TfVPS32 Regulates Cell Division in the Parasite *Tritrichomonas foetus*

Running head: *Tritrichomonas foetus* Cell Division

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ABSTRACT

The flagellated protist *Tritrichomonas foetus* is a parasite that causes bovine trichomonosis, a major sexually transmitted disease in cattle. Cell division has been described as a key player in controlling cell survival in other cells, including parasites but there is no information on the regulation of this process in *T. foetus*. The regulation of cytokinetic abscission, the final stage of cell division, is mediated by members of the ESCRT (endosomal sorting complex required for transport) machinery. VPS32 is a subunit within the ESCRTIII complex and here, we report that TfVPS32 is localized on cytoplasmic vesicles and a redistribution of the protein to the midbody is observed during the cellular division. In concordance with its localization, deletion of TfVPS32 C-terminal alpha helices (α5 helix and/or α4-5 helix) leads to abnormal *T. foetus* growth, an increase in the percentage of multinucleated parasites and cell cycle arrest at G2/M phase. Together, these results indicate a role of this protein in controlling normal cell division.

KEYWORDS: *Tritrichomonas foetus*; ESCRTIII complex; VPS32 protein; cytokinesis; cell division

INTRODUCTION

Tritrichomonas foetus is a flagellated parasitic protozoan that infects the bovine genital tract where it causes a sexually transmitted disease in bovine (Rae and Crews 2006) and large bowel diarrhea in domestic cats (Gookin et al. 1999, Levy et al. 2003, Yao and Koster 2015). *T. foetus* has a worldwide distribution and causes significant economic losses due to bovine trichomonosis (Mardones et al. 2008, Ondrak 2016, Rae and Crews 2006, Michi et al. 2016). In the bull, infection normally persists for years without clinical signs. In contrast, trichomonosis in female cattle ranges from clinically almost unapparent infections to severe manifestations of disease (vaginitis, placentitis and pyometra) that result in infertility, early embryonic death or abortion in cattle (Rhyan et al. 1988, Mickelsen et al. 1986). Despite these serious health-related and

economic consequences, biological processes important for *T. foetus* growth and survival is not defined and the understanding of these events is imperative prerequisite for any intervention strategy.

Although cell division has been described as a key player in controlling cell survival in other cells, including parasites (Hammarton et al. 2003), there is no information on the regulation of this process in *T. foetus*. The most basic function of the cell cycle is to duplicate accurately the vast amount of DNA in the chromosomes and then segregate the copies precisely into two genetically identical daughter cells. This is a complex process involving multiple proteins that could be thought as novel targets to be analyzed in the context of diseases control (Hammarton et al. 2003). The regulation of cytokinetic abscission, the final stage of cell division where the two daughter cells are separated, is mediated by the endosomal sorting complex required for transport (ESCRT) machinery. The ESCRT machinery, composed of four distinct complexes (0, I, II, III), drives a diverse collection of membrane remodeling events, including the biogenesis of multivesicular bodies, release of enveloped viruses, reformation of the nuclear envelope and finally cytokinetic abscission (Raiborg and Stenmark 2009, Roxrud et al. 2010, Henne et al. 2011, Slagsvold et al. 2006, Wegner et al. 2011). While some ESCRT components are only required for specific processes, the assembly of ESCRT-III polymers on target membranes is mandatory for every process (Adell et al. 2016). Interestingly, ESCRT-III function in cell division seems to be critical throughout evolution (Samson et al. 2008) as several members of ESCRT-III were shown to support normal cell growth in Archaea (Lindas et al. 2008), yeast (Kohler 2003) and humans (Carlton and Martin-Serrano 2007, Morita et al. 2007). The studies performed in *S. cerevisiae* have largely contributed to our current understanding on ESCRT-III complex assembly. In yeast, there are six ESCRT-III related proteins (VPS2, VPS24, VPS20, SNF7, DID2/VPS46 and VPS60) proposed as regulatory members. Humans share these "core" subunits but have four additional SNF7 (CHMP4A-D) and two VPS2 (CHMP2A and B) paralogues (Kranz et al. 2001, Babst et al. 2002, Saksena et al. 2007). In humans, the deletion of the C-terminal autoinhibitory domain of CHMP3 prevents cytokinesis (Dukes et al. 2008) and CHMP4C acts as an essential regulator the abscission checkpoint (Carlton et al. 2012). Studies in Archaea have revealed roles for ESCRT-III in cell division (Samson et al. 2008, Snyder et al. 2013). Although a clear role in cell division has been demonstrated for several members of the ESCRT-III complex, it is interesting to note that the role of the most abundant protein of the complex, VPS32 (known as SNF7 in yeast or CHMP4B in humans), has not been addressed yet. In this context and as a step toward further understanding the role of ESCRT-III, we have examined the role of VPS32 in *T. foetus* cell division. Interestingly, our data indicate a key role for this protein in the final cytoplasm abscission and provide support for a model in which the α-helical coiled-coil domains at the C-terminal tail of VPS32 mediates this function. To the best of our knowledge, this is the first study to explore molecular aspects of cell division in the parasite *Tritrichomonas foetus*.

