The trypanosome flagellar pocket

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Abstract | Trypanosomes are important disease agents and excellent models for the study of evolutionary cell biology. The trypanosome flagellar pocket is a small invagination of the plasma membrane where the flagellum exits the cytoplasm and participates in many cellular processes. It is the only site of exocytosis and endocytosis and part of a multiorganelle complex that is involved in cell polarity and cell division. Several flagellar pocket-associated proteins have been identified and found to contribute to trafficking and virulence. In this Review we discuss the contribution of the flagellar pocket to protein trafficking, immune evasion and other processes.

Flagellum
A tail-like structure emerging from the cell. In trypanosomes the flagellum consists of a 9 + 2 microtubule configuration axoneme and a paraflagellar rod. The whole structure is attached to the cell body and anchored through connections to the flagellar attachment zone, a specialization of a subset of microtubules subtending the plasma membrane.

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Trypanosomatids belong to the order Kinetoplastida, which includes both free-living and parasitic species. Research generally concentrates on disease-causing species, but there are many other interesting aspects about the biology of trypanosomes. For example, glycosylphosphatidylinositol (GPI) membrane anchoring, *trans*-splicing, RNA editing and eukaryotic polycistronic transcription of protein-coding genes were first described in trypanosomes. With several complete genome sequences, effective reverse genetics and a divergent evolutionary position, trypanosomes have emerged as an important model for the study of protozoan biology (FIG. 1).

This Review focuses on *Trypanosoma brucei*, the causative agent of human African trypanosomiasis and several animal diseases[1]. In the absence of therapeutic intervention, as is increasingly more common, human African trypanosomiasis is characterized by two main pathological phases. In the early phase, trypanosomes are found in the systemic circulation, tissue spaces and lymphatics. High circulating parasitaemia and the presence of by-products of immune defence against these parasites (primarily trypanosome molecules following cell lysis) lead to overall debilitation of the host immune system and multiple organ complications[1]. In later stages the parasite invades the central nervous system by crossing the blood–brain barrier, probably at the choroid plexus, but mechanistic details of this process remain unclear[2–4]. The late stage is accompanied by bipolar behavioural changes, lethargy, coma and ultimately death. Disease progression depends on both the parasite subspecies and host factors, and disease pathology is so complex that the mechanism by which parasite–host interactions lead to organ damage and immune suppression remains unclear. Disease progression is similar in some cattle, but, remarkably, many animals that are native to Africa, including several indigenous breeds of livestock, are trypanotolerant[5–7].

T. brucei is transmitted by tsetse flies between mammalian hosts and is responsible for at least 50,000 deaths each year. Livestock disease causes an unquantified impact on economic prosperity across sub-Saharan Africa. Although the death toll has fallen recently, resistance to available drugs is increasing, and there are poor prospects for the development of new therapeutic agents in the near future[1,8,9]. A promising lead compound (known as DB289; see REF. 10) was recently withdrawn owing to toxicity, and this was a major setback. The global burden of trypanosome-related problems is widened considerably if *Trypanosoma evansi* and *Trypanosoma equiperdum* are taken into account, as *T. evansi* is an important agricultural parasite throughout the subtropics. Both *T. evansi* and *T. equiperdum* are essentially petite mutants of *T. brucei*, having lost part or all of their mitochondrial genome, but are transmitted by biting dipterans[11].

The trypanosome life cycle has at least 10 developmental forms, with proliferative stages in both mammalian and insect hosts[12–14] (FIG. 1). Each transition is preceded by a pre-adaptation; at least in crossing from mammal to insect, this includes growth arrest[15,16]. Both transitions (that is, insect to mammal and vice versa) are accompanied by remodelling cell architecture, intracellular transport, primary metabolism and major changes in gene expression. Mammalian forms have high endocytosis and recycling activity, but in most insect forms the levels of these activities are lower and are accomplished by changes in the expression of specific components. The three proliferative forms express distinct cohorts of surface proteins (TABLE 1). All parasite forms are spindle-shaped with a single flagellum emerging through the flagellar pocket (FP), a small plasma

membrane invagination at the base of the flagellum. The FP is close to the basal body, which is itself attached to the kinetoplast (the mitochondrial genome)[17,18] (FIG. 2). The basic cellular architecture is highly conserved with other Kinetoplastids, for example *Leishmania* spp. and *Trypanosoma cruzi*.

T. brucei remains in the host bloodstream, lymphatic system and tissue spaces, placing a high burden on immune evasion mechanisms. The primary survival strategy of the parasite is antigenic variation, which is achieved by sequential expression of antigenically distinct variant surface glycoproteins (VSG)[19], and as a result this precludes any prospect for facile vaccination. A second mechanism is efficient removal of surface-bound immune factors, including immunoglobulins[20–22], through capping and internalization. Additional processes include restriction of invariant receptors in the FP, rendering them inaccessible to host immune effectors, and expression of receptors that can sequester factors secreted by innate immune cells[23–25]. In this Review we describe the architecture of the trypanosome FP and highlight the contribution of this organelle to processes such as protein trafficking, cell signalling and immune evasion.

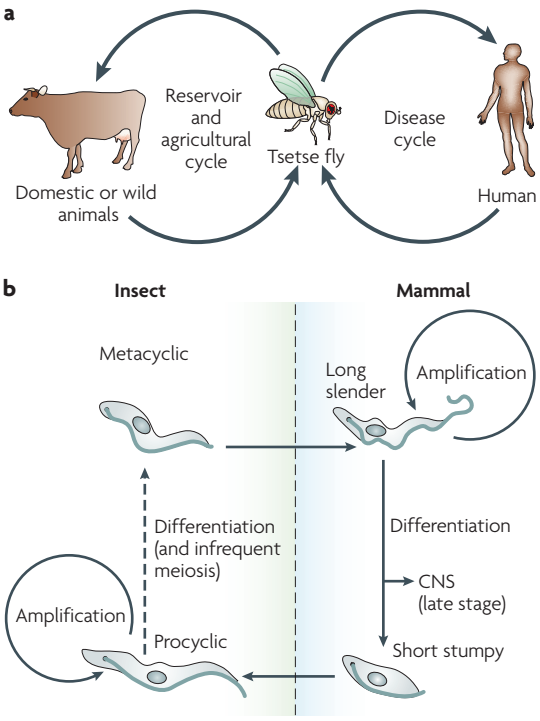

Figure 1 | **Life cycles of trypanosomes. a** | Dual cycle between the definitive host (the tsetse fly), humans and animal reservoirs. The animal reservoirs encompass both sylvatic (native wild) and domesticated agricultural animals, providing a double challenge to control; a difficult to eradicate natural reservoir of parasites and a negative impact on susceptible domestic species. **b** | Simplified life cycle emphasizing the alternation between amplification and differentiation. Infected mammals harbour high numbers of dividing long slender forms of the parasites in their bloodstream and tissue spaces. Long slender forms differentiate to a short stumpy form, which is pre-adapted to the insect vector and is growth arrested. At some point invasion of the central nervous system (CNS) occurs, with serious clinical implications. Transfer of the short stumpy form to the insect vector in a blood meal is accompanied by rapid differentiation to the procyclic form, which re-enters the cell cycle. Multiple differentiation stages, with a non-obligatory meiotic and sexual phase, are required to generate the mammalian infective metacyclic forms (not shown). Metacyclic forms reside in the salivary glands and are also growth arrested. Transfer of these parasites back to a mammalian host during feeding completes the life cycle. See text for discussion of the multiple changes that accompany progression through the life cycle. Several stages have been omitted from this figure for clarity.

