The EMBO Journal Vol. 22 No. 19 pp. 4991–5002, 2003

Clathrin-mediated endocytosis is essential in *Trypanosoma brucei*

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Supplementary data: Figures S1-4 and Video V1.

Results

Induction is extremely sensitive to tetracycline: The severity of the BigEye phenotype suggested that repression of dsRNA transcription must be very efficient, otherwise BigEye cells would have been detectable in uninduced cultures. Additionally, the lethality of the clathrin RNAi would have most likely rendered it impossible to obtain transformants. BigEye cells were used to directly titre the level of induction *versus* tetracycline concentration, as the BigEye phenotype can be easily scored by light microscopy. Following a 16 hour induction significant BigEye phenotype was achieved with 3nM tetracycline, and 50% of the cells became BigEye at ~10nM (Fig. S2). These data suggest that; (i) the repressor system is efficient as very few BigEye cells are seen in uninduced cultures, and (ii) that levels of tetracycline in the medium (derived from the foetal calf serum) are negligible, as introduction of very small amounts of tetracycline resulted in a large proportion of induced cells.

Measurement of exocytosis of VSG: The analysis of arrival of VSG at the cell surface is based on experiments described in Bangs *et al.* 1986, demonstrating that VSG on the surface is accessible to GPI-phospholipase C (GPI-PLC), whilst that within the endomembrane system is protected by membrane. Therefore, material recovered from the supernatant fraction following GPI-PLC digestion contains soluble VSG (sVSG) cleaved from the plasma membrane, whereas internal VSG remains membrane associated (mfVSG) and therefore separates into the pellet fraction.

SDS-PAGE analysis of recovered material taken at time points throughout the chase period clearly demonstrated a shift in the location of the majority of the radiolabelled VSG, from the pellet fraction early in the chase period to the soluble fraction (20 mins into the chase) (Fig. S2A). This shift from mfVSG to sVSG reflects the arrival of newly synthesised VSG at the cell surface. A continued increase in the proportion of sVSG recovered was observed at later time points during the chase period, until at 60 mins into the chase period, when all radiolabelled VSG detected was present in the soluble fraction (data not shown).

Upon plotting the proportion of sVSG at each time point it was found that despite the higher background in the noninduced cells, VSG is actively transported to the surface with similar kinetics in both induced and uninduced cells (Fig. S2C). This is in marked contrast to the apparent lack of endocytic activity detected in the induced cells (Fig. 3 and Fig. S4). These data indicate that a defect in arrival of the major cell surface protein at the surface cannot be detected,

suggesting that there is no severe alteration to exocytic flux.

Co-staining of trypanosomes with Con A and endocytic compartment markers: To investigate further the disruption of Con A receptor internalisation from the flagellar pocket of BSF cells lacking functional clathrin, markers for several endocytic compartments were used to co-stain BSF clathrin RNAi cells following Con A uptake and thereby determine whether Con A bound receptors were present in various compartments of the endocytic system.

Following uptake at 37^{o}C Con A is present throughout the endocytic system in uninduced cells, as determined by co-localisation with tomato lectin (TL) (which binds to polylactosamine glycans present on endosomal glycoproteins). Co-localisation with anti-p67 Ab (a lysosomal marker) also demonstrates that Con A has been delivered to the lysosome in these cells. In contrast, no co-localisation is detected between Con A and TL or anti-p67 Ab in induced cells, indicating that transport beyond the flagellar pocket is severely disrupted in BSF cells lacking functional clathrin (Fig. S4B). In parental cells Con A is delivered to the TbRAB5A early endosome at 12^{o}C. Therefore, this endocytosis assay was repeated at 12^{o}C on both uninduced and induced cells, which were then stained with anti-TbRAB5A Ab to identify this compartment (Fig. S4C). In uninduced cells there is clear co-localisation of Con A with the TbRAB5A endosomes, whereas the Con A taken up by induced cells does not co-localise with the TbRAB5A compartment, but remains associated with the enlarged flagellar pocket. This lack of co-localisation between ConA and the early endosomal compartments in BSFs lacking functional clathrin suggests that the disruption in transport of endocytosed material in these cells occurs very early in the endocytic pathway.

Anti-VSG antibody capping is defective in BigEye cells: Trypanosomes have a highly active system for removal of surface-bound antibody (O'Beirne *et al.* 1998, Pal *et al.* 2002, Balber *et al.*1979); this may be an immune evasion mechanism, but whatever the function, the system is dependent on the activity of endocytic system GTPases (Jeffries *et al.* 2001, Pal *et al.* 2002).

