

Both of the Rab5 subfamily small GTPases of *Trypanosoma brucei* are essential and required for endocytosis

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Abstract

Endocytosis is an essential process in *Trypanosoma brucei* and all evidence suggests it is exclusively clathrin-mediated. The trypanosome genome encodes two Rab5 proteins, small GTPases that play a role in very early stages of endocytosis. In the mammalian bloodstream stage TbRAB5A localises to compartments containing internalised antibody, variant surface glycoprotein (VSG) and transferrin, whilst TbRAB5B localises to compartments containing the transmembrane protein ISG₁₀₀. Dominant-active forms of TbRAB5A stimulate endocytosis in procyclic forms and alter the kinetics of anti-VSG antibody and transferrin turnover in bloodstream stages. Similar mutants of TbRAB5B increase fluid phase uptake in procyclic cells but do not significantly affect endocytosis in bloodstream forms. Here, we use RNA interference to evaluate the relative importance of TbRAB5A and TbRAB5B and show that both GTPases are essential in the bloodstream form. Depletion of either TbRAB5A or TbRAB5B results in morphological abnormalities, including enlargement of the flagellar pocket, consistent with a potent block to endocytosis. Also, RNAi compromises transferrin accumulation in both cases but induces distinct patterns of mislocalisation of endosomal markers. Finally, RNAi of either TbRAB5A or TbRAB5B results in a decrease in levels of clathrin. Taken together, these data indicate that both TbRAB5A and TbRAB5B are required for endocytosis in trypanosomes and demonstrate that there are multiple essential endocytic routes in this organism.

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1. Introduction

Trypanosoma brucei, the causative agent of African sleeping sickness in humans and Nagana in cattle, possesses a highly active endocytic system that has been implicated as important for uptake of nutrients (lipoproteins and transferrin [1,2]), sensitivity to serum lytic factors [3,4], turnover of the variant surface glycoprotein (VSG) coat [5] and also in removal of surface immune complexes [6,7]. Importantly,

the endocytic system is strongly developmentally regulated, being most active in the mammalian infective stage [8], suggesting a specific role in this part of the life cycle.

Recent work has identified a highly efficient recycling system for VSG [9,10]; the pathway involves a clathrin-dependent endocytic event followed by delivery to a sorting endosome, where VSG is negatively sorted by a second clathrin-dependent step, and finally recycling and delivery back to the surface via a TbRAB11-containing transport structure. Additional data suggest that the sorting endosome contains at least one member of the trypanosomal Rab5 family [7,11,35], which in higher eukaryotes mediate early endocytic transport processes as well as sorting events. A similar pathway is also taken for endocytosed anti-VSG immunoglobulin and transferrin, although kinetic measurements indicate the presence of potentially distinct aspects in recycling of VSG, antibody and transferrin [7].

Abbreviations: BSF, bloodstream form; BiP, binding protein; Con A, *Canavalia ensiformis* lectin (concanavalin A); DAPI, 4',6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; FITC, fluorescein; IFA, immunofluorescence analysis; ISG, invariant surface glycoprotein; ORF, open reading frame; TbCLH, clathrin heavy chain; VSG, variant surface glycoprotein

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The basis for the developmental regulation of endocytosis in trypanosomes is currently unknown. Neither the biological purpose of this highly active pathway, nor the molecular events underpinning the differential activity of the various life forms have been fully defined. Several molecular alterations have been documented that correlate with endocytic activity; specifically expression of both clathrin and TbRAB11 are greatly increased in the bloodstream form [8,12]. As both of these molecules are important mediators of the recycling system, it is likely that their expression profile in some manner is used to control endocytic flux.

An additional alteration that accompanies increased endocytic flux in the bloodstream form is greater complexity of the Rab5 endosome population. The trypanosome genome encodes two Rab5 proteins, TbRAB5A and TbRAB5B [13]. These proteins have evolved independently of the multiple Rab5 isoforms present in other eukaryotic lineages, i.e. *Saccharomyces cerevisiae* and *Homo sapiens*, suggesting that trypanosome Rab5 isoforms evolved to fulfill an organism specific function. In the procyclic stage, both TbRAB5A and TbRAB5B colocalise to perinuclear structures [13], whilst in the bloodstream form the locations of these two proteins are distinct. Indeed, TbRAB5A colocalises with internalised antibody and transferrin, whilst TbRAB5B colocalises with ISG₁₀₀, a polytopic membrane protein of unknown function [14]. These data suggest distinct roles for TbRAB5A and TbRAB5B.

Overexpression of both wildtype or constitutively active forms of TbRAB5A in procyclic cells leads to an increase in endocytosis of low density lipoprotein (LDL) and Lucifer yellow [11], and similar experiments in bloodstream stage cells indicate a role in intracellular transport of immunoglobulin and transferrin [7]. By contrast, whilst constitutively active TbRAB5B overexpression in procyclic cells is specifically associated with increased fluid phase uptake, attempts to determine a function for TbRAB5B in BSF by these approaches have been without success [7], suggesting that either TbRAB5B has a role fully redundant and subservient to TbRAB5A in this life stage, or that the assays used were unable to ascribe a function to TbRAB5B. Using RNA interference, we set out to determine if TbRAB5B had any detectable function. We show that TbRAB5A and TbRAB5B are both essential genes, and that each has a role in the trypanosome endocytic system.

2. Methods and materials

2.1. Maintenance of parasite cell lines

The BSF90-13 Lister 427 bloodstream form cell line [15] was routinely cultured in HMI9 medium supplemented with 10% tetracyclin-free foetal bovine serum (FBS) (Autogen Bioclear). To maintain the T7 responsive phenotype, cells were grown in the continuous presence of 5 µg ml⁻¹ Hygromycin B and 2.5 µg ml⁻¹ Geneticin (Sigma). Cells were

maintained at concentrations between 1 × 10⁴ and 2 × 10⁶ ml⁻¹. PTT procyclic line cells (gift of Philippe Bastin, Paris) were cultured in SDM79 medium supplemented with 10% FBS, 7.5 µg ml⁻¹ haemin, 25 µg ml⁻¹ Hygromycin B and 25 µg ml⁻¹ Geneticin. For growth curves, triplicate cultures were initiated at 1 × 10⁵ ml⁻¹ with or without tetracyclin at a concentration of 1 µg ml⁻¹. Cell concentration was determined using a Z2 Coulter counter (Beckman).