MATERIALS AND METHODS

Parasites cultures

T. foetus strain CH1 was cultured in Diamond's Trypticase-yeast extract-maltose (TYM) medium (Diamond 1957) supplemented with 10% horse serum and 10 U/ml penicillin/ 10 µg/ml streptomycin (Invitrogen). Parasites were grown at 37 °C and passaged daily.

In silico identification of putative *VPS32* genes in *T. foetus*

Trichomonas vaginalis genome database (http://www.trichdb.org) and *T. foetus* cDNA sequences (Gen-Bank EST database as Tf30924 cDNA library *Tritrichomonas foetus*) were used to identify

putative ESCRT-III genes. *T. vaginalis* genome was used based on the high sequence similarity with *T. foetus* genome. Sequence similarity searches were performed by BLAST using human, yeast and *Entamoeba histolytica* ESCRT-III protein sequences as queries. Database searches and sequence comparisons were performed using blastn, blastx, and BLAST two-sequence programs (www.ncbi.nlm.nih.gov/BLAST). Multiple alignments of the amino acid sequences for VPS32 were performed using PSI-Coffee alignment program (Di Tommaso et al. 2011) (Figure S1). The secondary structure of TfVPS32 was predicted using the Jpred program (http://www.compbio.dundee.ac.uk/~www-jpred/submit.html).

Plasmid construction and exogenous protein expression in *T. foetus*

The TfVPS32 full length, TfVPS32α5 (in which 18 amino acids from the extreme C-terminal were deleted) and TfVPS32α4/5 (in which 51 amino acids from the extreme C-terminal were deleted) constructs were generated using primers with *NdeI* and *KpnI* restriction sites engineered into the 5′- and 3′- forward and reverse primers respectively (Table S1). PCR fragments were generated using standard procedures and the resulting fragments were then cloned into the Master-Neo-$(HA)_2$ plasmid (Delgadillo et al. 1997) to generate constructs to transfect *T. foetus*. Additionally, TfVPS32 full length and TfVPS32α5 were ligated into expression vector pTvGFP (based on pTagvag2 with a Trichomonas codon-optimized green fluorescent protein or EGFP-tag replacing the HA) (Kusdian et al. 2013). Electroporation of *T. foetus* was carried out as described previously (Delgadillo et al. 1997) with 50 μg of circular plasmid DNA. Transfectants were selected with 100 μg/ml G418 (Sigma).

Immunolocalization experiments

Parasites were incubated at 37°C on glass coverslips for 4 hours and then fixed and permeabilized in cold methanol for 10 min. The cells were washed and blocked with 5% (v/v) FBS in PBS for 30 min, incubated with a 1:500 dilution of anti-HA primary antibody (Covance, Emeryville, CA, USA) diluted in PBS plus 2% (v/v) FBS, washed and then incubated with a 1:5,000 dilution of Alexa Fluor conjugated secondary antibody (Molecular Probes). The coverslips were mounted onto microscope slips using ProLong Gold antifade reagent with DAPI (4′, 6′-diamidino-2-phenylindole) (Invitrogen). All observations were performed on a Nikon E600 epifluorescence microscope. Adobe Photoshop (Adobe Systems) was used for image processing.

Parasite growth assay

The kinetic of growth curves were performed using EpNeo-HA (an empty vector), TfVPS32 full length-HA, TfVPS32α5-HA and TfVPS32α4/5-HA transfected parasites. For these experiments, $1x10^6$ trophozoites were inoculated in 10 ml of TYM medium and incubated at 37 °C for 72 hours. After inoculation, cell counts were recorded every 6 hours using a hemocytometer. Growth rates were determined as the natural logarithm of the change in the density of parasites per milliliter at time *t* compared with that at time zero (initial inoculum) by the following equation: growth rate = [ln*CC* (*t*) – ln*CC* (0)]/ (*t* - 0), where *CC* (*t*) and *CC* (0) are the parasites counts per milliliter at time *t* and time zero, respectively, and *t* is the time of incubation (Nix et al. 1995). The experiments were performed three times, in triplicate.