Upon binding of anti-VSG antibody, rapid capping and delivery to the flagellar pocket ensues, followed by internalisation and degradation of the ligand, plus recycling via a TbRAB11-dependent pathway (O'Beirne *et al.*1998, Jeffries *et al.* 2001). Even at 4˚C significant capping is observed, which is technically difficult to prevent in living cells (Fig. S4D). The presence of the clathrin RNAi plasmid has no effect on this process, as at 4^{o}C both parental and uninduced clathrin RNAi cells demonstrate Ab accumulation at the FP and some endosomal structures. At

37°C the Ab is mainly present within the endomembrane system and stains extremely strongly for this region of the cells. By contrast, in induced BigEye cells, a very low level of accumulation of antibody is observed at the flagellar pocket in cells at 4˚C, whilst at 37˚C this residual antibody remains associated with the pocket, and does not proceed deeper into the cell. Hence this pathway is also compromised by the ablation of clathrin heavy chain expression. Further, the capping process appears to be less efficient than in parental or noninduced cells (Fig. S4D), suggesting that capping itself may also rely on an active endocytic system.

Materials and methods

Tetracycline inducibility assay: The appearance of the BigEye phenotype was used to test the sensitivity of BSF CLH RNAi cells to inducibilty by tetracycline. BSFp2T7^{Ti}CLH cells were cultured for 16h in the presence of serially diluted concentrations of tetracycline and the incidence of the BigEye phenotype in live cells was scored by counting 50 cells per sample on a hemocytometer under light microscopy.

VSG exocytosis assay: 5 x 10^7 mid-log phase BSF cells were washed once in labelling medium (DMEM without methionine or cysteine (Sigma) supplemented with 10% dialysed foetal calf serum and 25mM Hepes (pH 7.4)) pre-warmed to 37°C. After centrifugation (800 x g, 10 mins, RT) the cells were re-suspended in 1ml labelling medium and incubated (37°C, 5% CO_2) for 15 mins to starve the cells of methionine and cysteine. The cells were pulse labelled at 37°C for 7 mins by adding [^{35}S] methionine and [^{35}S] cysteine pro-mix (Amersham Biosciences, >1000 Ci/mM) to a final concentration of 200μCi/ml, then chased for up to 1h (37°C, 5% CO_2) by diluting the labelled cells 1:10 with pre-warmed complete HMI9 (containing 30μg/ml non-radioactive methionine and 182μg/ml non-radioactive cysteine). At intervals during the chase period 1ml aliquots of cells were removed from the sample and placed on ice to prevent continued exocytosis. Each aliquot was then processed as follows;

Cell lysis and GPI-phospholipase C digestion: Following centrifugation in a microfuge (20000 x g, 20s, 4°C) the cell pellets were washed once in 1ml ice cold PBS/1mg/ml BSA, re-centrifuged as before, then resuspended in 920μl of hypotonic lysis buffer (10mM Tris-HCl, pH7.5) to lyse the cells. After 5 mins on ice the lysates were incubated at 37°C for 10 mins to enable endogenous GPI-phospholipase C (GPI-PLC) to convert susceptible membrane form VSG (mfVSG) to soluble VSG (sVSG). To separate mfVSG from sVSG the lysates were centrifuged

for 10 mins in a microfuge (20000 x g, 4°C), after which, 900µl of supernatant (containing sVSG) was transferred to a new eppendorf. The pellet fraction (containing mfVSG) was washed once with 1ml ice cold hypotonic lysis buffer then resuspended in 1ml ice cold 1 x sample lysis buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40) and incubated on ice for 25 mins to lyse the membranes. 90µl of 10 x sample lysis buffer and 10µl of NP-40 was added to the supernatant fraction to make sure that all the samples were in the same buffer. The lysates were then cleared by centrifugation for 15 mins (20000 x g, 4°C) and the supernatants transferred to new eppendorfs for Con A binding. 5mM iodoacetamide, 0.1mM N α-p-tosyllysine chloromethyl ketone (TLCK) and 1µg/ml leupeptin were included in each lysis buffer to minimize proteolysis.