2.2. RNAi constructs and transfections

TbRAB5A and TbRAB5B sequences corresponding to the entire open reading frame (ORF), were first verified using RNAi to ensure lack of sufficient homology to produce RNAi cross-suppression [16], and then the ORFs excised from the plasmids pXS519-TbRAB5A and pXS519-TbRAB5B, respectively [11], by digestion with *Bam*HI and *Hind*III, and inserted into p2T7Ti [17]. For transfection, plasmids were digested with *Not*I, ethanol precipitated and resuspended in sterile water. The BSF90-13 and PTT lines were transfected by electroporation with 5 µg DNA as described [11]. After electroporation, BSF cells were transferred to fresh medium and incubated for 166 h before addition of 2.5 µg ml⁻¹ Zeocin (Invitrogen). Transfected BSF90-13 cells were grown in the presence of the selective drug for 1 week and then cloned. PTT cells were selected with Zeocin for 4 weeks.

2.3. Western blotting

Cells were washed once in PBS and resuspended in SDS sample buffer. Cells were loaded on 12% SDS PAGE gels at 1 × 10⁷ cell equivalents per lane. After separation, proteins were transferred onto Hybond nitrocellulose paper (Amersham Biosciences). Blots were blocked with PBS/5% milk/0.1% Tween 20 for 1 h at room temperature, then incubated overnight at 4 °C in the presence of affinity purified rabbit anti-TbRAB5A, mouse anti-TbRAB5B or rabbit anti-TbCLH antibodies at a concentration of approximately 1 µg ml⁻¹ in blocking buffer [7,8]. Blots were washed in PBS/0.1% Tween 20 then incubated with peroxidase conjugated goat anti-rabbit or anti-mouse IgG (Sigma) for 1 h. Proteins were visualised by ECL. To ensure equality of loading, blots were stripped and reprobed with antibody to TbBiP [18]. Band intensity was quantified using NIHimage software.

2.4. Uptake of FM4-64

Microscopic analysis of FM4-64 uptake and recycling was carried out by a modification of the assay described by Wiederkehr et al. [19]. Cells induced with 1 µg ml⁻¹ tetracyclin for 18 h were harvested, washed twice in TES buffer (120 mM NaCl, 5 mM KCl, 3 mM MgSO₄, 16 mM Na₂HPO₄, 5 mM KH₂PO₄, 30 mM TES, 10 mM glucose, 0.1 mM adenosine) then resuspended at a concentration of 1 × 10⁷ ml⁻¹ in TES. Cells were pre-equilibrated for 20 min

at 4 °C or 37 °C, then 20 µM FM4-64 (Molecular Probes) was added. Cells were incubated for 5 min in the presence of FM4-64, then placed on ice, washed once in TES buffer and fixed for 1 h at 4 °C with 4% paraformaldehyde. Fixed cells were washed in PBS, adhered to poly-L-lysine slides (Sigma) and mounted with Vectashield containing DAPI (Vectalabs).

2.5. Immunofluorescence

Cells were fixed in 4% paraformaldehyde, adhered to poly-L-lysine slides (Sigma) and permeabilised with 0.1% Triton X-100. Slides were blocked with 10% goat serum in PBS. For staining of TbRABs, slides were incubated overnight at 4 °C with anti-TbRAB5A and TbRAB5B antibodies diluted to 2 µg ml⁻¹ in blocking buffer containing 0.02% sodium azide. All other primary antibody incubations were carried out for 1 h at room temperature. After washing with PBS, slides were incubated with Texas-Red conjugated goat anti-rabbit or -mouse IgG (Molecular Probes) for 1 h at room temperature. Slides were washed and mounted with Vectashield containing DAPI (Vector Laboratories). Cells were examined under a Nikon Eclipse E600 microscope and images were captured using a Photometrix Coolsnap FX camera controlled with Metamorph software (Universal Imaging Corp.) and assembled in Photoshop 7.0.1 (Adobe Inc.).

2.6. Uptake of transferrin and concanavalin A

Cells were incubated for 18 h with 1 µg ml⁻¹ tetracyclin, harvested and washed once in serum free HMI9 medium containing 1% BSA. Cells were resuspended in HMI9/BSA at a concentration of 1 × 10⁷ ml⁻¹ and incubated at 4 °C or 37 °C for 20 min. Either FITC-transferrin (10 µg ml⁻¹) (Molecular Probes) or FITC-Con A (10 µg ml⁻¹) (Vectalabs) were added and the cells incubated for various times up to 30 min. Uptake was stopped by placing on ice. Labelled cells were washed in HMI9/BSA at 4 °C, then fixed and adhered to slides as described above. Slides were either mounted immediately or counterstained with antibody.

2.7. Electron microscopy

For transmission electron microscopy, cells were fixed in suspension by adding chilled 5% glutaraldehyde (TAAB) and 8% paraformaldehyde (Sigma) in PBS in a 1:1 ratio to the growth medium containing trypanosomes. The final dilutions were therefore 2.5% glutaraldehyde and 4% paraformaldehyde. Cells were fixed on ice for 10 min, centrifuged at 10,000 rpm for 5 min in 2 ml eppendorfs, the supernatant carefully replaced with fresh fixative for a further 50 min without disturbing the pellet, rinsed in 0.1 M sodium cacodylate and post fixed in 1% osmium tetroxide (TAAB) in the same buffer at room temperature for 1 h. After rinsing in buffer, cells were then dehydrated in an ethanol series, adding 1% uranyl acetate at the 30% stage, followed by propylene oxide and then embedded in Epon/Araldite 502 (TAAB) and

finally polymerized at 60 °C for 48 h. Sections were cut on a Leica Ultracut T ultramicrotome at 70 nm using a diamond knife, contrasted with uranyl acetate and lead citrate and examined on a Philips CM100 transmission electron microscope.

