The Role of the PI(3,5)P₂ Kinase TbFab1 in Endo/Lysosomal Trafficking in *Trypanosoma brucei*.

Julia K. Gilden¹, Khan Umaer², Emilia Kruzel², Oliver Hecht¹, Renan O. Correa¹, John M. Mansfield¹, and James D. Bangs^{2*}

¹ Department of Bacteriology, University of Wisconsin-Madison, Madison, WI, USA

² Department of Microbiology Immunology, School of Medicine and Biomedical Sciences, University at Buffalo (SUNY), Buffalo, NY, USA

* Corresponding Author: James D. Bangs, Ph.D.
Department of Microbiology & Immunology
Jacobs School of Medicine & Biomedical Sciences
University at Buffalo, 138 Farber Hall
3435 Main Street
Buffalo, NY 14214

Julia K. Gilden:	julia.gilden@gmail.com
Khan Umaer:	khanumae@buffalo.edu
Emilia Kruzel:	emiliakr@buffalo.edu
Oliver Hecht:	ohecht@wisc.edu
Renan O. Correa:	renanocorrea@gmail.com
John M. Mansfield:	john.mansfield@wisc.edu
James D. Bangs:	jdbangs@buffalo.edu

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Abbreviations: MVB: Multivesicular Body, ESCRT: Endosomal Sorting Complex Required for Transport, ILV: Intraluminal Vesicle, LE: Late Endosome, ISG: Invariant Surface Glycoprotein, PIP: Phosphoinositide, BSF: Bloodstream Form, TL: Tomato Lectin,

ABSTRACT (200)

Protein trafficking through endo/lysosomal compartments is critically important to the biology of the protozoan parasite *Trypanosoma brucei*, but the routes material may take to the lysosome, as well as the molecular factors regulating those routes, remain incompletely understood.

Phosphoinositides are signaling phospholipids that regulate many trafficking events by recruiting specific effector proteins to discrete membrane subdomains. In this study, we investigate the role of one phosphoinositide, PI(3,5)P₂ in *T. brucei*. We find a low steady state level of PI(3,5)P₂ in bloodstream form parasites comparable to that of other organisms. RNAi knockdown of the putative PI(3)P-5 kinase TbFab1 decreases the PI(3,5)P₂ pool leading to rapid cell death. TbFab1 and PI(3,5)P₂ both localize strongly to late endo/lysosomes. While most trafficking functions were intact in TbFab1 deficient cells, including both endocytic and biosynthetic trafficking to the lysosome, lysosomal turnover of an endogenous ubiquitinated membrane protein, ISG65, was completely blocked suggesting that TbFab1 plays a role in the ESCRT-mediated late endosomal/multivesicular body degradative pathways. Knockdown of a second component of PI(3,5)P₂ metabolism, the PI(3,5)P₂ phosphatase TbFig4, also resulted in delayed turnover of ISG65. Together, these results demonstrate an essential role for PI(3,5)P₂ in the turnover of ubiquitinated membrane proteins and in trypanosome endomembrane biology.

1. Introduction

Trypanosoma brucei subspecies are parasitic protozoa transmitted by tsetse flies to a wide variety of mammalian hosts, causing Human African Trypanosomiasis (HAT or African Sleeping Sickness) in man and nagana in cattle and other livestock. Untreated infections can persist for months or years before the host ultimately succumbs to the disease. The parasite lives free in the bloodstream and tissues of the infected mammal and avoids the host adaptive immune system by a process of antigenic variation [1, 2]. Essential to survival in this hostile environment is the parasite's endolysosomal system. Endocytosis is accelerated in the bloodstream form relative to other developmental stages [3, 4], and contributes to parasite nutritional needs, as well as evasion of humoral immune responses [3, 5-8]. Paradoxically, the lysosome also represents a point of vulnerability for *T. brucei*, and is a target of both the human innate immune system and some chemotherapeutic strategies [9, 10].

The path of endocytic cargo to the lysosome for degradation in *T. brucei* is generally similar to that of other model eukaryotes in that subcompartments are marked by characteristic small regulatory GTPases called Rabs. Cargo is endocytosed from the flagellar pocket, a specialized subdomain of the plasma membrane, in clathrin-coated vesicles [3, 4], which subsequently fuse with TbRab5-positive early endosomes [11, 12]. From there, endocytosed material can be sorted either to TbRab11-positive recycling endosomes for return to the cell surface [11, 13], or to TbRab7-positive late endosomes for delivery to the single terminal lysosome and subsequent degradation [11, 14, 15]. Late endosomal material can also be diverted for recycling, presumably via the recycling endosome [11].