Recovery of VSG from cell lysates: Concanavalin A-sephrose 4B (Sigma) was used to recover glycoproteins (the majority of which are VSG) from the cell lysates. To aid binding to Con A, $MnCl_2$ and $CaCl_2$ were added to each sample to a final concentration of 1mM. 10µl of a 50% slurry of ConA-sepharose 4B in Con A wash buffer (10mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM $CaCl_2$ and 1mM $MnCl_2$) was added to each sample and mixed for 1h at 4°C. After mixing, the Con A-sepharose with bound glycoproteins were centrifuged and washed as follows: three times in ice cold Con A wash buffer, then once in ice cold PBS. The samples were centrifuged for 1 min in a microfuge (100 x g, 4°C) after each wash. Following the final wash the beads were resuspended in PBS and 2 x SDS-PAGE sample buffer (2 x = 100mM Tris-HCl (pH 6.8), 2% SDS, 80 mM dithiothreitol, 20% glycerol, 0,1% bromophenol blue) so that the final sample contained 1 x 10^5 cell equivalents/µl. The recovered proteins were separated on 10% SDS-PAGE gels, loading 1 x 10^6 cell equivalents per lane. The gels were stained with coomasie blue, soaked in EN^{3}HANCE (NEN Life Sciences), then dried down and exposed to Kodak Biomax MR-1 film to identify metabolically labelled proteins. Autoradiograms were scanned using a Heidelberg 1200 scanner, and images converted to TIFF format and imported into Image J (NIH). The VSG bands were quantitated, together with background subtraction (adjacent area from the same lane), and data expressed as percent recovered in the soluble fraction (released).

Con A uptake assay and co-stain with endocytic compartment markers: Essentially the same protocol described in the materials and methods was used for the Con A uptake assay, except that 100 µg/ml biotinylated Con A (Vector Laboratories, Burlingame, CA) was used instead of FITC-Con A for cells co-stained with anti-p67 Ab or tomato lectin (TL). Following fixing and

adherence to the slides, the cells were permeabilised with 0.1% triton-x-100 for 10 mins then blocked in PBS/10% goat serum for 1h. In order to visualise the biotinylated Con A, Texas red streptavadin (Vector Laboratories, Burlingame, CA) was added to the cells at a 1:500 dilution in PBS/10% goat serum for 1h, then washed off with PBS. Anti-p67 Ab (which localises to the lysosome) or FITC-Tomato lectin (Sigma) (which stains the entire endosomal system) were added to these cells at a dilution of 1:500, at RT for 1h. Cells that had taken up FITC-Con A were incubated at 4^{o}C O/N with Anti-TbRAB5A Ab (to identify the early endosomal compartments) at 1:50 dilution. These slides were then washed with PBS before adding the appropriate secondary Ab diluted according to manufacturer's instructions (Oregon green anti-mouse Ab (Molecular Probes, Oregon, USA) to the slide stained with anti-p67 Ab or Texas Red anti-rabbit Ab (Molecular Probes, Oregon, USA) to the slide stained with anti–TbRAB5A). All cells were stained with DAPI at 0.5μg/ml to visualise the nucleus and kinetoplast.

Anti-VSG Ab uptake assay: To follow internalisation of surface bound anti-VSG antibody, 3 x 10^7 bloodstream form cells/ml were incubated in HMI9 containing anti-VSG 221 antiserum diluted 1:100 (Pal et al., 2002) at 4^{o}C or 37^{o}C for 5 mins. Prior to the addition of the antibody, the cells were pre-incubated for 10 mins at the appropriate temperature for the assay. After incubation in the presence of the antibody the cells were transferred to ice, washed with ice cold VPBS before fixing in 3% PFA/VPBS for 1 h at 4^{o}C. After fixing, the cells were permeabilised with PBS/0.1% triton-X-100, adhered to poly-L lysine slides then blocked in PBS/10% goat serum before staining the cells with Cy3 anti-rabbit IgG to identify internalised anti-VSG antibodies.

Supplementary literature cited

Bangs JD, Andrews NW, Hart GW, Englund PT. Posttranslational modification and intracellular transport of a trypanosome variant surface glycoprotein. J Cell Biol. 1986 103:255-63.