Maintenance of cellular architecture

The spindle shape of the trypanosome results from a spiralled subpellicular array of interlinked microtubules that must accommodate growth and cell division[18,26,27]; remodelling of the cytoskeleton facilitates the distinct morphologies of the various developmental forms[28,29]. Subpellicular microtubules are nucleated at points in the microtubule array and orientated with their plus ends at the posterior pole[26]. A single flagellum originates at the basal body with the plus ends of the axonemal microtubules anteriorally disposed and contributes to the positioning of the basal body[30,31] (FIG. 3). The flagellum exits the cell through a gap in the microtubule array and a corresponding invagination of the plasma membrane, which forms the FP. The flagellum runs along the cell body in a left-handed spiral towards the anterior pole and is held in place by the flagellum attachment zone (FAZ), which is composed of punctate transmembrane junctions[32]. Four cytoplasmic microtubules are nucleated close to the basal body and run around the flagellar pocket and along the entire FAZ to the anterior cellular pole. As the plus ends of this microtubule quartet are at the anterior end, they have the opposite polarity to the subpellicular array microtubules. The basal body is physically connected to the kinetoplast, the mitochondrial DNA, by a tripartite attachment complex spanning both mitochondrial membranes[17].

Replication of the flagellum, which is an integral part of the cell cycle, has been described in greater detail for the form for the parasite that resides in insects (procyclic form). The new flagellum emerges from the FP, grows and extends along the cell body, the tip remaining adjacent to the old flagellum. In procyclics the tip is held to the side of the old flagellum by the flagellar connector, a transmembrane complex of unknown composition[33,34]. Once the flagellar connector reaches a fixed point along the old flagellum, migration stops but growth of the new flagellum continues, and the basal bodies separate[35], with the new basal body and FP moving towards the posterior pole of the cell. If the growth of the new flagellum axoneme is restricted, the daughter FP has an incorrectly formed collar and, as a result, proteins that are usually restricted to the FP diffuse into the plasma membrane[36].

The cellular morphology differs between distinct forms of trypanosomes with regard to the relative positions of the nucleus, kinetoplast (and therefore

Basal body
An organelle that is formed from the centriole and, in the case of many flagellates, that acts as the base of the flagellum from which the axoneme is built. There are two basal bodies per cell, only one of which gives rise to a flagellum. Duplication of basal bodies is a key step in early mitosis.

Variant surface glycoprotein
(VSG). A ~60kDa glycosyl-phosphatidylinositol-anchored glycoprotein that is expressed on the surface of African trypanosomes at extreme density. By sequentially expressing distinct VSG genes, trypanosomes achieve antigenic variation.

Facile vaccination
Vaccination against bacterial infections using heat killed bacteria or surface proteins, which can be regarded as facile (that is, easy), at least conceptually. Such approaches have not been successful against many protozoan parasites.

Subpellicular array
A regularly spaced microtubule array that is located just beneath the plasma membrane in trypanosomes and is responsible for maintaining the shape of the cell.

Axoneme
The inner core of the flagellum and cilia in eukaryotes. Most possess nine outer ring microtubule doublets and an inner central pair of microtubules.

Table 1 | **Trypanosome surface and intracellular transport systems**

Molecule or activity	Trypomastigote	Procyclic trypomastigote	Epimastigote	Refs
Surface protein expression				
VSG	50,000,000*	Undetectable	ND	–
Procyclin	Undetectable	10,000,000*	ND	–
BARP	Undetectable	Undetectable	Expressed‡	88
ISG65	70,000*	Undetectable	ND	31
ISG75	50,000*	Undetectable	ND	31
SRA	Expressed‡	Undetectable	ND	40
Transferrin receptor	2,300*	Undetectable	ND	73,89
Trafficking protein expression				
Clathrin	High	Low	Low	51
RAB5A and RAB5B	Low	Low	Low	81
RAB11	High	Low	Low	11
EpsinR	10,000*	10,000*	ND	72
***Activity*§**				
Exocytosis	Moderate	Moderate	Moderate	16
Recycling	High	Moderate	High	16
Endocytosis	Very high	Moderate	High	16

The data are restricted to the major known proliferative stages. Estimated copy numbers are indicated by an *. ‡Quantitative data are not available. §Estimates are based on a combination of direct measurements or from the expression of marker antigens that are thought to correlate with the activity through the specific trafficking pathway[8]. 'Moderate' indicates a steady state activity that is sufficient to deliver molecules to the cell surface to fulfil biosynthetic requirements, or, in the case of endocytosis and recycling a measurable level of activity. 'High' and 'very high' are flux rates that are in excess of this level. ISG, invariant surface glycoprotein; ND, not determined; SRA, serum resistance-associated protein; VSG, variant surface glycoprotein.

basal body) and flagellum exit point along the anterior–posterior axis[37] (FIG. 4). There is no evidence for depolymerization of the subpellicular microtubules in *T. brucei* as they remain remarkably stable throughout the cell cycle and the cell grows by the intercalation of new microtubules and not by the rearrangement of existing elements[27]. The movement of the FP along the anterior–posterior axis that occurs during the differentiation of bloodstream to procyclic forms of the parasite is achieved by the extension of the cytoskeleton, which moves the posterior pole away from the FP[28]. Transition from trypomastigote to epimastigote form (FIG. 4) occurs through movement of the nucleus towards the posterior pole, passing the FP[29].

FP architecture

The FP is a prominent and complex structure that can be easily imaged by electron microscopy (FIG. 5). There is considerable organizational complexity and precise positioning of the FP with respect to cytoskeletal elements and other organelles; investigations of markers, cell cycle positioning and three-dimensional reconstruction have provided a detailed view of these associations and organization while further highlighting complexity in the FP itself[38].

The FP can be subdivided into several subdomains. The basal body may be used to define one pole of the overall structure. The FP membrane and luminal volume are asymmetric, and the organelle is precisely associated with the Golgi complex and cytoskeletal elements. The FP lumen is filled with a carbohydrate-rich matrix of poorly defined composition and unknown function. The pro-basal body is positioned close to the basal body in interphase cells. Moving distal from the basal body, the axoneme asymmetrically invades the FP membrane and lumen. A transitional zone separates the basal body from the axoneme, which has the classical 9+2 microtubule core. The paraflagellar rod (PFR) runs parallel to the axoneme, where the flagellum exits the FP to the flagellar tip; the PFR is also found in Euglenida, and, although it is not unique to trypanosomes, it is probably restricted to these two orders[39]. The precise spatial interaction between the flagellum and the cell body may depend on the reverse polarity of the microtubule quartet[26,40]. At the neck of the FP, the microtubule quartet integrates into the subpellicular array and becomes associated with the FAZ. Therefore, these microtubules seem to define an axis for the entire flagellum and its associated structures.