In yeast and animals, transmembrane proteins can be turned over by trafficking through a specialized late endosome called the multivesicular body (MVB), and progress through this organelle is mediated by the stepwise assembly of ESCRT (Endosomal Sorting Complex Required for Transport) proteins [16-18]. Such proteins are marked for turnover by

monoubiquitinylation of lysine residues in their cytoplasmic domains, and once in an endosomal compartment such ubiquitinated species are aggregated laterally by ESCRT0 and ESCRT1 protein complexes. Areas of aggregation then invaginate via the action of ESCRT2 complex, and ESCRT3 complex is responsible for subsequent scission into intraluminal vesicles (ILVs). Ultimately the spent ESCRT machinery is disassembled by an ATPase, Vps4, which provides the sole energetic input to the system [19].

Characteristic MVBs with distinct ILVs are not morphologically obvious in trypanosomes, but clear orthologues of the ESCRT1-3 machinery are evident in the *T. brucei* genome, two of which (TbVps23 and TbVsp4) have been characterized [15, 20]. Both colocalize with TbRab7 to the late endosome (LE) consistent with MVB-type functions [15]. Ubiquitinylation has also been shown to be important for turnover of two trypanosome invariant surface glycoproteins, ISG65 and ISG75 [21-23]. Both are Type I membrane glycoproteins with lysine residues in their cytoplasmic domains that are subject to ubiquitinylation. Initially, based on RNAi silencing of TbVps23, it was argued that the classic ESCRT pathway was responsible for ubiquitin dependent lysosomal targeting and degradation of ISG65 [20]. However, we were unable to reproduce that finding, and in contrast found that silencing of TbVps4 actually accelerated turnover of ISG65, as did silencing of TbRab7 [15]. These results suggest that the role of the trypanosomal ESCRT machinery in regard to ISGs is rescue from lysosomal targeting by retrieval and recycling from the late endosome. Whether this is dependent on ubiquitinylation is unknown. Such a role for ESCRT, which has many cellular functions besides MVB formation, is not without precedent - ESCRT is critical for recycling of EGF receptor in some mammalian cell lines [24].

These potentially important distinctions between the endosomal pathways in *T. brucei* and other eukaryotes has sparked interest in studying other mediators of the pathway, and in trying to understand the complete network of routes that endocytosed material may take to the lysosome or back to the flagellar pocket. One such mediator is the signaling phospholipid,

phosphatidylinositol (3,5) bisphosphate (PI(3,5)P₂). Like other phosphoinositides (PIPs), PI(3,5)P₂ sits in the cytoplasmic leaflet of membranes and recruits effector proteins to carry out localized functions on those membranes. This lipid is produced when a kinase, Fab1p in yeast or PIKfyve in mammals, phosphorylates PI3P on the 5 position of the inositol ring [25, 26]. Depletion of the kinase in many systems leads to dysregulation of the vacuole or lysosome, including loss of acidification, morphological changes, disrupted trafficking of ubiquitinated cargo, and severe growth defects [25-32].

In this study we examine the role of PI(3,5)P₂ in *T. brucei* by disrupting the enzymes that control its metabolism. We find that while steady-state levels of PI(3,5)P₂ are very low in *T. brucei*, as in other eukaryotes, the lipid is profoundly important for turnover of ISG65 and for parasite survival.

2. Materials and methods

2.1 Parasite cultures Lister 427 BSF parasites were grown in HMI9 media with 20% fetal bovine serum [33], and with antibiotics as appropriate for maintaining transgenes. Tetracycline inducible cell lines, produced in the single-marker BSF background [34], were grown in HMI9 supplemented with tet-free fetal bovine serum (Atlanta Biologicals). For experiments, cells were harvested at mid-late log phase (BSF, 0.5x10⁶ - 10⁶).

2.2 RNAi and tagging constructs dsRNA against TbFab1 and TbFig4 was performed using the dual promoter tetracycline-responsive p2T7i vector [35]. 930 (TbFab1) or 700 (TbFig4) base pair regions of the genes were PCR amplified with flanking 5' BamHI and 3' HindIII cloning sites for insertion into p2T7i. Primers were (restriction sites in lower case): TbFab1 5': ggatccTTGGAAGTTGTGAGGGCTATTT; TbFab1 3': aagcttAATAAGCGTGTCTGGGTAACG, TbFig4 5': ggatccACGGTATCCACTTCCAGTGC, TbFig4 3':