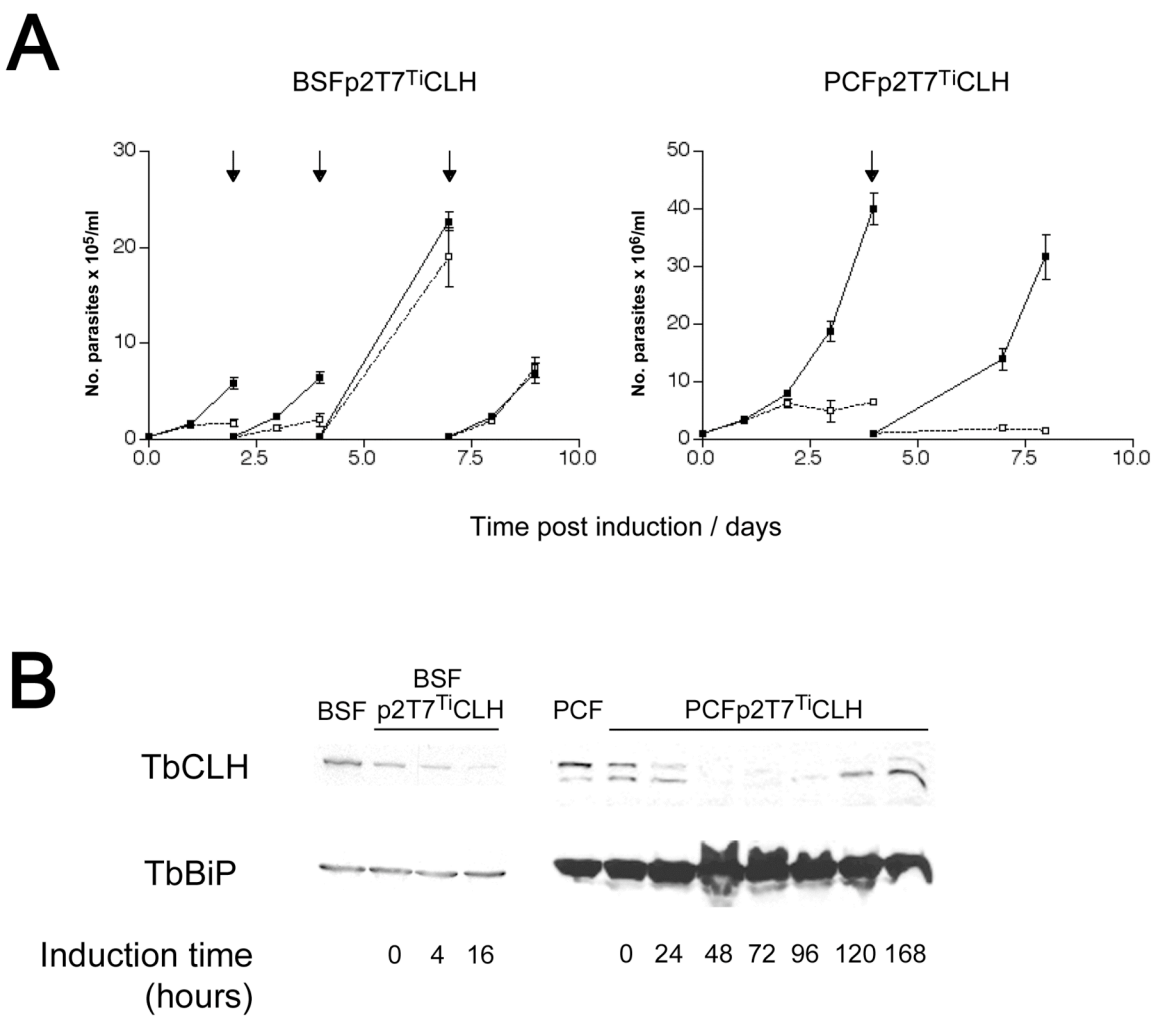


Figure S1: Depletion of clathrin heavy chain by RNAi is lethal in procyclic and bloodstream forms of *T. brucei*. A; Growth curves for bloodstream (BF4p2T7^{Ti}CLH) and procyclic (29-13p2T7^{Ti}CLH) trypanosomes containing the p2T7^{Ti}CLH RNAi plasmid. Solid symbols denote uninduced cultures and open symbols cultures induced with tetracycline (on day 0). Proliferation was monitored with a Coulter Counter at regular intervals, and cells were maintained under mid-log growth conditions by periodic subculturing (indicated by arrow). Note that both induced and uninduced cultures were passaged at the same time. Data are from an experiment where cultures were set up in triplicate (data points are the mean, $\pm$ standard deviation), and are representative of multiple experiments. B; Western blot analysis of clathrin expression under RNAi induction. The leftmost lane in each set is a control lysate from parental cells. Aliquots of cultures were taken at the indicated times, solubilised and analysed by Western blot following separation of proteins by SDS-PAGE. A clear decrease in clathrin heavy chain expression is apparent in both procyclic and bloodstream cells. Membranes were re-probed with antibody against TbBiP as a loading control. Note that the clathrin heavy chain is susceptible to proteolysis, which gives rise to the doublet in the procyclic lanes (Morgan *et al.* 2001).

