path up the cell between the old and new flagella. The presence of zoids, 1K2N cells and multinucleated cells are indicative of defects in cytokinesis. However, the fact that all of these cell types still possess a flagellum suggests that correct positioning of the cleavage plane between the old and new flagella is still occurring. We know that growth of an attached flagellum is essential for correct spatial segregation of organelles (Kohl et al. 2003; LaCount et al. 2002; Moreira-Leite et al. 2001) and it is likely that defects in flagellum attachment are leading to the loss of spatial organisation of the kinetoplasts and nuclei in dividing cells. Indeed it is tempting to speculate that the FAZ zone may

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

also be involved in nuclear positioning and that FAZ filament defects and mis-alignments in the FAZ1 induced cells may result in defective positioning of the two nuclei during the cell cycle.

The identification of FAZ1 provides the first insights to components of the FAZ filament. The FLA1 proteins provide a view of the transmembrane connection, but it will be interesting to ascertain the identity of the FAZ proteins which are located on the flagellum side of the complex in order to study how assembly and connection are coordinated.

Methods

Cell culture: Procyclic *T. brucei* cells were cultured at 28 °C in SDM-79 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum as described previously (Brun and Schönenberger 1979).

RNA interference (RNAi): A 600 bp fragment of the repeat region of ORF Tb927.4.3740 was amplified by polymerase chain reaction (PCR) and two copies were inserted into an inducible vector pHD430 in opposing directions to potentially form a dsRNA in a hairpin structure (Bastin et al. 2000). This vector was transformed into a tetracycline-repressor-expressing procyclic *T. brucei* cell line (PTP) (Wirtz and Clayton 1995) and positive clones were selected. RNAi was mediated by addition of 1 μg/ml doxycycline to the culture medium. For the growth curve cells were cultured at a starting concentration of 1×10^6 cells/ml. Every 24 h the growth rate was measured using a Schärfe Systems CASY-1 cell counter and cells diluted back to 1×10^6 cells/ml with fresh doxycycline added to culture medium of the induced cells.

Immunolocalisation: Cells were settled onto glass slides and extracted with 1% NP-40 in 100 mM PIPES, pH6.9, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA. The cytoskeletons were fixed in methanol for 30 min followed by antibody labelling with L3B2, DOT1 or CD10. Labelling was visualised by FITC-conjugated anti-mouse IgG (Sigma). Slides were examined and counts made on a Zeiss Axioplan 2 microscope.

Electron microscopy: Cells were fixed in 2% paraformaldehyde, 2% glutaraldehyde and 0.2% picric acid in 100 mM phosphate buffer (pH 7.2), post-fixed and processed for TEM as described previously (Sherwin and Gull 1989a). Preparation of negatively stained whole mount cytoskeletons for TEM was also carried out using previously established methods (Sherwin and Gull 1989a).

Bioinformatics: Data mining was carried out using Genedb (Hertz-Fowler et al. 2004). Sequence logos were produced by WebLogo version 2.8.2 http://weblogo.berkeley.edu/.

Acknowledgements

This work was initiated at the University of Manchester and completed in Oxford. We thank members of the Gull lab in both institutions for generous and constructive discussions and specifically thank Gary Lawson for assistance with electron microscopy. The work was funded by The Wellcome Trust and the EP Abraham Trust. Keith Gull is a Wellcome Trust Principal Research Fellow.

References

Baron DM, Kabututu ZP, Hill KL (2007) Stuck in reverse: loss of LC1 in *Trypanosoma brucei* disrupts outer dynein arms and leads to reverse flagellar beat and backward movement. J Cell Sci **120**: 1513—1520

Bastin P, Sherwin T, Gull K (1998) Paraflagellar rod is vital for trypanosome motility. Nature **391**: 548

Bastin P, Ellis K, Kohl L, Gull K (2000) Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system. J Cell Sci **113**: 3321—3328

Birkett CR, Parma AE, Gerke-Bonet R, Woodward R, Gull K (1992) Isolation of cDNA clones encoding proteins of complex structures: analysis of the *Trypanosoma brucei* cytoskeleton. Gene **110**: 65—70

Broadhead R, Dawe HR, Farr H, Griffiths S, Hart SR, Portman N, Shaw MK, Ginger ML, Gaskell SJ, McKean PG, Gull K (2006) Flagellar motility is required for the viability of the bloodstream trypanosome. Nature **440**: 224—227