2.8. Endocytosis of transferrin

Bovine *holo*-transferrin (Sigma) was iodinated using IodoBeads reagent (Pierce) following the manufacturer's instructions. Endocytosis was monitored essentially as described previously [11], but with the following modifications. Bloodstream form cells were induced for 18–24 h with tetracycline, harvested and washed with serum-free HMI9/1% BSA. Aliquots of these cultures were taken for Western analysis to confirm knockdown of the relevant TbRAB5 isoform. Cells were resuspended in serum free HMI-9 medium supplemented with 1% bovine serum albumin at 2 × 10⁷ cells/ml for 30 min at 37 °C to empty transferrin receptors. Cells were then pulsed with ¹²⁵I-transferrin (typically ~2 µg at a specific activity of 3 × 10⁶ cpm/µg), and aliquots (0.5 ml) withdrawn at varying times. Aliquots were added to pre-cooled microcentrifuge tubes in a cold block on ice containing 0.5 ml HMI-9/1% BSA/600 µg ml⁻¹ unlabelled transferrin. Cells were washed twice by pelleting in a refrigerated microfuge for 30 s at top speed and resuspending in PBS/0.5% BSA, and then accumulated radioactivity determined by γ-counter. Data are represented as the difference between cell-associated radioactivity at *t* = 0 and 40 min.

3. Results

3.1. Both TbRAB5A and TbRAB5B are essential in bloodstream forms

When RNAi was induced for either TbRAB5 isoform in bloodstream cells, a dramatic loss of viability was observed (Fig. 1). For TbRAB5A, protein expression became undetectable at 24 h, whilst residual protein could be detected for TbRAB5B. No change was observed in expression of the ER protein BiP or in expression of the related Rab proteins TbRAB4 and TbRAB11 (data not shown), indicating that the loss of expression was specific; hence both proteins are rapidly turned over and appear to be required for continued replication. A specific block in cell cycle progression could not be detected by analysis of nuclear and kinetoplast karyotype (data not shown).

3.2. Depletion of TbRAB5A and TbRAB5B results in an enlarged flagellar pocket

Depletion of either TbRAB5 isoform resulted in the emergence of abnormal cells. At later times in induced cultures, these abnormal forms predominate (data not shown). For TbRAB5A, the appearance of a phase-light vacuolar structure

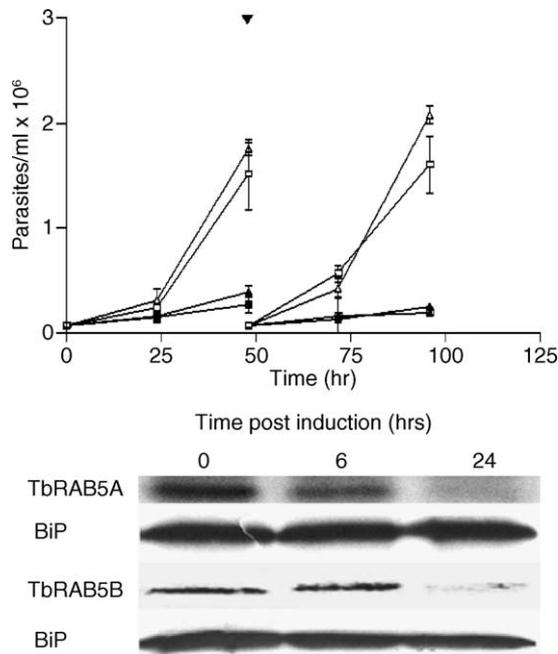


Fig. 1. RNA interference of TbRAB5A or TbRAB5B results in growth cessation. Growth curves for cells transformed with the p2T7 RNAi constructs for TbRAB5A (squares) and TbRAB5B (triangles) in the presence (closed) and absence (open) of $1 \mu\text{g ml}^{-1}$ tetracycline. Cell number was determined by Coulter counter at the indicated times of triplicate cultures. Induction was begun at time = 0. Arrowhead indicates dilution of cultures to initial concentration to avoid overgrowth of uninduced cells. Lower panel shows suppression of TbRAB5A and TbRAB5B protein expression by RNAi. Cultures were sampled at 0, 6 and 24 h, cells were lysed and proteins separated by SDS-PAGE followed by Western blotting for TbRAB5A, TbRAB5B and for TbBiP as loading control.

at the posterior end of the cell was observed at 12 h, with a progressively more pronounced morphology accumulating at later times of induction (Fig. 2A). A similar, although less severe, morphology was observed with RNAi of TbRAB5B and in this case a vacuole appeared at ~24 h. Electron microscopy demonstrated that the vacuolar structure was the flagellar pocket (Fig. 2B), as evidenced by the presence of both a flagellum cross-section and matrix material within the lumen of the structure. A rather more extreme and rapid enlargement of the pocket was obtained with the TbRAB5A RNAi compared to the TbRAB5B RNAi. At 18 h, approximately 43% of cells lacking TbRAB5A expression displayed an enlarged flagellar pocket when examined by EM ($n = 150$), whilst in the case of TbRAB5B RNAi, only 15% of the induced cells were positive for morphological defect ($n = 85$). As the flagellar pocket is the sole site of endocytic and exocytic pathway interaction with the plasma membrane, these data are consistent with a major role in membrane transport for both of these proteins. The effect is broadly similar to the BigEye phenotype reported for RNAi of the trypanosome clathrin heavy chain [20], where endocytosis, but not exocytosis, was inhibited. However, unlike the BigEye cells the flagellar pocket membrane is not perfectly circular, but folded and crenulated; this is more apparent for TbRAB5B

RNAi than TbRAB5A and may reflect the severity of the defect in membrane transport. The observation that depletion of either protein is lethal demonstrates that the respective roles of TbRAB5A and TbRAB5B are non-redundant. This is in sharp contrast to studies in *S. cerevisiae*, where knockout of individual Rab5 homologues (Ypt51, Ypt52 and Ypt53) is non-lethal and suggestive of significant redundancy [21].

3.3. Endocytosis is altered in TbRAB5 RNAi cells

FM4-64 is a lipid-binding probe that can be used to follow endocytosis in *T. brucei* [20]. Cells were induced for 18 h and allowed to take up FM4-64. In both lines, uninduced cells showed clear labelling of multiple vesicles in the early endocytic compartment between the nucleus and flagellar pocket, but after induction the dye appeared to enter the cell but was limited to regions close to the enlarged flagellar pocket (Fig. 3A). This suggests that both TbRAB5A and TbRAB5B play a role in the early stages of endocytic trafficking and is the first indication that TbRAB5B may be involved in bloodstream form endocytosis in addition to the role already established for TbRAB5A [7,11].