There are several clear boundaries in the FP that demarcate distinct structural subdomains. The two most prominent are the FP collar (which is found at the neck of the FP, where the flagellum exits) and the point where the flagellum enters the FP (the collarette, which is proximal to the basal body). Both subdomains are associated with organized structures that are connected across the membrane and cytoskeleton. The FP collar is an electron-dense annulus and only one of its components, Bilbo1, has been identified so far. Bilbo1 is essential for FP formation, as its suppression leads to loss of the FP, with the flagellum protruding from the cell through an apparently undifferentiated membrane[41].

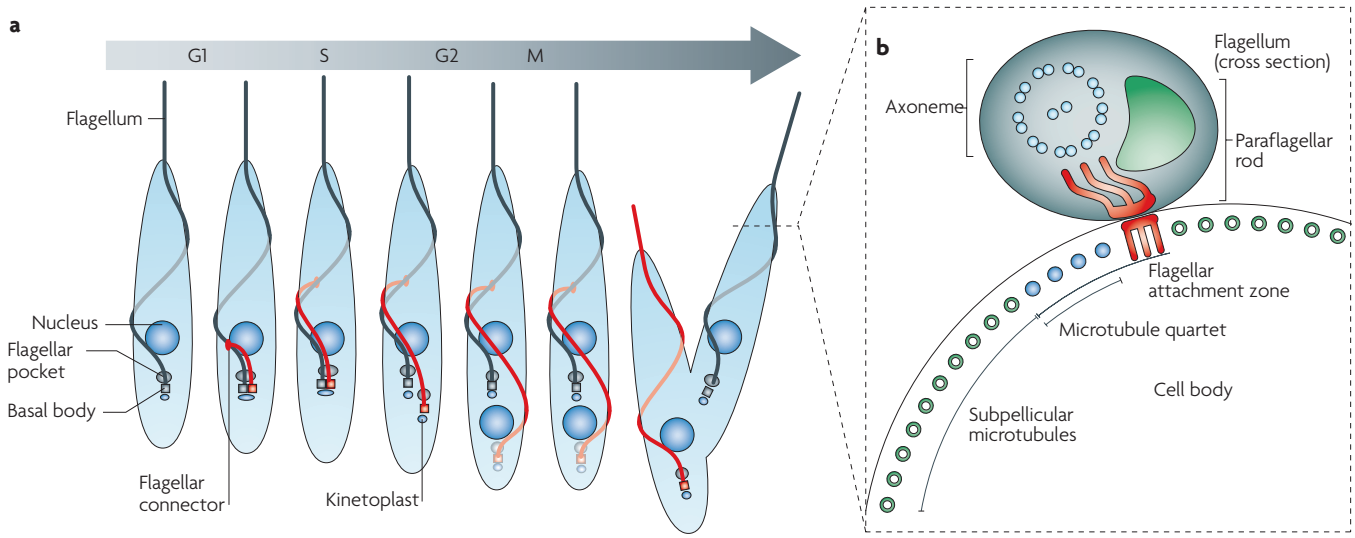

Figure 2 | **Morphology and positioning of cytoskeletal structures during the cell cycle. a** | Schematic of the cellular morphology of procyclic forms of trypanosomes during the cell cycle. The new basal body and flagellum are shown in red. The distal tip of the new flagellum is attached to the old flagellum by the flagellar connector. The anterior pole is at the top. Nuclei of the old cell are aligned in the vertical plane for reference. **b** | Cross section of the cell looking towards the anterior pole, showing the distribution of microtubules in the subpellicular array in the cell body, the axoneme microtubules, the paraflagellar rod in the flagellum and the flagellar attachment zone[32]. The polarity of microtubules in the axoneme and the microtubule quartet (shown in blue) is minus end towards the viewer and plus end away. The remaining microtubules in the subpellicular array have the opposite polarity.

Furthermore, suppression of Bilbo1 leads to the disruption of both exocytosis and endocytosis as well as incorrect positioning of the new flagellum during cytokinesis. Importantly, although normal FP morphology depends on flagellum biosynthesis, this process is apparently normal in these cells[42]. These observations indicate that the FP collar segregates FP resident proteins, as shown in cells in which Bilbo1 has been silenced by RNA interference[41], and probably also facilitates the close apposition of membranes at the FP neck, which contains the FP matrix.

The point where the flagellum enters the FP is also associated with an annulus, the collarette, which consists of transitional fibres that are located on the outside of the flagellum membrane — that is, in the FP lumen. These fibres are in register with the nine outer microtubule doublets of the axoneme, and fibres connect the two rings. Presumably the collarette serves to anchor the membrane and the axoneme and potentially is a positioning mechanism for the flagellum in the context of the cytoskeleton. This probably explains why flagellum biogenesis is essential for correct FP morphology[42].

This association of cytoskeletal and membrane elements presents a clear challenge during cell division because these structures have to be faithfully segregated. Positioning of the FAZ and flagellum are crucial. Replication of the basal body and kinetoplast occurs early in the cell cycle, rapidly followed by duplication of the Golgi complex[27,43]. FP duplication is also an early event and is completed before extensive growth of the new flagellum beyond the neck region of the FP. New flagellum growth is guided initially by the flagellar connector. Coordination between flagellum growth and the rest of the cell is partly facilitated by the intimate connection between the FAZ, flagellum and integration of the FAZ microtubule quartet in the subpellicular array. How these events are coordinated structurally or temporally and how the complex of structures that are associated with the FP physically segregate remains poorly understood.

In addition to the endosomal and recycling apparatus, secretory system organelles are associated with the FP. Specifically, endoplasmic reticulum membranes are closely apposed to the FP, which seems more extensive on its bulged side[38] (FIG. 4). The Golgi complex is invariably associated with the bulged side of the FP and maintains a discrete *cis* and *trans* orientation[38,44]. The association between the Golgi complex and the FP is likely to facilitate high fidelity single organelle inheritance. The trypanosome Golgi complex is associated with a bilobe cytoskeletal element, which shares at least one component, Centrin2, with the basal body[45]. Sharing of components between the Golgi complex and basal body suggests a mechanism by which Centrin2 acts to coordinate the basal body, Golgi complex and FP duplication. Identification of a Golgi complex cytoskeleton in higher eukaryotes suggests that the platform delimiting the Golgi complex may be elaborate[46], and it will be of interest to determine how similar trypanosome and mammalian Golgi cytoskeletons are, as this will provide insight into the evolution of the Golgi complex and into organelle inheritance mechanisms. Duplication and segregation of the Golgi complex requires the action of a Polo-like kinase and *T. brucei* Vps34, which indicates that this process integrates with signalling pathways[47,48].