Brooks AS (1978) Ultrastructure of the flagellar attachment site in three species of trypanosomatids. Trans Am Microsc Soc **97**: 287—296

Brun R, Schönenberger M (1979) Cultivation and in vitro cloning or procylic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Trop **36**: 289—292

Davidge JA, Chambers E, Dickinson HA, Towers K, Ginger ML, McKean PG, Gull K (2006) Trypanosome IFT mutants provide insight into the motor location for mobility of the flagella connector and flagellar membrane formation. J Cell Sci **119**: 3935—3943

Gull K (1999) The cytoskeleton of trypanosomatid parasites. Annu Rev Microbiol **53**: 629—655

Hertz-Fowler C, Peacock CS, Wood V, Aslett M, Kerhornou A, Mooney P, Tivey A, Berriman M, Hall N, Rutherford K, Parkhill J, Ivens AC, Rajandream MA, Barrell B (2004) GeneDB: a resource for prokaryotic and eukaryotic organisms. Nucleic Acids Res **32**: D243—D339

Kohl L, Gull K (1998) Molecular architecture of the trypanosome cytoskeleton. Mol Biochem Parasitol **93**: 1—9

Kohl L, Robinson D, Bastin P (2003) Novel roles for the flagellum in cell morphogenesis and cytokinesis of trypanosomes. EMBO J **22**: 5336—5346

Kohl L, Sherwin T, Gull K (1999) Assembly of the paraflagellar rod and the flagellum attachment zone complex during the *Trypanosoma brucei* cell cycle. J Eukaryot Microbiol **46**: 105—109

LaCount DJ, Barrett B, Donelson JE (2002) *Trypanosoma brucei* FLA1 is required for flagellum attachment and cytokinesis. J Biol Chem **277**: 17580—17588

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005

Moreira-Leite FF, Sherwin T, Kohl L, Gull K (2001) A trypanosome structure involved in transmitting cytoplasmic information during cell division. Science **294**: 610—612

Nozaki T, Haynes PA, Cross GA (1996) Characterization of the *Trypanosoma brucei* homologue of a *Trypanosoma cruzi* flagellum-adhesion glycoprotein. Mol Biochem Parasitol **82**: 245—255

Robinson DR, Sherwin T, Ploubidou A, Byard EH, Gull K (1995) Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle. J Cell Biol **128**: 1163—1172

Rocha GM, Brandao BA, Mortara RA, Attias M, de Souza W, Carvalho TM (2006) The flagellar attachment zone of *Trypanosoma cruzi* epimastigote forms. J Struct Biol **154**: 89—99

Ruiz-Moreno L, Bijovsky AT, Pudles J, Alves MJ, Colli W (1995) *Trypanosoma cruzi*: monoclonal antibody to cytoskeleton recognizes giant proteins of the flagellar attachment zone. Exp Parasitol **80**: 605—615

Sherwin T, Gull K (1989a) The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations. Phil Trans R Soc Lond B Biol Sci **323**: 573—588

Sherwin T, Gull K (1989b) Visualization of detyrosination along single microtubules reveals novel mechanisms of assembly during cytoskeletal duplication in trypanosomes. Cell **57**: 211—221

Souto-Padron T, Reyes MB, Leguizamon S, Campetella OE, Frasch AC, de Souza W (1989) *Trypanosoma cruzi* proteins which are antigenic during human infections are located in defined regions of the parasite. Eur J Cell Biol **50**: 272—278

Weise F, Stierhof YD, Kuhn C, Wiese M, Overath P (2000) Distribution of GPI-anchored proteins in the protozoan parasite *Leishmania*, based on an improved ultrastructural description using high-pressure frozen cells. J Cell Sci **113**: 4587—4603

Wirtz E, Clayton C (1995) Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. Science **268**: 1179—1183

Woods A, Sherwin T, Sasse R, MacRae TH, Baines AJ, Gull K (1989) Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. J Cell Sci **93**: 491—500

Woodward R, Carden MJ, Gull K (1995) Immunological characterization of cytoskeletal proteins associated with the basal body, axoneme and flagellum attachment zone of *Trypanosoma brucei*. Parasitology **111**: 77—85

Please cite this article as: Vaughan S, et al. A Repetitive Protein Essential for the Flagellum Attachment Zone Filament Structure and Function in *Trypanosoma brucei*, Protist (2007), doi:10.1016/j.protis.2007.08.005