In order to confirm that uptake is blocked in the induced cells and that the inhibition is a genuine effect of TbRAB5 RNAi on endocytosis rather than rearrangement of the endocytic compartment, cells were labelled with the mannose-binding lectin, concanavalin A (Con A), a highly useful reagent for the analysis of general membrane protein endocytosis [22]. At 4°C , Con A accumulates in the flagellar pocket, but at 37°C the lectin is endocytosed, and accumulates within the lysosome, as defined by antibody against p67 [23]. In induced TbRAB5A or TbRAB5B RNAi cells, Con A was localised to the flagellar pocket at 4°C (data not shown) and whilst taken up at 37°C , did not appear to progress as efficiently as in the uninduced cells (Fig. 3B). This was confirmed by counterstaining cells that had internalised Con A at 37°C with antibody to p67. In uninduced cells, the bulk of internalised Con A colocalised with p67, but in induced TbRAB5A RNAi cells the majority of Con A failed to reach the lysosome as colocalisation was not obtained between Con A and p67. A pronounced decrease in Con A lysosomal delivery was also apparent for the TbRAB5B RNAi cells. Quantitation of the degree of Con A and p67 colocalisation revealed a profound block in transport in both cells (Fig. 3C). These data suggest a partial block in endocytosis in cells lacking either Rab5 isoform.

3.4. Transferrin endocytosis

The analysis above is highly suggestive of a significant defect in endocytic activity as a consequence of suppression of TbRAB5A or TbRAB5B. However, neither FM4-64 nor Con A represent naturally transported ligands for trypanosomes. Therefore, the effect of TbRAB5 RNAi on BSF transferrin uptake was determined. By fluorescence, uptake of

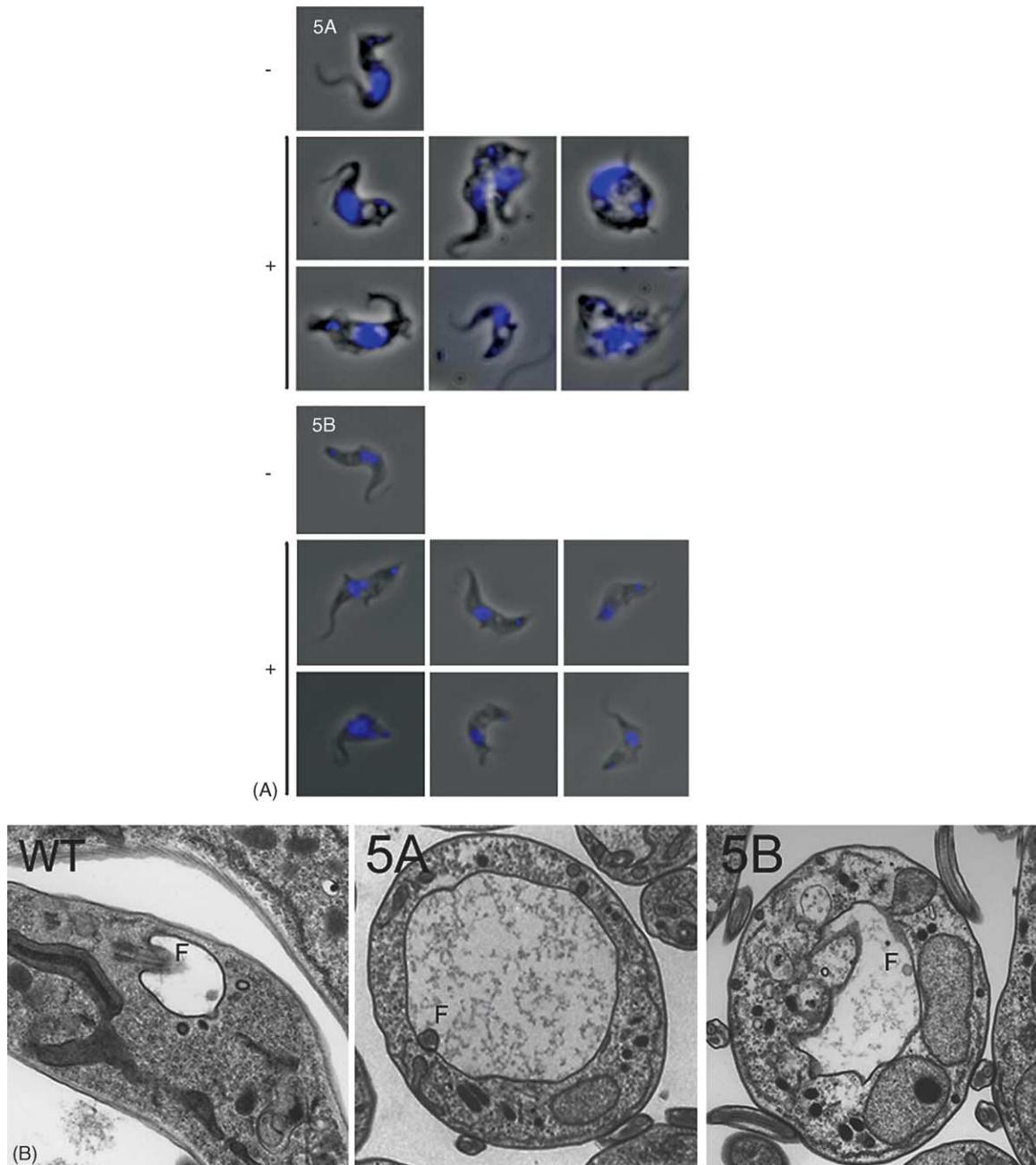


Fig. 2. Gross morphological abnormalities accompany loss of TbRAB5A and TbRAB5B protein. Panel A: Phase and DAPI fluorescence images for RNAi of TbRAB5A and TbRAB5B. For each set of images, “+” denotes induced, “–” denotes uninduced. For TbRAB5A, the leftmost pair of induced images is from cultures exposed to tetracycline for 12 h, and the remainder for 24 h. All induced images for TbRAB5B are from cultures induced for 24 h. Panel B: Ultrastructural analysis by EM of wildtype 427 BSF and cells under RNAi for TbRAB5A and TbRAB5B for 18 h. Note the presence of the enlarged flagellar pocket in both cases. The severity of the morphological defect for TbRAB5A is somewhat greater and more rapid in onset than for TbRAB5B. F denotes flagellum.