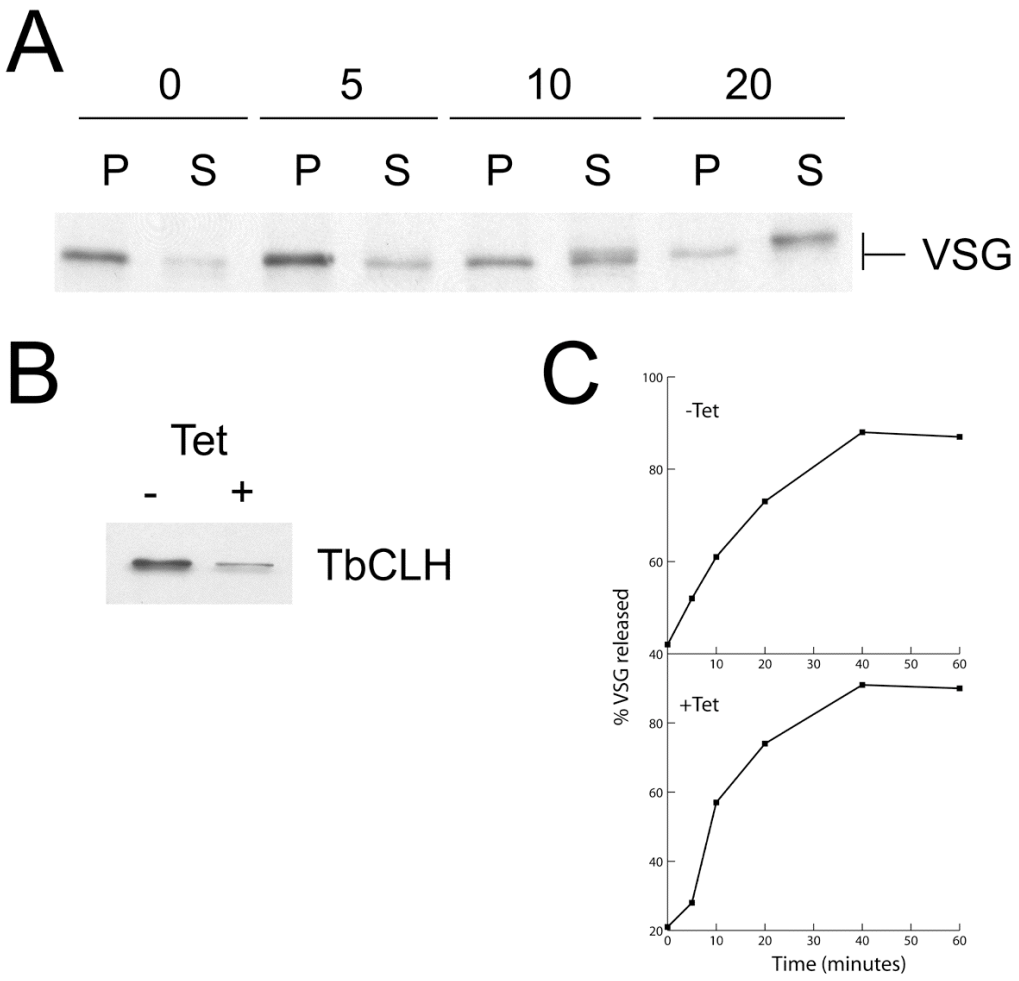


Figure S2: VSG export is not impaired by suppression of TbCLH. Panel A; Pulse-chase kinetic analysis of arrival of VSG at the surface. Cells, and cells following induction, were pulse-labelled for 5 minutes and then chased for the indicated periods prior to quenching on ice and lysis of the cells in hypotonic media. Following incubation at 37˚C for 15 minutes, lysates were fractionated into soluble (S) and pellet (P) fractions by centrifugation in a microfuge. Concanavalin A-binding glycoproteins were recovered from the lysates following solubilsation with TX100 (1%) and collected with ConA-agarose. Recovered material was analysed by SDS-PAGE. In separate experiments it was shown that ConA –agarose and antiVSG221 antisera precipitated a band of the apparent molecular weight by SDS-PAGE (data not shown). The protocol is based on experiments described in Bangs *et al.* 1986, demonstrating that VSG on the surface is accessible to GPI-PLC, whilst that within the endomembrane system is protected by membrane. Analysis was continued for 60 minutes, but only the first 20 minutes are shown here. Panel B; Western analysis of an aliquot of the cells used for the kinetic analysis, probed with antibody to TbCLH to demonstrate suppression of the targeted gene product. Panel C;