Polarized trafficking

Subpellicular array microtubules are too closely spaced to allow transport vesicles to access the plasma membrane; this restricts membrane traffic interchange with the cell surface to the FP. The FP membrane has a distinct proteome (TABLE 2), as several polypeptides are restricted to this structure, and most probably includes factors that function as receptors or docking sites for transport intermediates. The endomembrane system is highly polarized, and apart from the endoplasmic reticulum, which is distributed throughout the cytoplasm, the secretory and endocytic organelles are all located between the nucleus and kinetoplast and the single lysosome is normally located close to the nucleus (FIG. 3). In addition to the Golgi complex, which has *cis*- and *trans*-faced vesicular complexes[43,49,50], there are a multivesicular body-like structure[51] and differentiated sorting and recycling endosomes[52]. The Golgi complex has a cytoskeleton, but it is not known whether the other endomembrane compartments are associated with such structures, as cytoplasmic microtubules have never been observed. Overall, this is a conventional endomembrane system, albeit with a coordinated compact spatial distribution that is directed towards the FP.

Representatives of most paralagous protein families that are required for specificity of vesicle transport are present in trypanosomes, which is consistent with the overall configuration of the endomembrane system being established early in eukaryotic evolution[53,54]. There are sixteen Rab and Rab-related proteins as well as six Arf and Arf-like proteins encoded in the trypanosome genome, all of which mediate the specificity of vesicle fusion and cytoskeletal and coat protein interactions[55,56]. The functions of most trypanosome Rab proteins have been assigned, and these include roles in exocytotic, endocytic, recycling and retrograde pathways. Only two Arf proteins, ARL1 and ARF1, have known functions; as Arf protein evolution is lineage specific, these

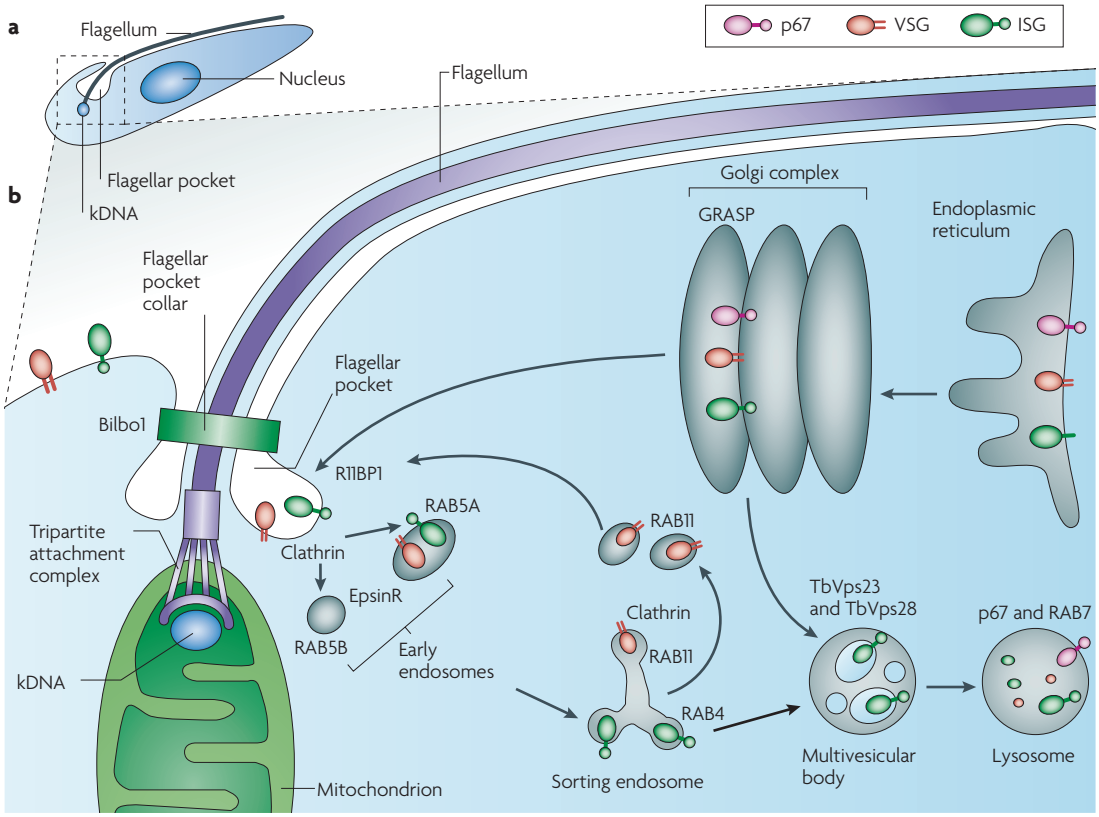

Figure 3 | **Architecture of the trypanosome, focusing on the flagellar pocket. a** | Schematic of a trypanosome, illustrating positions of the flagellar pocket, the flagellum emerging from the pocket and running along the trypanosome cell towards the anterior end, the nucleus and the kinetoplast DNA (kDNA). **b** | The expansion shows the single copy organelles requiring faithful replication and segregation at cytokinesis. These structures include the flagellum, the flagellar pocket, the kinetoplast, the mitochondrion, the Golgi complex, the nucleus and possibly the lysosome. The diagram is highly schematic and mostly based on the morphology in fixed cells. Endocytic and recycling trafficking pathways are shown, and the molecular markers that are used to define each compartment are indicated: Rab proteins; R11BP1 (a coiled-coil protein interacting with RAB11 that localizes to the flagellar pocket); and Vps23 and Vps28 (trypanosome orthologues of ESCRT (endosomal sorting complex required for transport) gene products). Evidence for other factors are discussed in the text. The trafficking routes of three proteins are shown: variant surface glycoprotein (VSG) is recycled, invariant surface glycoprotein 65 (ISG) is endocytosed by a ubiquitin-dependent mechanism and p67 is sorted by a dileucine-dependent mechanism. Arrows show probable movement of material between compartments. GRASP, Golgi reassembly-stacking protein.