FITC-transferrin was impaired in both cell lines after induction, but the impact of TbRAB5A RNAi was more severe than for TbRAB5B RNAi (Fig. 3D).

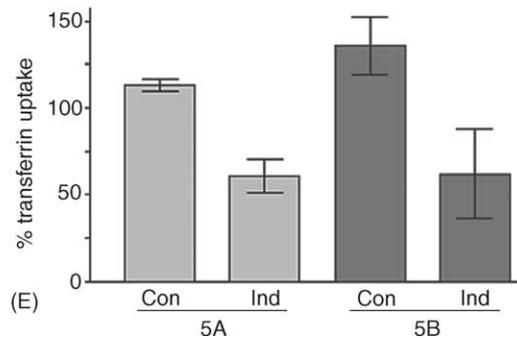
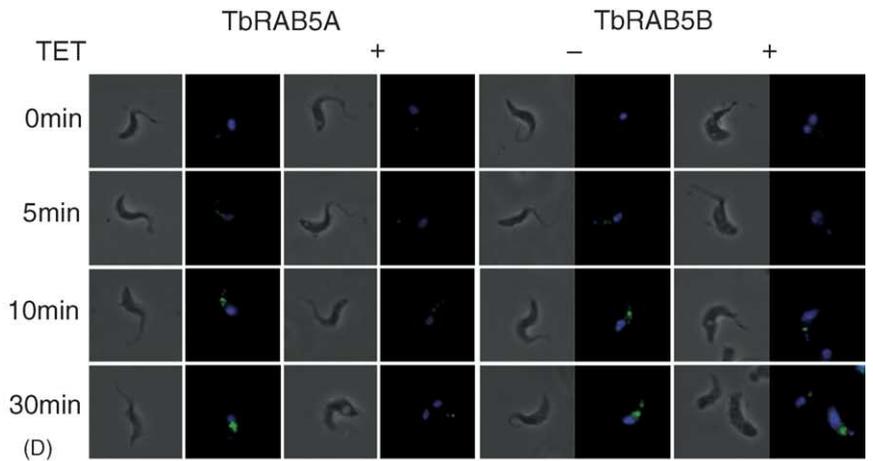
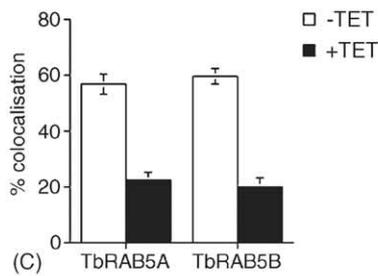
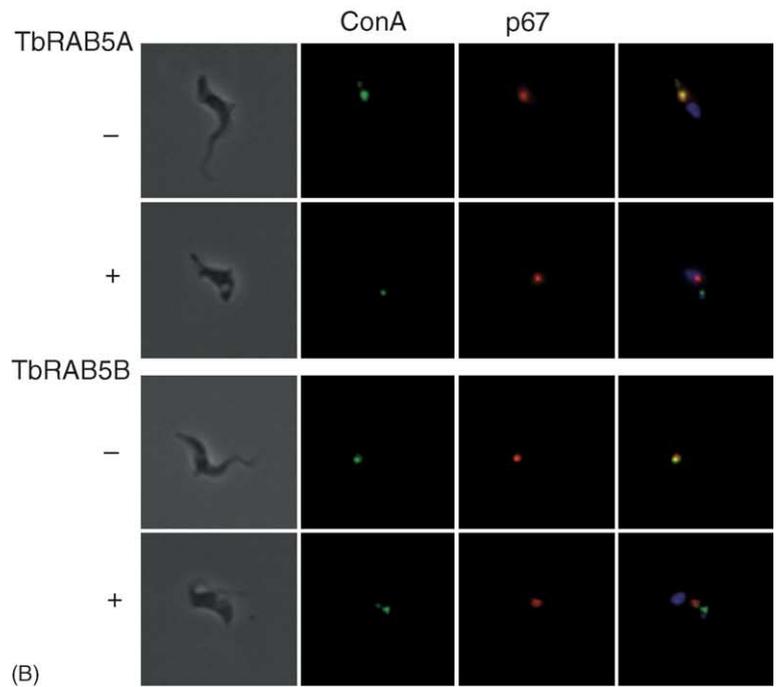
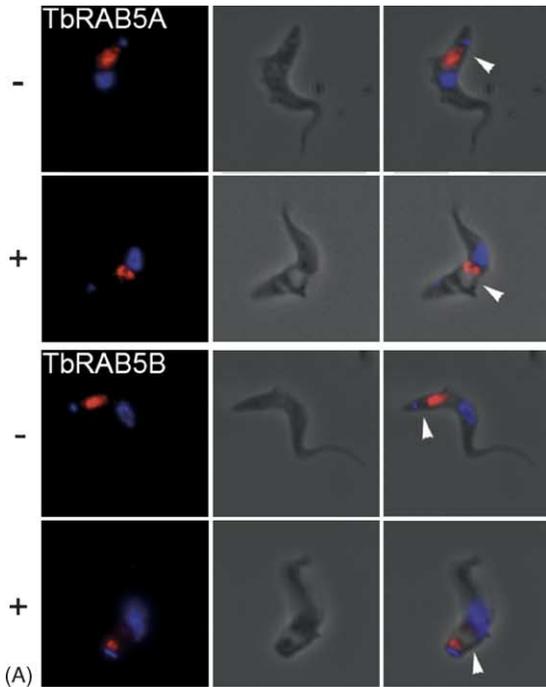
To obtain a more quantitative indication of the influence of RAB5 RNAi on macromolecular uptake, we analysed accumulation of ^{125}I -radiolabelled transferrin. Cells were induced for 18–24 h and then subjected to analysis. Induction was

monitored by Western blotting for the respective TbRAB5 protein and was suppressed as expected (data not shown). From prior work, we selected a 40 min pulse period [11] for our analysis and compared transferrin accumulation in induced and uninduced cells with a nontransfected parental 427 bloodstream form cell line (Fig. 3E). Accumulation of transferrin by BSF cells under RNAi for TbRAB5A or TbRAB5B

was substantially decreased to less than 60% of the wild-type level. IFA staining showed that induction of TbRAB5A or TbRAB5B RNAi does not appear to alter localisation of ESAG6/7, the transferrin receptor (data not shown). The reduction in transferrin uptake is therefore likely to be the result of a blockade to endocytosis.