Quantitation of the data in panel A (plus later time points from the same experiment). Autoradiograms were scanned using a Heidelberg 1200 scanner, and images converted to TIFF format and imported into Image J (NIH). The VSG bands were quantitated, together with background subtraction (adjacent area from the same lane), and data expressed as percent recovered in the soluble fraction (released). Despite the higher background in the noninduced cells, it is clear that VSG is actively transported to the surface with similar kinetics in both induced and uninduced cells. This is in marked contrast to the apparent lack of endocytic activity detected in the induced cells. The analysis has been performed three times, with essentially the same results.

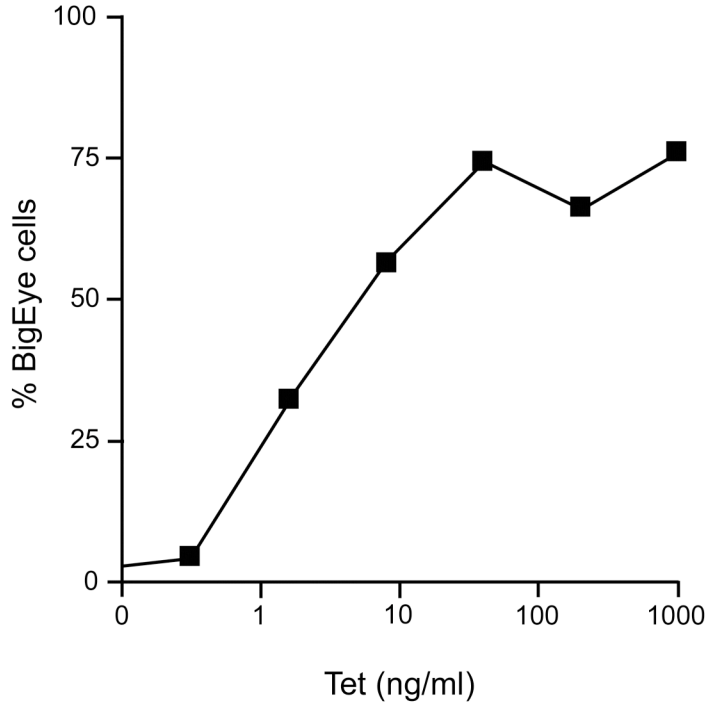
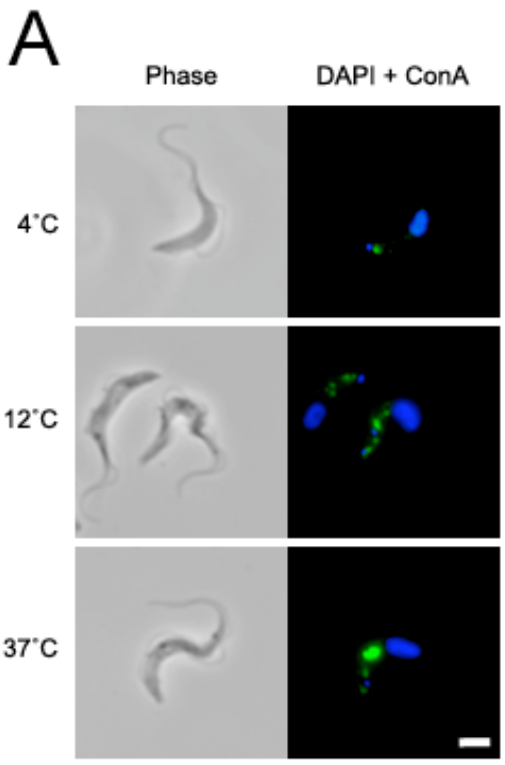
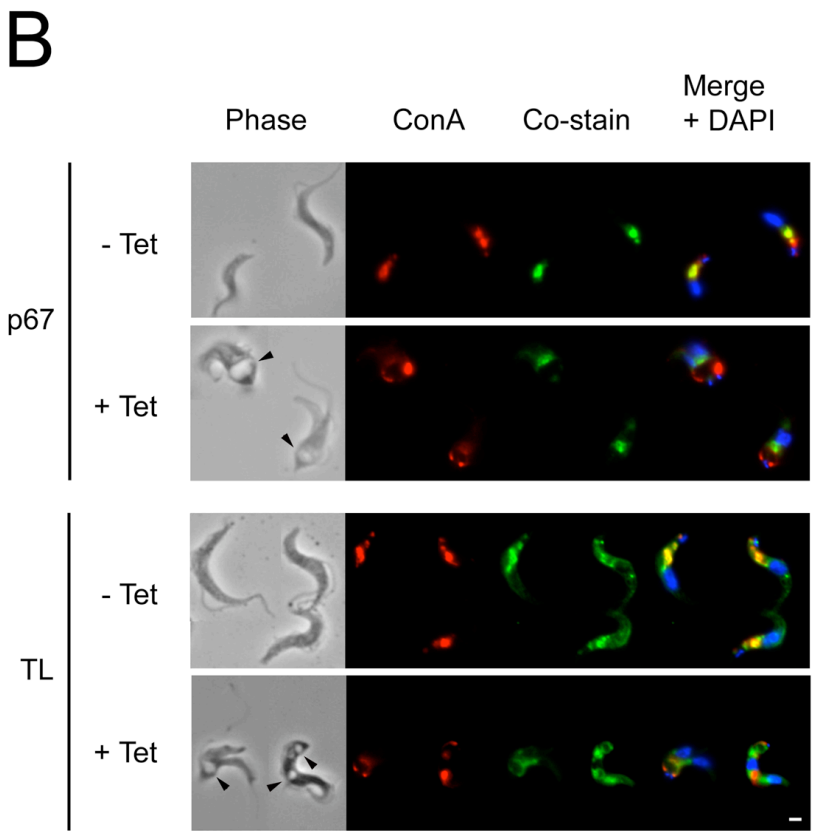
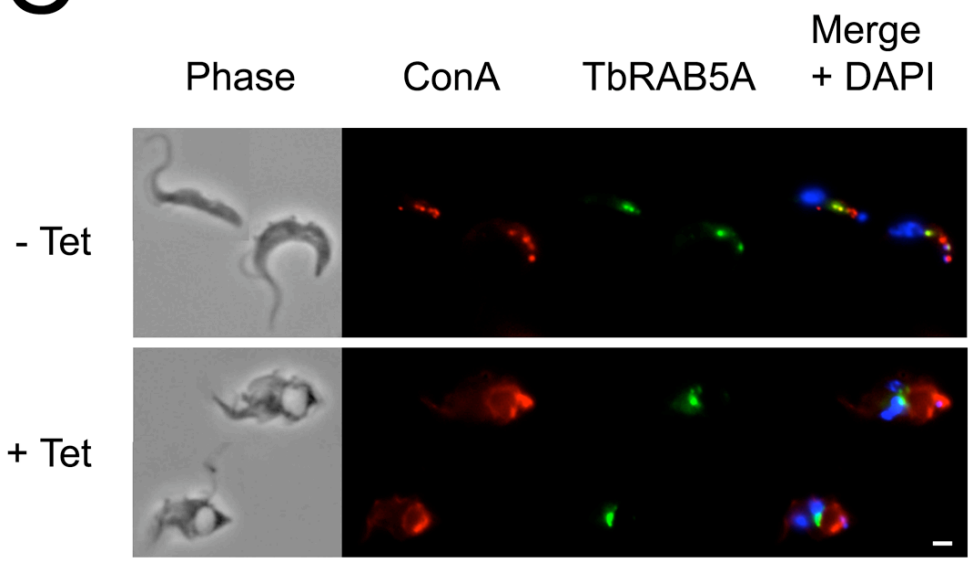