Similar experiments were also performed with pTvGFP, TfVPS32 full length-GFP, TfVPS32α5-GFP parasites.

Nuclear staining

DAPI staining was used to determine the number of nuclei per cell. Parasites were incubated at 37 °C on glass coverslips for 4 hours and were then fixed and permeabilized in cold methanol for 10 minutes. The cells were washed 3 times in PBS and incubated with 300 nM DAPI stain

solution for 5 minutes, protected from light. After 3 washes with PBS, the coverslips were mounted onto microscope slides using fluoromont mounting media.

Electron microscopy

Parasites were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Cells were post-fixed in 1% OsO4 and 0.8% potassium ferricyanide, dehydrated in acetone series and infiltrated in Epon. Polymerization was carried out for 72 h. Thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined with a Tecnai G2 Spirit transmission electron microscope.

Flow cytometry

Parasites (5×10^6) transfected with EpNeo-HA, TfVPS32 full length-HA, TfVPS32α5-HA and TfVPS32α4/5-HA were harvested to determine the relative cellular DNA content and the distribution during the various phases of the cell cycle. Parasites were fixed in 5 ml of ice-cold 100% EtOH, and incubated at 4 °C overnight. Thereafter, each sample was washed in 1ml PBS containing horse serum (HS) 2% v/v, resuspended in 1 ml PBS with 180µg/ml RNase A to digest RNA and 2% v/v HS and incubated for 30' at 37 °C. Then, samples were stained with 25 µg/ml propidium iodide (PI) solution and were incubated for 30' at 37 °C prior to flow cytometer analysis. Samples were analyzed using a fluorescence-activated cell sorter (FACScan, BD Bioscience) with appropriate filter sets. The data was analyzed using FlowJo 7.6 software.

RESULTS

A putative VPS32 was identified from *T. foetus* database

Taking into account the importance of ESCRT-III complex in the regulation of cell division in other cells, we hypothesized that the most abundant member of the complex, VPS32, could be a key player in the regulation of *T. foetus* cell division. A putative *T. foetus* VPS32 was retrieved using the *T. foetus* cDNA sequences (available in the Gen-Bank EST database as Tf30924 cDNA library *Tritrichomonas foetus*) and compared its sequences with that the *T. vaginalis* genome database (http://www.trichdb.org) due to the high sequence similarity between both parasites (Huang et al. 2013). Interestingly, only one putative TvVPS32 was identified in *T. vaginalis* genome (accession number TVAG_459530). The gene encoding for TfVPS32 was cloned from *T. foetus* genomic DNA and sequenced. The sequence analysis revealed that TfVPS32 (Gen Bank accession number KX426376) coding region comprises 615 bp and encodes a 204 amino acids protein containing the classical features described for VPS32 genes from other organisms: according to Pfam database predictions, TfVPS32 contains a conserved protein domain family Snf7 (Fig. 1) and the classical basic N-terminal region followed by a smaller C-terminal acidic domain. Analysis of predicted secondary structure using the Jpred program indicated that TfVPS32 contains four alpha helices connected by a relatively long linker to a fifth short predicted helix at the C-terminus (Fig. 1).

TfVPS32FL and its C-terminal helix truncated versions localize to the midbody during *T. foetus* cell division

The C-terminal acidic halves of ESCRT-III proteins have been shown to play critical roles as regulatory domains that modulate protein function in mammalian cells and yeast (Shim et al. 2007, Capalbo et al. 2012, Sciskala and Kolling 2013). Therefore, we investigated whether the C-terminal helix of *T. foetus* VPS32 contributes to its function by deleting one (TfVPS32α5) or two (TfVPS32α4/5) predicted alpha helices from the C-terminal end of TfVPS32 (Fig. 1) and monitored the effects of these deletions. To this end, we deleted 18 (TfVPS32α5) or 51 amino acids (TfVPS32α4/5) considering the presence of a putative MIM (microtubule-interacting and transport interacting motif) within the alpha helix 4 of TfVPS32. The TfVPS32α4/5 construct