3.5. Tomato lectin-binding proteins accumulate at the flagellar pocket in TbRAB5 RNAi

A number of proteins that are resident within endosomal compartments, including ISG₁₀₀, have been shown to bind to *Lycopersicon lycopersicum* lectin [24], presumably due to



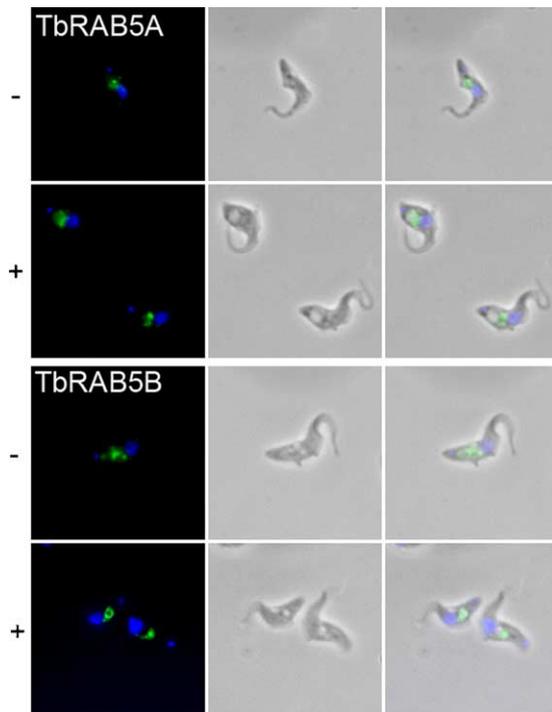


Fig. 4. Localisation of tomato lectin reactive determinants is altered by TbRAB5B RNAi. Immunofluorescence of TbRAB5A and TbRAB5B RNAi cells stained with FITC-labelled tomato lectin. Some accumulation of the lectin ligand is seen in the flagellar pocket of TbRAB5B RNAi cells. Immunofluorescence is in green, DAPI (DNA) in blue. For each set of images, “+” denotes induced for 18 h, “–” denotes uninduced. All images show representative examples, and the experiments have been performed at least twice.

polylactosamine *N*-glycan processing of the underlying glycoprotein. In parental bloodstream form cells, tomato lectin staining is localised to the endosomal compartments (Fig. 4) residing between the nucleus and kinetoplast. In cells with TbRAB5B RNAi, lectin-binding is found closely associated with the flagellar pocket, whilst RNAi of TbRAB5A has less impact on distribution of tomato lectin reactive sites. This suggests that the trafficking of tomato lectin-binding proteins in *T. brucei* depends on both Rab5 isoforms, but TbRAB5B may play a more significant role. However, in neither case was the localisation to the enlarged flagellar pocket complete,

consistent with the partial inhibition observed with Con A as an endocytic tracer.

3.6. Clathrin is expressed at decreased levels in TbRAB5A and TbRAB5B RNAi cells

In order to determine if the effects of TbRAB5 suppression on morphology and endocytosis were mediated through interactions with components of the endocytic coat system, we examined cells for clathrin expression levels. Western analysis indicated that clathrin expression was significantly decreased by RNAi of TbRAB5A or TbRAB5B (Fig. 5A). Depletion was much slower in onset than by direct RNAi of clathrin, as befits a synthetic interaction as opposed to direct suppression (Fig. 5B). By immunofluorescence there is an apparent accumulation of clathrin around the flagellar pocket, most noticeably in the TbRAB5A depleted cells (Fig. 5C). Immunoblot confirmed the continued presence of clathrin coats of membrane figures subtending the flagellar pocket (Fig. 5D), suggesting that the ability to initiate formation of clathrin-coated pits is not compromised in cells lacking expression of TbRAB5A. The apparent quantitative discrepancy between immunoblot and microscopic analysis is probably due to heterogeneity in the population. We have previously observed that complete ablation of clathrin expression results in rapid cell death, hence biasing the observable population towards those cells still retaining some clathrin [20].

4. Discussion

In higher eukaryotes, the Rab5 GTPase subfamily are involved in early events in endocytosis, including the very first steps of membrane internalisation, in sorting in the early endosome and also in phagocytosis [25]. The complex function of Rab5 proteins is reflected in the large number of effector proteins with which these GTPases interact, including tethering factors, of which EEA1 is the best characterised example, components of the SNARE fusion machinery and phosphatidylinositol kinases, e.g. [26,27]. Rab5 is present on both clathrin-coated vesicles and early endosomes and promotes fusion by coordinating the interactions of effector proteins

Fig. 3. RNAi of TbRAB5 isoforms alters endocytosis. Panel A: Uptake of FM4-64 in uninduced (–) and induced for 18 h (+) TbRAB5 BSF lines. Panel B: Fluorescence micrographs of TbRAB5A and TbRAB5B RNAi cells following exposure to Con A for 30 min at 37 °C, counterstained with monoclonal antibody against p67. Lectin fluorescence is in green, anti-p67 in red and DAPI (DNA) in blue. For each set of images, “+” denotes induced for 18 h, “–” denotes uninduced. All images show representative examples, and the experiments have been performed at least three times. Panel C: Quantitation of overlap between p67 and Con A signals. The percentage of total green fluorescence colocalising with red was measured using Metamorph Imaging Software. Data represents the mean values above background for at least 30 cells ± S.E.M. Student’s *t*-test indicates differences between induced and uninduced populations are significant: $p < 0.001$. Panel D: Transferrin endocytosis is decreased in TbRAB5A and TbRAB5B RNAi cells. Uptake of FITC-transferrin (green) was monitored at different time points in cells induced for 18 h, “+”, and uninduced, “–” cells. After incubation, cells were washed, fixed in 4% paraformaldehyde, and counterstained with DAPI (blue) before mounting. Panel E: Uptake of iodinated transferrin. Following induction, bloodstream form trypanosomes were washed and incubated in medium containing ^{125}I -transferrin. Aliquots were withdrawn at various times and cells washed to remove unbound transferrin and the amount accumulated by the cells determined by γ -counting. Data were normalised to 100% for untransfected parental cell line, and are shown for a 40 min uptake period. For both TbRAB5A and TbRAB5B RNAi, a significant decrease in accumulation of ^{125}I was detected. The experiment shown is representative of two. Data are the mean of two duplicate determinations and the variance between replicates are shown by error bars. Data for TbRAB5A are shown in light bars and for TbRAB5B in dark bars.