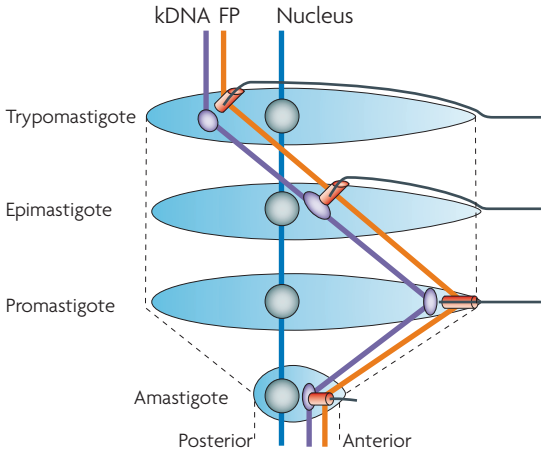

Figure 4 | **Life cycle repositioning of flagellar pocket in relation to other organelles.** The morphology is defined by the positions of the flagellum emergence point and the flagellar pocket (FP; shown in orange), nucleus (shown in blue) and kinetoplast DNA (mitochondrial kDNA; shown in purple) along the anterior–posterior axis. The *Trypanosoma brucei* life cycle is restricted to trypomastigote and epimastigote forms, but amastigotes and promastigotes are found in other Kinetoplastids, for example *Leishmania* spp.. In addition to these forms, the dimensions of the various morphologies are variable between distinct stages. Image is modified with permission, from *Nature* REF. 37 © (1966) Macmillan Publishers Ltd. All rights reserved.

proteins have no higher eukaryote orthologues[57,58]. In *T. brucei* SNARE (soluble *N*-ethylmaleimide-sensitive factor accessory protein receptor) proteins, which mediate membrane fusion, have not been analysed, but studies in *Leishmania* spp. indicate that they are present in essentially all major endomembrane organelles[59]. *T. brucei* have no obvious orthologues of the SNARE proteins SNAP25 (synaptosomal-associated protein 25) or R-type brevin, but most SNARE proteins are highly divergent and few can be assigned orthologous relationships. Furthermore, trypanosomes have proteins that are part of major coat systems. Although little is known about the location and function of adaptin complexes[60], adaptor protein 1 (AP1) knockdown has been shown not to affect lysosomal delivery but to result in a pleiotrophic phenotype that includes morphological defects, cell cycle progression and accumulation of electron-dense membrane-bound cytoplasmic structures, and AP3 is required for acidocalcisome biogenesis[51,61]. The absence of localization data for the trypanosome adaptins is an important gap to understanding their functions. Moreover, both coatomer complexes are present in trypanosomes; COPI localises to the Golgi complex and COPII to the endoplasmic reticulum, which is consistent with higher eukaryotes[62,63]. Exocytosis requires two Rab proteins, RAB1A and RAB2, which localize to the endoplasmic reticulum exit sites and the Golgi complex[64]. Finally, genes encoding tethering factors, including COG (conserved oligomeric Golgi complex) and HOPS (hepatocyte odd protein shuttling protein; also known as TMUB1), that are involved in transport vesicle docking are also present in trypanosomes[65].

It is clear that trypanosomes have a conventional endomembrane system, albeit with a polarized arrangement that is well placed to serve the FP. However, the trypanosome endomembrane system has novel features. First, unlike higher eukaryotes, all endocytosis is clathrin dependent[66,67], and this is supported by studies in which clathrin and RAB5 have been knocked down and by the observation that trypanosome genomes lack components of clathrin-independent pathways[52,67,68]. Inhibiting endocytosis is accompanied by massive internal ballooning of the FP; this spectacular phenotype is the result of imbalanced endocytosis and exocytosis[66]. Second, in the bloodstream stage, dynamin does not seem to be required for endocytosis[69], although this has been questioned by studies in the procyclic form, in which dynamin knockdown leads to an enlargement of the FP[70]. Third, although actin is clearly involved in endocytosis[71], the absence of genes encoding any obvious factors that might connect actin and clathrin-mediated endocytosis suggests a distinct mechanism.

Exclusive clathrin-mediated endocytosis indicates a streamlined internalization mechanism. The packing density of VSG at the plasma membrane, which is thought to approach the maximum possible, probably precludes further concentration in clathrin-coated pits[50,52]. AP2, which mediates cargo concentration into clathrin-coated pits in higher eukaryotes, is absent from *T. brucei*, probably owing to this inability to concentrate VSG. Several other Trypanosomatids, including *Leishmania* spp., have AP2, but all four subunits are lost from *T. brucei* and other trypanosomes that express VSG, eliminating the basic mechanism of cargo concentration in clathrin-coated pits. In higher eukaryotes dephosphorylation of AP2 before clathrin uncoating could retard the rates of membrane internalization, and the incorporation of dynamin into the assembling clathrin coat could delay budding of clathrin-coated vesicles. Therefore, loss of AP2 in trypanosomes could also be an adaptation for extremely efficient endocytosis. All of these considerations suggest selection for rapid non-selective internalization of FP membrane during the evolution of African trypanosomes.

The only characterized factor that might act as an endocytic adaptor is an ENTH-domain family member, EspinR[72]. EpsinR seems to have a general involvement during endocytosis, as EpsinR knockdown leads to defective uptake of many proteins that are anchored to the cell membrane by GPI or transmembrane domains. Therefore, endocytosis in trypanosomes is probably adapted for non-selective and extremely rapid uptake.

Sorting signals

Although initial uptake at the FP is non-selective, there is evidence for sorting in subsequent trafficking pathways. Following uptake, sorting of internalized molecules and endomembrane components occurs at tubulated endosomes[52]. VSG itself is rapidly returned to the surface by RAB11-positive vesicles. Sorting endosomes might

Orthologue
Homologous sequences that are separated only by a speciation event. If genes are orthologous they share common ancestry, and this allows inference of similar function when comparing two distinct lineages.

Acidocalcisome
An electron-dense acidic membrane-bound cytoplasmic organelle containing a matrix of pyrophosphate and polyphosphates with bound calcium and additional cations. Acidocalcisomes are found in a wide range of eukaryotes and prokaryotes.