was generated as the MIM has been demonstrated as regulator of specific binding in other proteins (Obita et al. 2007). We transfected *T. foetus* with constructs encoding HA- or GFP-tagged truncated version of TfVPS32 and we compared the distribution of each version with that of its full-length counterpart by immunostaining and epifluorescence microscopy. Using an anti-HA antibody, TfVPS32 full length (TfVPS32FL) showed to be localized at intracellular vesicles (Fig. 2); consistent with previous studies in other cells (Shim et al. 2007, Peck et al. 2004). Like TfVPS32FL, the truncated version TfVPS32α5 and TfVPS32α4/5 also localized in intracellular vesicles (Fig. 2). Similar results were obtained with a truncated version TfVPS32α5-GFP tag (Figure S2).

Members of the ESCRT-III have been involved in regulating the final stages of the cytoplasmic abscission in other cells (Dukes et al. 2008, Morita et al. 2007). Interestingly, we observed that TfVPS32FL as well as TfVPS32α4/5 are localized at the midbody during the cellular division in *T. foetus* (Fig. 3A and Figure S3). These data demonstrate that the C-terminal alpha helix is not necessary for targeting the protein to its final destination and might be suggesting a possible role for TfVPS32 in parasite cell division.

VPS32α5 and VPS32α4/5 affects *T. foetus* normal growth

Homologs of ESCRT-III were shown to support normal cell growth in other organisms such as Archaea (Lindas et al. 2008, Samson et al. 2008), yeast (Kohler 2003) and humans (Morita et al. 2007). Similarly, we noted that parasites transfected with truncated versions TfVPS32α5 and TfVPS32α4/5 grew more slowly. Hence, we decided to investigate the kinetic of growth of parasites transfected with TfVPS32α5 and TfVPS32α4/5 compared with TfVPS32FL and Empty vector (EpNeo) transfected parasites (Fig 3B). As shown in figure 3B, parasites transfected with EpNeo and TfVPS32FL showed maximum growth around 30 hours while parasites TfVPS32α5 and TfVPS32α4/5 showed maximum growth after 42 hours; indicating a clear delay in reaching their maximum of growth. Similar data have been obtained with parasites pTvGFP, TfVPS32FL-GFP and TfVPS32α5-GFP (Figure S4). Likewise, when the growth rate was calculated, TfVPS32α5 and TfVPS32α4/5 have a lower growth rate (6.36% and 6.54% respectively) compared to EpNeo and TfVPS32FL parasites (9.80% and 9.50% of respectively). These results suggest that TfVPS32 may play a role in regulating normal cell growth.

TfVPS32 regulates normal cell division

Taking into account the specific localization during cell division and the delay in cell growth observed when parasites are transfected with VPS32α5 and VPS32α4/5; we decided to analyze the number of nuclei per parasites to evaluate possible alterations of normal cell division related to C-terminal deletions. Parasites transfected with EpNeo, TfVPS32FL, TfVPS32α5 or TfVPS32α4/5 were stained with DAPI and the number of nuclei per cell was quantified under the fluorescence microscope (Fig. 4A and 4B). Interestingly, a significant increase of binucleated cells, 16% and 15% were found in TfVPS32α5 and TfVPS32α4/5 transfected parasites respectively, compared to 5% and 2% of binucleated cells in TfVPS32FL and EpNeo parasites, respectively (Fig. 4A). Interestingly, we also found 4% of parasites with more than two nuclei when parasites are transfected with TfVPS32α5 and TfVPS32α4/5 (Fig. 4A and 4B). Importantly, multinucleated cells were not found when transfected with EpNeo or TfVPS32FL versions (Fig. 4A). Similar as observed in Figure 2, TfVPS32α4/5 is localized at intracellular vesicles in multinucleated cells (Fig. 5A).

During unfavorable environmental conditions, the trophozoites, which are polar and flagellated, can adopt a spherical shape and internalize their flagella, in a form known as “pseudocystic” (Pereira-Neves et al. 2003). This “pseudocystic form” is able to undertake nuclear division to

form multinucleated cells (Pereira-Neves et al. 2003, Pereira-Neves and Benchimol 2009). In order to evaluate if the multinucleated cells observed in TfVPS32α5 and TfVPS32α4/5 correspond to pseudocyst forms of the parasite, we analyzed the location of flagella in multinucleated cells using anti-tubulin antibody (Fig. 5B). Multinucleated parasites showed external flagella, indicating that are not pseudocystic forms (Fig. 5B). Additionally, these results were confirmed by TEM where we also observed the presence of multinucleated cells with external flagella (Fig. 5C). These results indicate a role for TfVPS32 in cell division wherein the C-terminal alpha helix modulates protein function.