aagcttTTGGCAAGAGTGGAGCTTTT. The ENTH domain of yeast Ent3p (plasmid pFL670, [36]) was a gift of Professor Sylvie Friant (University of Strasbourg). 5' AclI and 3' EcoRI sites were added by PCR and the product was cloned into the ClaI and EcoRI sites of the constitutive pXS6^{3xHA} vector (neomycin selection) [14], generating an in frame C-terminal 3xHA-tag fusion. The plasmid was linearized with NotI for insertion into the rDNA locus. In situ chromosomal C-terminal HA-tagging of TbFab1 was performed using the pXS6^{3xHA} vector. 454 bp at the 3' end of the open reading frame and 539 bp of the immediate 3' UTR were amplified and sequentially cloned into pXS6^{3xHA} using the 5' HindIII/3' EcoRI and 5' PacI/3' SacI sites, respectively. Primers were: TbFab1 ORF 5', aagcttGAACCGTGTAGCGCGTGAG; TbFab1 ORF 3', gaattcAGCCAAACTGCATGAATCCCCT 3' UTR 5', ttaattaaCACACCGTCAACACCACCAG; 3' UTR 3', gagctcGTTTAACTCACTTCCTCGTTGCCG. As experimental contingency indicated that robust detection of the tagged protein required tagging of both alleles, the construct was prepared with both neomycin and puromycin resistance cassettes. Both plasmids were linearized with HindIII and SacI for chromosomal tagging. The TY-Rab7 in situ tagging construct has been described previously [14]. All constructs were verified by sequencing.

2.3 Transfection and cloning of cell lines Linearized plasmids were transfected using Mirus Ingenio transfection solution on an Amaxa Nucleofector. Transfected parasites were then plated by limiting dilution and cultured with appropriate antibiotics to select for stably transfected clones.

2.4 PIP extraction and head group analysis Trypanosomes were cultured overnight in inositol deficient media DMEM (US Biological, Salem MA) supplemented with 36 mM sodium bicarbonate, 1 mM hypoxanthine, 50 µM bathocuproine, 1.5 mM cysteine, 0.2 mM 2-

mercaptoethanol, 1 mM pyruvate, 0.16 mM thymidine, 20% FBS, and 100 U/mL penicillin and streptomycin in the presence of 10 μ Ci/mL [³H]myo-inositol (American Radiolabeled Chemical, St Louis, MO; 30-80 Ci/mmol). To assess the effect of TbFab1 knockdown on PI(3,5)P₂ production, tetracycline was included throughout the labeling period. Starting densities were 2x10⁵/ml for control cells and 4x10⁵/ml for knockdown cells, which have impaired growth compared to controls. Labeled cells were then washed 3x in buffered saline with glucose and final pellets were flash frozen in a dry ice/ethanol bath. Isolation of glycerolPIP head groups was based on the protocol described in [37]. Pellets were extracted in methanol:chloroform:2.4 M HCl (125:250:10) and lipids were back-extracted in methanol:1M HCl:chloroform (235:245:15). After drying under N₂, lipids were resuspended in fresh methylamine reagent (methanol:40% methylamine:1-butanol at 1:1:0.1) and incubated at 50°C for 1 hour for deacylation. Head groups and fatty acids were separated by extraction with butanol/petroleum ether/ethyl formate (20:4:1). Purified glyceroylPIP (gPIP) head groups were then dried and resuspended in 10 mM (NH₄)₂PO₄ pH 3.8 for analysis. gPIPs were separated by anion exchange HPLC on a Beckman System Gold (Beckman Coulter Life Sciences, Indianapolis, IN) using an analytical 25-cm Partisil 5 SAX column (Hichrom, Reading, UK) and the following gradient of (NH₄)₂PO₄, pH 3.8 at a flow rate of 1 mL per minute: 10 mM for 5 min, 10-125 mM over 40 min, and 125 mM to 1.0 M over 10 min. Fractions were collected every 0.7 minutes, and [³H]gPIPs were detected by liquid scintillation counting. To generate PIP₂ standards *S. cerevisiae* cells were labeled for 20 hours in inositol-free YPD media with 10 μ Ci/mL [³H]myo-inositol. Labeled cells were washed, resuspended and incubated in hyperosmotic zymolyase buffer containing 30 mM DTT for 15 minutes. Next, cells were centrifuged and resuspended in zymolyase buffer with 67 U/mL zymolyase and incubated at 30°C for 40' with shaking. Resulting spheroplasts were centrifuged, snap frozen, and PIPs were extracted and treated as described above.