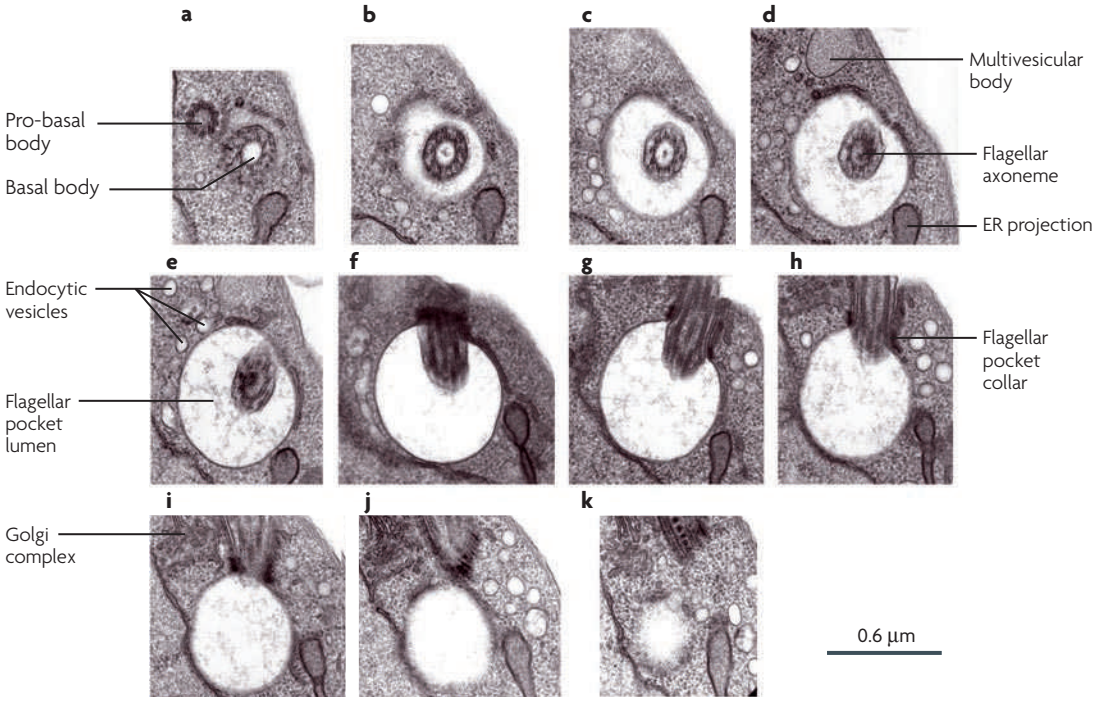

Figure 5 | **Morphology and integration of the flagellar pocket with the trafficking system.** Serial sections through the flagellar pocket of *Trypanosoma brucei* insect form. Sections are approximately 70 nm thick. They were stained with osmium tetroxide–uranyl acetate and visualized on a Phillips CM100 transmission electron microscope. Sections are oriented so that the plasma membrane is at top right. The images are ordered so that **a** is at the deepest point of the flagellar pocket and **k** is the shallowest section. The identity of prominent organelles surrounding the pocket are indicated. **a** | Flagellar basal body (9+0 microtubule configuration) and the second basal body are visible as is an extension of the endoplasmic reticulum (ER) that continues through the entire series. A few vesicular profiles are also visible. **b**,**c** | The axoneme proper with a 9+2 microtubule structure becomes visible and there are increased numbers of vesicles associated with the pocket. **d**,**e** | A multivesicular body is visible at the top in **d** and also membrane-associated with the face of the pocket, which in **e** resolves into two profiles that are split by the path of the emerging flagellum. **f**–**i** | An extensive tubulated vesicle is visible, as is the electron-dense flagellar pocket collar, which is also visible in **g**, **h** and **i**. Note the close juxtaposition of the flagellum membrane and the flagellar pocket membrane in **g**. **i**–**k** | A portion of the Golgi complex is visible at the top left. Material in the lumen of the pocket is carbohydrate rich, but has not been fully characterized. Image courtesy of D. Goulding, Imperial College London, UK.

partition VSG from other molecules using a mechanism involving clathrin-coated boutons, which seems to involve dilution of the VSG[52]. Interestingly, VSG and the transferrin receptor, which are both GPI-anchored, are actively recycled[73]; this indicates that recycling might be the default route taken by lipid-anchored molecules. A distinct pathway involving ubiquitylation of cytoplasmic lysines operates for type I transmembrane proteins. This is consistent with a mechanism of clathrin-mediated segregation of transmembrane proteins away from the main VSG recycling pathway[74–76].

Sorting of the lysosomal protein p67, a lysosome-associated membrane glycoprotein (LAMP) analogue, depends on a dileucine motif. p67 has a short cytoplasmic domain containing a classical dileucine sequence that is embedded in an acidic region which is required for lysosomal targeting[51,77]. p67 is essential and required for normal lysosomal morphology, as evidenced by its knockdown[78]. Furthermore, cysteine-rich acidic integral membrane protein (CRAM), a transmembrane protein that localizes to the FP, contains a multifunctional sorting signal that participates in both anterograde export from the endoplasmic reticulum and endocytosis[79]. An additional sorting mechanism that has been identified only in *T. brucei* involves an endosomal- and FP-targeting pathway that depends on the modification of N-linked oligosaccharides with poly-*N*-lactosamine; this pathway has been observed for the sorting of p67 and transferrin receptor[80–82]. The basis for selection for modification by poly-*N*-lactosamine is unclear, and assembly occurs on a standard trimannosyl chitobiose N-glycan. It is unclear whether there is a peptide signal-based mechanism analogous to that required for mannose-6-phosphate modification. How this motif contributes to sorting is also unknown, but there seems to be some compartmentalization between endosomal subpopulations as well as an ability to inhibit endocytosis of poly-*N*-lactosamine-bearing proteins using soluble lactosamine polymers, suggesting a role for a lectin-like factor[80].

Clathrin-coated bouton
(from the French for button). A small bud or button-like structure that extends from the central region of the sorting endosome and where clathrin is preferentially localized. It is thought that the differential morphology and protein composition allows the structure to selectively sort proteins.

Surface expression at the FP

The high rate of endocytosis and recycling that takes place in trypanosome mammalian forms does not occur in most insect stages. In higher eukaryotes transcriptional modulation is used as a mechanism to alter trafficking activity[83,84] but, with the exception of

Table 2 | **Locations of important surface proteins in *Trypanosoma brucei***

Protein	Bulk membrane (96.98% total area)*	Flagellar pocket (2.4% total area)	Refs‡
VSG§¶	+	+	NA
ISG65§	+	+	99
ISG75§	+	+	99
Procyclin‖	+	+	NA
AT1#	+	?	NA
SRA§¶	–	+	90
Transferrin receptor§¶	–	+	23,73
LDL receptor**	–	+	81
HPHBR§¶	–	+	25
CRAM	–	+	79

*A confident assessment of the presence of the protein is denoted by +, and – indicates a robust indication that expression level is below the detection limit. ‡A citation is given were there is a clear, and recent, first description of the localization. NA denotes proteins for which it is difficult ascribe the first formal report. §Expressed in bloodstream stages only. ‖Expressed in procyclic forms only. ¶GPI-anchored proteins. #Presumed to be present on the bulk membrane based on the behaviour of other polytopic membrane proteins in trypanosomes, but has not been formally shown. **Staining for this receptor is clearly detectable on the bulk membrane and the flagellar pocket, but the receptor itself has not been identified. CRAM, cysteine-rich acidic integral membrane protein; ISG, invariant surface glycoprotein; GPI, glycosylphosphatidylinositol; LDL, low density lipoprotein; NA, not available; HPHBR, haptoglobin–haemoglobin receptor; VSG, variant surface glycoprotein.

clathrin, in *T. brucei* developmental changes in endocytosis are not accompanied by substantial alterations in mRNA levels for most trypanosome endocytic components[85]. Manipulation of the levels of RAB5 by overexpression or knockdown leads to alterations in clathrin expression, but these are at the protein and not mRNA level[81,85]. The mechanism for this regulation of clathrin expression is unknown, but given an enormous difference in copy numbers of RAB5 and clathrin proteins, it is probably mediated by an indirect process (for example, polymerization or targeting) rather than formation of stoichiometric complexes. This phenomenon does not seem to extend to cells from higher eukaryotes. Overall, these observations suggest that distinct mechanisms controlling endocytosis probably operate in trypanosomes.

In contrast to changes in early endocytosis, delivery of molecules to the lysosome seems to be constant[86] and efficient in both insect and mammalian stages. A return to high rates of endocytosis occurs in salivary gland metacyclic forms, which are pre-adapted for the colonization of mammals[16]. Mammalian infectivity is exclusively associated with high levels of endocytosis and VSG expression.