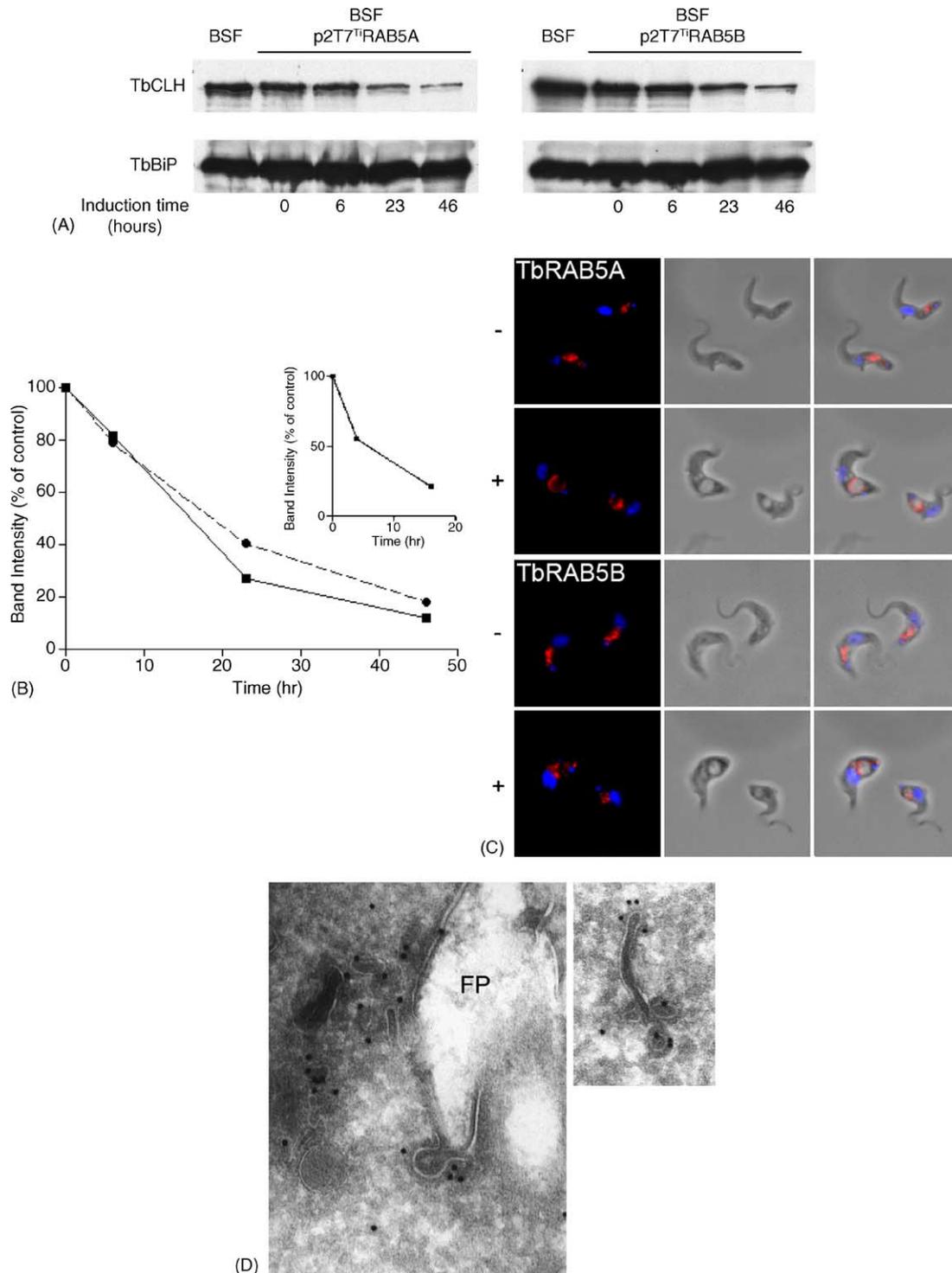


Fig. 5. TbRAB5 RNAi alters levels and localisation of clathrin. Panel A: Western analysis indicates a decrease in levels of clathrin in cells under RNAi for TbRAB5A and TbRAB5B. Lysates from cultures induced for the indicated times for TbRAB5 RNAi were separated by SDS-PAGE and clathrin detected by immunoblotting following transfer of protein to nitrocellulose membranes. Antibody to TbBiP was used as loading control. Panel B: Quantitation of intensity of clathrin heavy chain band in blots for shown in Panel A using NIHimage. Values for TbRAB5A (squares) and TbRAB5B (circles) RNAi represent pixels above background expressed as a percentage of control levels in uninduced cells. Clathrin values for BSF induced for clathrin RNAi (inset) are derived from previously published material and are included for purposes of comparison only [20]. Panel C: Immunofluorescence for clathrin heavy chain in cells under RNAi for TbRAB5A and TbRAB5B. For each set of images, “+” denotes induced for 18 h, “-” denotes uninduced. TbCLH immunofluorescence is in red (centre and right), phase at left, and DAPI in blue (right). Panel D: CryoEM of the flagellar pocket region showing clathrin-coated structures in cells under RNAi for TbRAB5A. Class I clathrin-coated vesicles budding from the flagellar pocket (left panel) are clearly visible, as are class II sorting clathrin-coated structures associated with internal endosomal structures (right panel) [9]. FP: flagellar pocket lumen.

on the vesicle and acceptor membranes [28]. However, the requirement for cytosolic Rab5-GDI for ligand sequestration into clathrin-coated pits *in vitro* and the ability of plasma membrane targeted mutants of Rab5 to interfere with internalisation of the EGF receptor point to an additional role in clathrin-coated vesicle formation [29,30].