Parasites transfected with TfVPS32α5 and TfVPS32α4/5 show larger cell size and cell cycle arrest at G2/M phase

As ploidy is proportional to cell size in some cell types (Galitski et al. 1999), we analyzed if multinucleated parasites were also larger in size by analyzing the forward scatter within the flow cytometry output. We compared populations of wild type *T. foetus* parasites, parasites transfected with EpNeo, TfVPS32FL and TfVPS32α4/5. Notably, the results obtained indicate that TfVPS32α4/5 parasites have a larger size when compared to the ones transfected with EpNeo, TfVPS32FL or wild type *T. foetus* (Fig. 6A). The increased size is in agreement with the higher number of cells with two or more nuclei in TfVPS32α4/5 parasites.

Insufficient cell division also leads to the accumulation of cells with a DNA content of 2C (or higher) if DNA synthesis occurs but cytokinesis fails. Thus, we measured cellular DNA content by flow cytometry after staining parasites with the fluorescent dye propidium iodide (PI). As can be seen in figure 6B, wild type parasites, parasites transfected with EpNeo and TfVPS32FL showed most of DNA content of 2C while a dramatic accumulation of cells with DNA content ~4C was observed in parasites transfected with TfVPS32α5 and TfVPS32α4/5 indicating that cell cycle is affected in these parasites. Four distinct phases of the cell cycle could be recognized in a proliferating cell population: G1, S (DNA synthesis phase), G2-and M phase (mitosis). However, G2 and M phase could not be discriminated based on their differences in DNA content. Specifically, we observed 19.57% and 23.31% of EpNeo and TfVPS32FL parasites, respectively in the G2/M stages compared to 43.14% and 58% in TfVPS32α5 and TfVPS32α4/5, respectively (Fig. 6C). Notably, the appearance of an increased ~4C population in TfVPS32α5 and TfVPS32α4/5 parasites suggest a possible arrest in G2/M (4C DNA) phase during cell cycle.

DISCUSSION

This study, the first to identify and characterize a protein from ESCRT-III complex in the parasite *T. foetus*, reveals a key role for one subunit of this complex, named VPS32, in cell division. Previous studies in animal cells and plants showed that vesicular membrane traffic is important for the successful completion of cytokinesis (Albertson et al. 2005, Dhonukshe et al. 2007, Otegui et al. 2005). Our experiments demonstrated that TfVPS32 localizes to the midbody in dividing cells and regulates division. Consistent with this, members of ESCRT-III complex (proteins CHMP2, CHMP4 and CHMP5) in humans were also detected at the midbody of dividing cells (Morita et al. 2007).

The regulation of cell division is mediated by the endosomal sorting complex required for transport (ESCRT) machinery. The C-terminal acidic halves of ESCRT-III proteins have been shown to play critical roles as regulatory domains that modulate protein function in mammalian cells and yeast (Shim et al. 2007, Capalbo et al. 2012, Sciskala and Kolling 2013). In this sense, truncation of the C-terminal alpha helix of CHMP3C localizes to the midbody and leads to multi-nucleation in human cells. Similarly, when *T. foetus* is transfected with TfVPS32 truncated

versions cytokinesis is affected as determined by an increase in the percentage of multinucleate cells, enlarged cells, arrest in G2/M stage of the cell cycle and defect in cell growth.

Taking into account these results, we propose that the TfVPS32 C-terminal truncated versions might have a dominant negative effect on endogenous TfVPS32. This could be explained by the mutants disrupting critical TfVPS32 tail-dependent interactions involved in cellular division, such as the interaction with the other VPS32 neighboring subunit or associated proteins. In this sense, it has been demonstrated that C-terminal halves of numerous ESCRT-III proteins modulate polymerization; a critical step in regulation of protein function (Shim et al. 2007, Henne et al. 2012).