2.5 qRT-PCR RNA was extracted using a Qiagen RNEasy Mini kit with on-column DNase digestion (Qiagen, Valencia, CA). cDNA was produced from 1-3 µg RNA using Superscript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) and anchored oligo-dT (IDT) according to the manufacturers instructions. cDNA was then diluted 1:10 in nuclease-free water and used for qPCR analysis. qPCR was performed using Power SYBR Green Master Mix (Thermo Fisher) on a BioRad CFX96 qPCR system. TbZFP3 (Tb927.3.720, nts 241-301) was used as the control amplicon. All calculations and normalizations were done using native Biorad software. Reactions were performed in technical triplicates, and means ± standard errors of the means (SEM) for three biological replicates are presented.

2.6 Immunofluorescence staining and microscopy Immunofluorescence staining was performed as described [14]. Briefly, cells were fixed with methanol/acetone and stained with the following primary antibodies diluted in blocking buffer: rat anti-130 HA (Roche Diagnostics, Indianapolis, IN), monoclonal anti-TY 1:2000 (UAB Hybridoma Facility, Birmingham, AL), rabbit anti-ISG65 (gift of Professor Mark Carrington, Cambridge University, UK), and monoclonal anti-p67 and rabbit anti-TbCatL [38]. AlexaFluor conjugated goat secondary antibodies (Molecular Probes, Eugene OR) were used as appropriate. Stained cells were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame CA). Images were acquired on a Zeiss Axiolmager M2 using a 100x oil immersion objective, with exposure times <500 msec, and images were analyzed and prepared using ImageJ software (NIH).

2.7 Radiolabeling and Immunoprecipitation Metabolic labeling and immunoprecipitation were performed as previously described [14]. Briefly, cells were pulsed radiolabeled in labeling media for 10 minutes with [³⁵S]Met/Cys (American Radiolabeled Chemicals), then chased in complete pre-warmed HMI9 for the times indicated. Clarified lysates prepared in RIPA buffer

and immunoprecipitated overnight at 4° C and resolved by SDS-PAGE. After drying, gels were used for phosphorimaging and images were collected using a Typhoon FLA 9000 (GE Healthcare Life Sciences) and quantitative analysis was performed in ImageJ (NIH).

2.8 Flow cytometry trafficking assays Assays measuring uptake and trafficking of labeled tomato lectin were performed as previously described [14, 15]. Binding and endocytosis were measured by incubating cells for 30' at 4°C (binding) or 37°C (uptake) in serum-free HMI-9 with 0.5 mg/mL BSA and 5 µg/mL Alexa488-conjugated tomato lectin (Molecular Probes). Trafficking to the lysosome was measured by labeling cells at 4°C for 30' in serum-free HMI-9 with 0.5 mg/mL BSA and 5 µg/mL FITC-conjugated tomato lectin (Jackson ImmunoResearch Laboratories, West Grove PA), washing, then chasing for the indicated times at 37°C. Cells were labeled with 5 µg/mL DAPI prior to analysis for dead-cell exclusion. Flow cytometry was performed on an LSR II with FACSDiva acquisition software (BD Biosciences), and analysis was done in FlowJo 8.8.7.

2.9 ISG65 turnover Cells were incubated with cycloheximide (100 µg/ml) to inhibit de novo protein synthesis. As indicated the lysosomal thiol protease inhibitor FMK024 (morpholinourea-phenylalanine-homophenylalanine-fluoromethyl ketone; 20 µM; MP Biomedicals, Aurora, OH) was included prior to and during cycloheximide treatment. Whole cell lysates were fractionated by SDS-PAGE, transferred using an Owl semi dry apparatus (Thermo Fisher Scientific) to Immobilon-P membranes (Millipore Corp., Bedford, MA), and blocked in a 5% milk solution. Membranes were incubated 1 hr with anti-ISG65 (1/50,000), washed, and incubated with anti-rabbit IgG:HRP (1/10,000, 1 hr). Blots were stripped and reprobed with rabbit anti-BiP. The final washed blots were visualized on X-ray film using Pierce SuperSignal West Pico substrate (Thermo Fisher Scientific), as described elsewhere [39]. Film was digitized to 16-bit grayscale

using a transparency scanner and bands quantified using the gel volume analysis function of ImageJ 1.46 (NIH, Bethesda, MD).

2.10 Statistical Analysis Statistical analyses were performed with Prism4 software (GraphPad Software, Inc., San Diego CA). Tests and numbers of replicates are indicated in the figure legends.