A total change in the abundant cell surface proteins accompanies the differentiation from mammalian to insect forms. Mammalian forms express VSG, a GPI-anchored dimer, whereas insect midgut forms express procyclins, which are GPI-anchored monomers. There is a further change in the salivary gland epimastigote, which expresses BARP, another GPI-anchored protein[87,88]. Less is known about the differences in the expression of FP components between these developmental forms. However, most proteins that are known to localize at the FP are restricted to one life stage or the other. This restriction indicates that many components of the FP are involved in interactions with factors from the individual hosts. For example, the FP components serum resistance-associated protein (SRA), transferrin receptor and the recently characterized haptoglobin (HP) –haemoglobin (HB) receptor (HPHBR) bind mammalian serum components and their expression is therefore restricted to the mammalian form of the parasite[25,89,90]. Thus, developmental remodelling of surface proteins is not restricted to abundant antigens and those distributed over the entire surface.

Specific localization of proteins to the FP membrane occurs by an unknown mechanism. Retention in the FP is saturable, and overexpression of transferrin receptor leads to escape to the bulk membrane[23]. It is possible that this restriction is a dynamic result of the high rate of endocytosis, which retains transferrin receptor, SRA and HPHBR in the FP and prevents their diffusion through a non-specific barrier functioning in a similar manner to mammalian cell corrals[91]. Importantly, the copy number of receptors that are restricted to the FP is extremely low compared with both invariant surface glycoproteins (ISGs) and VSGs. Selective retention in the FP is probably vital to the parasite, both in terms of efficient nutrient acquisition but also for immune evasion. Suppression of the FP collar leads to loss of FP architecture, thereby allowing escape of specific proteins onto bulk membrane[41], but this is a non-selective phenotype, and we have no insights into specific mechanisms or factors that allow the free passage of VSGs and ISGs but not of transferrin receptor and other proteins. As the bulk plasma membrane occupies a much greater area than the FP, and most experiments examining FP specificity have been carried out with microscopy, it is possible that even equivalent numbers of FP proteins on the surface have been overlooked, especially as trypanosomes cap receptors so efficiently. For example, if modest overexpression can lead to the escape of transferrin receptor from the FP, any potential diffusion barrier surrounding the FP is leaky[23]. In the absence of a reliable quantification of the copy numbers of FP proteins in both the FP and bulk plasma membrane or a full list of FP and FP collar proteins, the mechanism remains unclear.

Signalling pathways and the FP

The FP is involved in several signalling pathways, as observed by defects in FP morphology following disruption of these pathways. Indeed, both phosphoinositide 3-kinase (PI3K) and target of rapamycin (TOR) signalling pathways seem to interact with the FP. First, the trypanosome PI3K Vps34, which is important in endocytosis, is required for normal FP morphology, and Vps34 knockdown results in abnormal structure and accumulation of membrane-bound paracrystalline inclusions containing flagellar and PFR proteins[47]. Furthermore, knockdown of phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ) also leads to an enlarged FP together with inclusions, although these are distinct from the Vps34-induced PFR-containing structures[92]. Extensive accumulation of cytoplasmic

Paracrystalline inclusion
An inclusion in membrane-bound structures found in the lumen of the flagellar pocket that have small lattice-like structures separated by regions of less clear order. Such structures frequently arise by high concentrations of proteins accumulating and forming partially ordered aggregates.

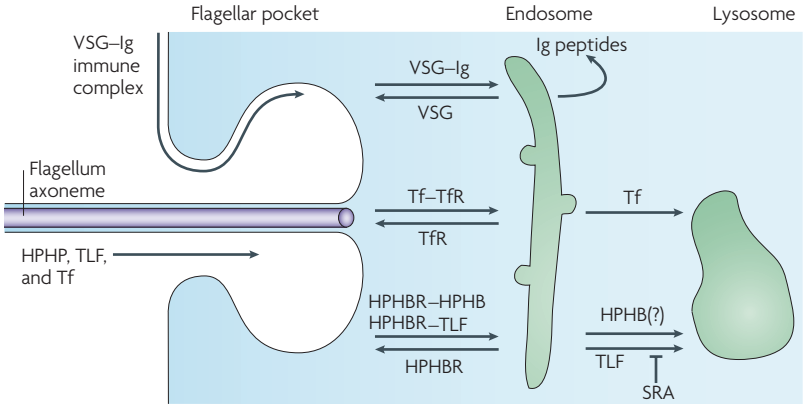

Figure 6 | **Trafficking during nutrient acquisition and immune evasion.** The trypanosome endosomal system is schematically reduced to three compartments: the flagellar pocket, a generic endosome and the terminal lysosome. Surface-bound VSG–immunoglobulin (Ig) complexes enter the pocket associated with the plasma membrane, whereas the haptoglobin–haemoglobin complex (HPHB), trypanosome lytic factor (TLF) and transferrin (Tf) probably enter the pocket through the fluid-phase. VSG-specific antibody degradation and VSG recycling pathways probably contribute to defence against the adaptive immune response. VSG–Ig immune complexes are efficiently endocytosed and dissociated. VSG is efficiently recycled to the surface and immunoglobulin is degraded[22–24]. Tf is taken up and is required for the provision of iron. The Tf–Tf receptor (TfR) complex is endocytosed and dissociates in endosomes. TfR efficiently returns to the cell surface, and Tf is delivered to the lysosome and degraded[21]. The *Trypanosoma brucei* haptoglobin–haemoglobin receptor (HPHBR)[25] is a mediator of innate immunity and potentially also nutrient acquisition. HPHBR binds HB that is released from haemolysed cells in complex with haptoglobin, but also binds TLF owing to the presence of an HP-related protein in TLF. Hence, HPHBR delivers lytic TLF to the parasite through endocytosis. As haem is retained by the parasite, it is likely that the HPHBR and cargo reach the lysosome, where haemoglobin is probably degraded. TLF only disrupts the lysosome in TLF-sensitive *T. b. brucei*. In TLF-resistant *T. b. rhodesiense* parasites serum resistance-associated protein (SRA) prevents TLF action[24,90,109].

vesicles is associated with mislocalization of CRAM, mirroring clathrin suppression defects[66,67]. Overall these studies potentially link phosphoinositide-based signalling to the function of the FP.

TOR kinases control cell growth in higher eukaryotes through the actions of two distinct protein complexes, TORC1 and TORC2, which are conserved in *T. brucei*. In mammals TOR regulates cell growth through several mechanisms, including sensing amino acid levels. Exposing trypanosomes to rapamycin leads to rapid decrease in cell growth and engorgement of the FP[93]. TORC2 is the only target of rapamycin in trypanosomes, and its knockdown perturbs actin localization and endocytosis; this explains the engorgement of the FP following rapamycin treatment. Overall, these observations indicate a sophisticated signalling platform that supports trafficking and FP dynamics. These signalling pathways are generally similar to those of higher eukaryotes, but there are important differences that reflect the specific requirements of trypanosomes for managing the function of the FP.