Despite the impressive degree of molecular characterisation of Rab5, it is still unclear as to what specific roles the three different members of the human or yeast Rab5 family play. In yeast, it has not been possible to dissect distinct functions for these proteins, whilst in mammals some specificity has been demonstrated. For example, activation of endocytosis via the EGF receptor requires Rab5a function, but not Rab5b or Rab5c [31]. The Rab5 isoforms also display differences in their phosphorylation potential [32] and inducibility by interferon- γ [33]. There are also subtle distinctions in the interactions between mammalian Rab5a and Rab5b with EEA1 that may underpin distinct functionality [34].

In *T. brucei*, the variation in sequence between the two Rab5 isoforms is much greater than that between the various mammalian forms and previous evidence has suggested distinct functions for the two enzymes [7,11]. This is based on differences in the kinetics of uptake in trypanosomes expressing mutant Rab5 proteins and on the presence of a different cargo set in the TbRAB5A and TbRAB5B endosome populations. Multiple and non-redundant routes through the early endosome is one potential interpretation of these data, and would indicate selection of distinct cargo molecules for specific routes. However, it is formally possible that these data reflect differences in the steady state distributions of molecules transiting the endosomal system, rather than truly distinct pathways. This latter possibility would imply considerable redundancy in function between TbRAB5A and TbRAB5B. Interestingly, expression of dominant mutant forms of TbRAB5A (GTP- or GDP-locked forms) leads to alterations in receptor-mediated endocytosis in both procyclic and bloodstream form, but mutant forms of TbRAB5B have no detectable transport phenotype in bloodstream forms and only alter fluid phase uptake in procyclic trypanosomes [7,11]. Taken together, the data indicate at best a minor role for TbRAB5B in endocytosis.

This study demonstrates that RNAi of either TbRAB5A or TbRAB5B is lethal in bloodstream forms, indicating essential and distinct functions for these two proteins. In addition, the phenotype observed is consistent with a role in membrane uptake from the flagellar pocket, as enlargement of the pocket, as observed here, can arise by ablation of endocytosis by RNAi against clathrin [20]. Interestingly, the phenotype for TbRAB5A and TbRAB5B is less severe than for the clathrin RNAi suggesting that whilst the vast majority of endocytosis from the pocket is clathrin-mediated, this transport may be dissected into more than one component utilising distinct TbRAB5 isoforms. As the TbRAB5A phenotype is the more rapid in onset, the TbRAB5A route may be quantitatively more important than the TbRAB5B route, consistent with previous data. Further, both TbRAB5A

and TbRAB5B RNAi cells have a defect in concanavalin A delivery to the lysosome. Even more compelling is the detection of a significant decrease in transferrin accumulation in cells suppressed for TbRAB5A or TbRAB5B. Taken together, these data demonstrate a clear role for both TbRAB5A and TbRAB5B in endocytosis.

The effect of TbRAB5A and TbRAB5B RNAi on the location of tomato lectin reactive proteins is intriguing, given that little effect is seen on the localisation of known TL-ligands, such as the transferrin receptor and ISG₁₀₀ and that TL staining has been shown to predominantly colocalise with TbRAB5A. However, the relative contributions of specific proteins to the TL signal and the mechanisms regulating their location are not known. The ability of TbRAB5B RNAi to induce a pattern of relocalisation of TL-binding sites absent in TbRAB5A RNAi suggests that TbRAB5B may regulate trafficking of hitherto uncharacterised TL-reactive proteins, or of other proteins which control sorting of polylysosamine *N*-glycan-modified proteins.

The surface coat of the *T. brucei* bloodstream form is subject to continuous, rapid turnover [10]. Recent work documenting endocytosis of VSG [9] indicates that the trypanosome endocytic system is dominated by clathrin-mediated pathways. The data presented here and in previous work indicates that these pathways can be subdivided into at least two distinct routes, characterised by dependence on TbRAB5A or TbRAB5B. The appearance of an enlarged flagellar pocket indicates a direct role for trypanosomal Rab5 proteins in the earliest stages of endocytosis, whilst the decrease in the levels of clathrin also suggests a close interaction between TbRAB5A and TbRAB5B and the clathrin coat system. The loss of the TbRAB5 proteins could lead to clathrin degradation through an increase in the protease susceptible pool of clathrin when budding and fusion of clathrin-coated vesicles is blocked. A direct association between Rab5 and clathrin expression levels has not been observed in other systems, but Rab5 and its effectors do interact with coated pits and have been linked functionally to the early stages of clathrin-coated pit formation [28–30]. A more indirect effect may also be responsible: recent work indicates Rab5-dependent signal transduction regulates actin remodelling in mammalian cells, and actin RNAi in *T. brucei* induces very similar physical changes in cell morphology as clathrin loss and also inhibits endocytosis [36,37]. Interactions between Rab5 and the actin cytoskeleton are mediated by the GTPase activating protein, RN-Tre [36]. If TbRAB5A and TbRAB5B share similar such effectors, RNAi of either protein might trigger the same cellular response. Interaction with clathrin via actin thus provides an alternative potential mechanism for the effects of TbRAB5 RNAi on early endocytosis.

It is not clear whether the effects of TbRAB5 RNAi on endocytosis and on cell morphology are attributable solely to the decrease in clathrin levels rather than being a direct result of Rab protein loss, or are due to a combination of the two phenomena. Unique features of the *T. brucei* endocytic apparatus, including rapid turnover rates, complete dependence

on clathrin-mediated uptake and lack of AP2-like complexes may combine to make the system particularly vulnerable to disruption via Rab5 knockout. Unfortunately, the requirement for both isoforms to maintain normal levels of clathrin limits further functional analysis of the two pathways using RNAi. However, the data show that the two TbRAB5 proteins are not redundant but are both essential to the maintenance of cellular homeostasis and survival. The extreme sensitivity of the trypanosome to factors interfering with internalisation and trafficking highlights the therapeutic potential of targeting drugs against components of the endocytic system.

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