It has been previously suggested that flagella provide motile forces that contribute to facilitate cytokinesis in trichomonads (Ribeiro et al. 2000). Interestingly, we observed that multinucleated parasites transfected with TfVPS32α5 or TfVPS32α4/5 with external flagella have a defect in cytokinesis. These result might be suggesting that the force provided by the flagella, although necessary, is not sufficient for a proper cell division and another mechanism might be contributing to regulate this process. In this sense, the loss or reduction of ESCRT function in human cells, *A. thaliana* and *S. acidocaldarius* frequently results in multinucleated cells, caused by a failure of cytokinetic abscission and probably subsequent cleavage furrow regression upon prolonged cytokinesis arrest (Schmidt and Teis 2012). However, these are some of several possible explanations; defining the mechanism underlying the observed abnormal cellular division awaits further studies.

Interestingly, a similar defect in cell division is observed when parasites are transfected with TfVPS32α5 or TfVPS32α4/5. These results indicate that the last 18 amino acids (eliminated in TfVPS32α5) are sufficient to modulate TfVPS32 function. In this regard, when the amino acids deleted in TfVPS32α5 are analyzed, none of the classical motif described in other ESCRT-III proteins is identified. Specifically, we could not identify an MxxLxxW motif described as important in regulating the interaction with Alix protein (Eikenes et al. 2015). Similarly, TfVPS32 lacks phosphorylation sites in the deleted fragment indicating that this post-translational modification is not responsible for observed effects in our mutants. The only recognizable motif identified in TfVPS32 C-terminal portion is the "microtubule-interacting and transport interacting motif" or MIM (EDELxxxxEE) that generally mediate VPS4 binding (Obita et al. 2007, Scott et al. 2005). However, this putative motif is present within the alpha helix 4 of TfVPS32. As the defect in cell division is also observed when TfVPS32α4/5 was transfected, we can speculate that the putative MIM is not responsible for observed effect.

An increase in ESCRT-III members has been observed through evolution (Leung et al. 2008). It has been postulated that different isoforms of ESCRT-III might have evolved to mediate different scission events. In humans CHMP4B (human isoform of VPS32) has been involved specifically as a key effector in abscission; whereas its paralogue, CHMP4C, is a component in the abscission checkpoint that delays abscission until chromatin is cleared from the intercellular bridge (Carlton et al. 2012, Capalbo et al. 2012). ESCRT-III subunits have been widely studied during cytokinesis in other eukaryotes and this study showing its participation in cell division of trichomonads. Interestingly, we have only identified one isoform of VPS32 in *T. foetus* suggesting that this protein might be responsible for regulating different scission event in the parasite cell division. Future studies are needed to establish the specific function of VPS32 within other stages of cell cycle progression in *T. foetus.*

Hence, findings obtained with *T. foetus* may be extrapolated to *T. vaginalis* as the mechanism of cell division in this human pathogen is still not studied. More importantly, due to the high cell

division rate and simple cultivation *in vitro, T. foetus* provide an excellent model to investigate novel roles of ESCRT machinery and other molecules involved in cytokinesis in general.

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FIGURE LEGENDS

Fig.1. Schematic representation of predicted secondary structure of TfVPS32 (TfVPS32FL) and TfVPS32 truncated versions (TfVPS32α5 and TfVPS32α4/5). Gray boxes correspond to predicted alpha helices. The classic domain SNF7 present in the protein members of the ESCRT-III complex (upper gray line).

Fig. 2. Sub-cellular localization of full-length and C-terminally deleted TfVPS32 protein. Cells exogenously expressing full length TfVPS32 (TfVPS32FL) and TfVPS32ΔCt (TfVPS32α5 and TfVPS32α4/5) fused to a haemagglutinin (HA) C-terminal tag were immunostained using a mouse anti-HA antibody and visualized by epifluorescence microscopy. PC, phase-contrast image. The nucleus (blue) was also stained with DAPI.

Fig. 3. **A.** Subcellular localization during cell division. Cells exogenously expressing TfVPS32FL and TfVPS32α4/5 during cell division with a C-terminal haemagglutinin (HA) tag were stained for immunofluorescence microscopy using a mouse anti-HA antibody. Note the subcellular localization at the midbody of cells in division in TfVPS32FL and TfVPS32α4/5 parasites. PC, phase-contrast image. **B.** Analysis on the *T. foetus* kinetic growth curve. Replication curves of parasites transfected with EpNeo (red), TfVPS32FL (light blue), TfVPS32α5 (blue), TfVPS32α4/5 (orange) were compared. Parasites count were collected at indicated times on the X axis. The results represent the average of three independent experiments and error bars represent standard deviations. **C.** Comparisons of growth rates of parasites transfected with EpNeo (red), TfVPS32FL (light blue), TfVPS32α5 (blue) and TfVPS32α4/5 (orange). Error bars represent standard deviations and asterisks denote statistically significant differences determined by ANOVA.