Immune evasion

T. brucei divide approximately every 6 hours in mammals, which is sufficient to rapidly overwhelm the host. Clearly, as infection can persist for years, mechanisms facilitating long-term host survival must operate[94] (FIG. 6).

In mammalian hosts, the trypanosome surface is covered by a dense monolayer of VSG[95]; at ~5×10^6 VSG dimers per cell[96] this density approaches the maximum possible by steric packing[97]. Antigenic variation is based on VSG sequence diversity, and different VSGs have only 15–20% amino acid sequence identity. *T. brucei* is readily killed by VSG-specific antibody-dependent complement cytotoxicity[98], and in fact it is thought that only VSG-specific immunoglobulins can result in antibody-mediated killing[99], which indicates that VSGs protect conserved plasma membrane proteins from immune recognition. At a population level, genetic and epigenetic switches result in changes in the VSG gene that is expressed, thereby generating distinct antigenic variants[100–102]. As a result, trypanosomes with new antigenic variants survive the immune response that was mounted against the previous variant. Parasitaemia in most natural mammalian hosts is regulated by a parasite density-dependent mechanism and fluctuates between 1×10^2 and 1×10^4 parasites per ml[103]. Many cells are arrested and susceptible to VSG-specific immunoglobulins. To counter rising VSG-specific immune responses trypanosomes can remove surface-bound immunoglobulin until a threshold of immunoglobulin concentration is exceeded and the trypanosome is killed. The overall effect allows an individual trypanosome cell to persist longer and increase the probability of transmission.

Clearance of VSG-specific immunoglobulins from the cell surface occurs by a unique mechanism. At 0°C, VSG-specific immunoglobulins uniformly label the whole cell surface. Subsequent incubation at 37°C results in the migration of immunoglobulins towards the posterior pole and into the FP[20,104,105], with a consequent reduction in antibody-dependent efficacy of killing[22,102]. The directional movement of VSG–immunoglobulin complexes towards the FP depends on hydrodynamic forces resulting from the forward motion of the trypanosome, dragging the immunoglobulins that rise above the plane of the VSG monolayer towards the posterior pole[22]. This mechanism depends on the extreme posterior location of the FP in the bloodstream form of trypanosomes.

Immunoglobulin removal is achieved through the high rate of endocytosis (FIGS 3,6). During this process VSG-specific bound immunoglobulins are internalized, segregated from VSGs and then degraded, while VSGs are recycled. Specifically, VSG–immunoglobulin complexes are endocytosed by RAB5A-containing vesicles[21,101]. VSG-bound immunoglobulins are then degraded to small peptides, with little proteolysis of the VSG[21,22,106]. The process is so efficient that antibody-aggregated cells can be separated[106]. The details of how immunoglobulins are selectively degraded remain unclear. Metacaspases are present in endosomal vesicles, but it is unclear whether they have a role in immunoglobulin degradation[107]; however, the process can be inhibited by the cysteine protease inhibitor K11777 (S. Natesan and M.C.F., unpublished observations). Therefore, the trypanosome has evolved a self-cleaning cell surface that is centred on immunoglobulin internalization through

the FP. As most trypanosome species that infect vertebrates do not express VSGs, it is unknown whether this mechanism represents an ancient adaptation that predates the evolution of the VSG coat and that originally served some other purpose, such as feeding, or whether it co-evolved with VSGs.

Most *T. brucei* isolates cannot infect humans and are killed through an innate immune-mediated mechanism[90]. This killing depends on the endocytosis of a specific high-density lipid particle containing apolipoprotein L1 (APOL1) and haptoglobin-related protein (HPR)[90,108]. HPR binds free serum HB, and trypanosome HPHBR recognizes the HPR–HB complex[25]. The entire high-density lipid particle is endocytosed, and APOL1 acts once the particle has been trafficked to the lysosome, where it compromises membrane integrity[109]. The function of the HPHBR at the FP is unclear, as both APOL1 and HPR are primate specific and *T. brucei* is predominantly a non-primate parasite[25].

There are two human infective subspecies, *T. brucei gambiense* in West and Central Africa and *T. brucei rhodesiense* in East Africa. The molecular mechanism of resistance to APOL1 killing for *T. b. rhodesiense* has been defined[110] (FIG. 6). *T. b. rhodesiense* is characterized by the presence of *SRA*[110–112], which is absent from all other *T. brucei* subspecies. *SRA* arose through an internal truncation of a VSG gene[113,114] and encodes a GPI-anchored dimer that is present in the FP and endosomes. SRA binds the carboxy-terminal region of APOL1 and is thought to neutralize the ability of APOL1 to disrupt the lysosome.

Is the FP an evolutionary trap?

The FP is common to many protists, in which most of the functions described above are irrelevant. For example, the FP of free-living trypanosomatids is not required for immune evasion. More importantly, in *Leishmania* spp. promastigotes and amastigotes the flagellum is not attached to the cell surface and therefore may not function as a template for cytokinesis. Thus, conceptually the presence of an FP is not required for the participation of the basal body and flagellum in directing cytokinesis. One original FP function was probably to provide access to the cytoplasm through the subpellicular microtubule array. Can a simple defined gap in the subpellicular array substitute for the FP in free-living protists?

Knockdown of the FP collar factor Bilbo1 leads to loss of the pocket invagination, disrupting trafficking and correct flagellum positioning during cytokinesis, without affecting flagellar biosynthesis[41]. Conversely, correct FP morphology requires flagellum biosynthesis[42]. The implication is that the FP is so tightly integrated with trafficking polarity, cell cycle progression and organelle positioning during cytokinesis that, regardless of functional requirements for an FP, it is now indispensable. The FP is therefore an evolutionary device that, in all likelihood, can never be lost from Kinetoplastids.

Conclusions

Understanding the importance of the trypanosome FP has seen major advances, which encompass greater molecular detail of acquired and innate immune evasion mechanisms, high resolution descriptions of structure, improved comprehension of the role of the cytoskeleton in coordinating organelle segregation and the first description of an FP collar component. The functions of new gene products that interact with the trafficking system, the flagellum or the cell division machinery are at least partially known, highlighting an interplay between many of these systems. Many gene products functioning in the context of the FP are essential, and their loss leads to gross morphological defects that fatally disrupt cellular functions. They are therefore particularly valuable therapeutic targets.

However, much remains unknown. A validated FP proteome is required to fully understand many FP functions, including protein sequestration and the total number and ligand-specificity of receptors. This will provide improved insight into interactions of the parasite with the host, and it is likely that additional surprises are in store. Furthermore, the FP is a valuable therapeutic target but has yet to be exploited, and this remains an important goal for the future.

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DATABASES
Entrex Genome Project: http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj
Trypanosoma brucei | *Trypanosoma cruzi*
UniProtKB: http://www.uniprot.org
p67 | RAB5 | SRA | transferrin receptor

FURTHER INFORMATION
Mark C. Field's homepage: http://homepage.mac.com/mfield/lab/Welcome.html

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