Figure S3: Induction of BigEye requires very low levels of tetracycline. BSFp2T7^{Ti}CLH cells were induced for 24 hours with varying concentrations of tetracycline. The incidence of the BigEye phenotype was determined by light microscopy of live cells, counting at least 50 cells per sample. The data are a representative experiment of two. Significantly, addition of 3nM tetracycline results in significant induction.


A
Phase
DAPI + ConA
4˚C
12˚C
37˚C


B
Phase
ConA
Co-stain
Merge
+ DAPI
p67
- Tet
+ Tet
TL
- Tet
+ Tet

C


Phase
ConA
TbRAB5A
Merge
+ DAPI
- Tet
+ Tet

D

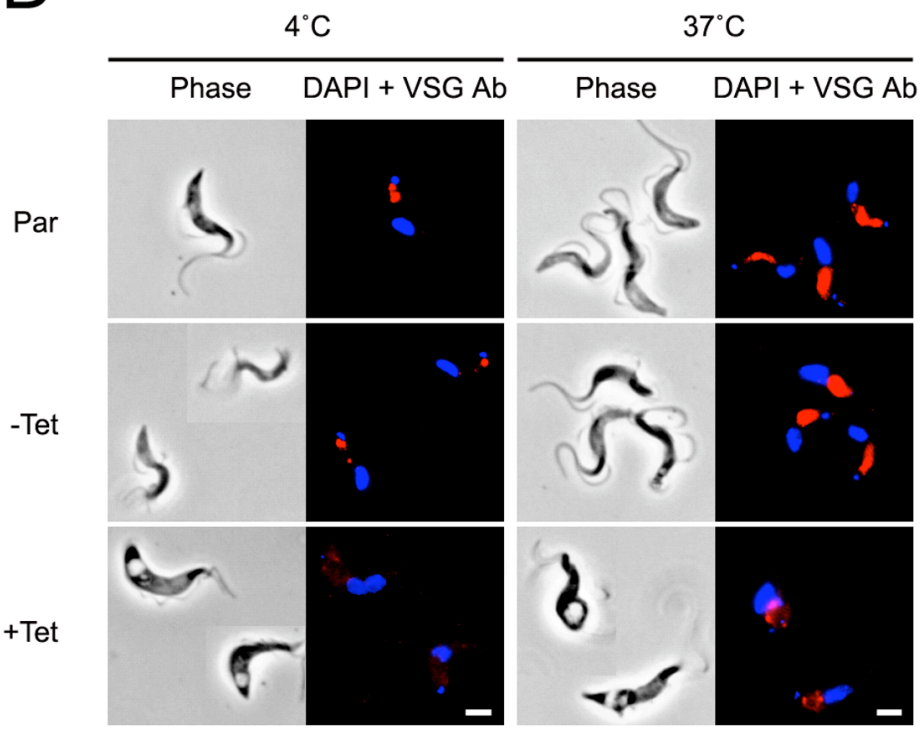

4˚C
37˚C
Phase
DAPI + VSG Ab
Phase
DAPI + VSG Ab
Par
-Tet
+Tet

Figure S4: Endocytosis is perturbed in BigEye cells. A; Concanavalin A uptake in parental bloodstream cells. At 4˚C, Con A is retained at the flagellar pocket. After a period at 12˚C, the lectin is internalised to the endocytic system, corresponding to the TbRAB5A early endosome. At 37^{o}C, the lectin is efficiently delivered to the terminal lysosome. B; Con A uptake by BSFp2T7^{Ti}CLH cells at 37^{o}C and co-stain with anti-p67 Ab or FITC tomato lectin (TL). C; Con A uptake by BSFp2T7^{Ti}CLH cells at 12^{o}C and co-stain with anti-TbRAB5A Ab. D; Antibody to VSG is not accumulated in clathrin-depleted cells. Anti-VSG Ab capping and internalisation by parental and BSF CLH RNAi cells at 4^{o}C and 37^{o}C. Panel A; Left image; phase, right image; fluorescence, DAPI (DNA) in blue, lectin in green. Panel B; Left image; phase, right images; fluorescence, Con A in red, co-stain (p67 or TL) in green. Merge; Con A and co-stain images merged along with DAPI (in blue), co-localisation between Con A and co-stain is shown in yellow. Arrow heads on phase images indicate enlarged flagellar pockets in induced cells. Panel C; Left image phase, right images; fluorescence, Con A in red, TbRAB5A in green (Con A and TbRAB5A images were pseudo-coloured red and green respectively to correspond to other images in this figure). Merge; Con A and TbRAB5A images merged along with DAPI (in blue), co-localisation between Con A and TbRAB5A is shown in yellow. Panel D; Left image; phase, right image; fluorescence, DAPI (DNA) in blue, antibody in red. In all panels; -Tet = uninduced BSFp2T7^{Ti}CLH cells, +Tet = BSFp2T7^{Ti}CLH cells induced for 16h with 1μg/ml tetracycline, Par = parental BSF 90-13 cells, scale bar = 2μm.

Video S1: BigEye cells retain motile behaviour. Phase contrast video microscopy of BSFp2T7^{Ti}CLH induced for 24 hours. Cells were observed at 40X under phase contract at ambient temperature using a Nikon Eclipse 300 microscope equipped with a Photometrics CoolSNAP *fx* CCD camera. Data were accumulated using Metamorph software V5.0 (Universal Imaging Corp) as individual frames, and the image stack converted to .mov format via Quicktime Pro V6.3 (Apple Computer). The video is encoded for playback at 30 fps.