Fig.4. **A.** Quantification of number of nuclei per parasite stained with DAPI. The percentages of parasites transfected with EpNeo, TfVPS32FL, TfVPS32α5, TfVPS32α4/5 with 1, 2 or more than 2 nuclei is shown. One hundred of parasites of each population were counted in triplicate in three independent experiments and the multinucleated parasites were considered as one cell. The results represent the average of three independent experiments and statistical significance was tested by Chi-square test (** p value <0.01). **B.** Multinucleated cells parasites overexpressing TfVPS32α5 (left panel) and TfVPS32α4/5 (right panel) were stained with DAPI (blue) and were visualized by epifluorescence microscopy. PC, phase-contrast image.

Fig.5. **A.** Immunofluorescence images showing multinuclear cells overexpressing TfVPS32α4/5 protein and co-stained with anti-HA (green), anti-acetylated tubulin (red). The nucleus (blue) was also stained with DAPI. PC, phase-contrast image.
B. Immunofluorescence images showing multinuclear cells overexpressing TfVPS32α4/5 protein and co-stained with anti-alpha tubulin (red). The nucleus (blue) was also stained with DAPI. Note the presence of extracellular flagella. PC, phase-contrast image.
C. Transmission Electron Microscopy (TEM) analysis of multinuclear cells of parasites expressing TfVPS32α4/5. Note the presence of more than two nuclei in each cell (N). The white arrows indicate the external recurrent flagellum (RF) and the black arrow indicate the three external anterior flagella (AF).

Fig.6. **A**. Flow cytometry analysis (FSC-H or forward scatter height) showing that parasites transfected with TfVPS32α4/5 (orange) showed larger cell size compared to wild type *T. foetus*, parasites transfected with EpNeo (red) and TfVPS32FL (light blue). **B.** Analysis of DNA content. Wild type *T. foetus* and parasites transfected with EpNeo (red), TfVPS32FL (light blue), TfVPS32α5 (blue) and TfVPS32α4/5 (orange) were stained with propidium iodide and their DNA content was analyzed by flow cytometry. Note the accumulation of cells with ~4C DNA content in parasites transfected with TfVPS32α5 and TfVPS32α4/5 compared to the accumulation of ~2C DNA content in wild type *T. foetus*, EpNeo and TfVPS32FL parasites. **C.** Analysis of cell cycle stages. The percentage of cells in the G1, S, and G2/M phases of the cell cycle of parasites transfected with EpNeo, TfVPS32FL, TfVPS32α5 and TfVPS32α4/5 are shown in grayscale. An arrest at G2/M phase was observed for parasites overexpressing TfVPS32α5 and TfVPS32α4/5. Parasites transfected with EpNeo and TfVPS32FL showed greater number of parasites in G1 and S phases of the cell cycle. A representative experiment is shown out of three experiments performed with similar results.

Figure S1. Sequence alignment showing the high degree of similarity between VPS32 proteins from different organisms. The alignment includes the open reading frame of HsCHMP4b (*H. sapiens*), ScSNF7 (*S. cerevisiae*), EhVPS32 (*E. histolytica*) and TfVPS32 (*T.foetus*).

Table S1. List of primers used in this study.

Figure S2. Sub-cellular localization of full-length and C-terminally deleted TfVPS32 protein. Cells exogenously expressing full length TfVPS32 (TfVPS32FL) and TfVPS32ΔCt (TfVPS32α5) fused to an EGFP C-terminal tag were immunostained using a rabbit anti-GFP antibody and visualized by epifluorescence microscopy. PC, phase-contrast image. The nucleus (blue) was also stained with DAPI.

Figure S3. Subcellular localization during cell division. Cells exogenously expressing TfVPS32FL during cell division with a C-terminal haemagglutinin (HA) tag were stained for immunofluorescence microscopy using a mouse anti-HA antibody. Note the subcellular localization at the midbody of cells in division in TfVPS32FL. PC, phase-contrast image.

Figure S4. Analysis on the *T. foetus* kinetic growth curve. Replication curves of parasites transfected with pTvGFP (red), TfVPS32FL-GFP (light blue), TfVPS32α5-GFP (blue) were compared. Parasites count were collected at indicated times on the X axis. The results represent the average of three independent experiments and error bars represent standard deviations.