3. Results

3.1 Measurement of PI(3,5)P₂ in bloodstream form cells In all eukaryotes studied, PI(3,5)P₂ is regulated by a multi-protein complex consisting minimally of a kinase, Fab1p (PIKfyve in mammals), a phosphatase, Fig4p, and a scaffolding protein, Vac14p [40-42]. A simple BLASTP search of the *T. brucei* reference genome (tritrypdb.org) with yeast sequences revealed clear gene orthologues for each of these proteins. TbFab1 (Tb927.11.1460, E 5e-36), TbFig4 (Tb927.11.5490, E 6e-62) and TbVac14 (Tb927.7.3530, E 6e-29) share significant homology in their enzymatic domains with their mammalian and yeast counterparts, and the domain structures of all three proteins are conserved across species (Fig. 1, Fab1 & Fig4 only). These observations suggest that the metabolism of PI(3,5)P₂ in *T. brucei* may be similar to that in other species.

PI(3,5)P₂ is typically present at very low abundance in most eukaryotes relative to other PIPs. However, its synthesis in yeast can be elevated by osmotic stress [43-45]. To determine whether PI(3,5)P₂ is present in *T. brucei*, we metabolically labeled bloodstream form (BSF) cells overnight with [³H]myo-inositol, extracted and saponified all phospholipids, and analyzed the resultant radiolabeled glycerol-PIP headgroups (gPIPs) by anion-exchange HPLC (Fig. 2A). Equivalently labeled and prepared gPIPs from osmotically stressed yeast were analyzed in parallel as chromatography standards. As expected, characteristic peaks representing

gPI(3,5)P₂ and a related species, gPI(4,5)P₂, were detected at similar levels in the yeast extracts [43, 45]. The identity of the gPI(4,5)P₂ species was confirmed by coelution in parallel chromatograms of authentic [³H]PI(4,5)P₂ added to unlabeled cells and prepared and fractionated identically (data not shown). A similarly strong peak representing gPI(4,5)P₂ is seen in the trypanosome extracts, and upon close examination a minor but reproducible peak corresponding to gPI(3,5)P₂ is also detectable. The relative abundances of gPI(3,5)P₂ and gPI(4,5)P₂ in trypanosomes are comparable to that typically observed in other organisms [42].

To determine whether TbFab1 is indeed responsible for production of PI(3,5)P₂ in trypanosomes, we used an RNAi knockdown approach. We expressed a tetracycline (tet)-inducible double stranded RNA construct in BSF cells. Induction for 24 hours resulted in an ~25% reduction in *TbFab1* mRNA, as measured by qRT-PCR (% of control 76.9±6.2, mean±S.D., n=3). Despite the modest reduction, within that time period growth of parasites ceased almost completely, and no recovery was observed within the first 72 hours (Fig. 2B). RNAi cells were labeled with [³H]myo-inositol during the first 18 hours of tet induction and gPIPs from control and knockdown cells were analyzed by HPLC (Fig. 2C). TbFab1 silencing had no effect on synthesis of gPI(4,5)P₂, but the minor gPI(3,5)P₂ species was reproducibly reduced to barely detectable levels. Collectively these data indicate that TbFab1 is a PI3P-5 kinase and is the major kinase responsible for PI(3,5)P₂ production in *T. brucei*. Importantly, gross morphology was unaltered at the 18hr time point, in particular there was no evidence of internal vacuolization or flagellar pocket swelling as judged by differential interference contrast microscopy (data not shown).

3.2 Localization of TbFab1 and PI(3,5)P₂ Fab1p in yeast and PIKfyve in mammals localize to the vacuole and lysosome, respectively, and we expect similar localizations in trypanosomes. To address this, we created a cell line in which both chromosomal alleles of *TbFab1* were fused in situ with a C-terminal 3xHA epitope tag (TbFab1:HA). Growth of this doubly tagged cell line

was unaffected indicating that the tag does not impair TbFab1 function (data not shown). TbFab1 is predicted to be a 156 kDa protein, although a spliced leader trapping dataset [46] indicates the use of an alternative internal trans-splice site within the TbFab1 ORF (see Fig. 1) followed by an in frame start codon, which would result in a much smaller 63 kDa protein. Indeed, western blotting of TbFab1:HA lysates revealed that the vast majority of TbFab1 is present as a 63 kDa species (Fig. 3, asterisk), although a full length species was specifically detected in the transformed cell line (Fig. 3, arrowhead). In addition, minor amounts of specific polypeptides were consistently seen in the 50-100 kDa range, presumably the result of degradation. Published transcriptomic datasets [47, 48] and our own RT-PCR analyses (data not shown) indicate that the full-length TbFab1 transcript is present. However, these immunoblot analyses indicate that the 156 kDa isoform constitutes only a tiny fraction of total steady state TbFab1.

Immunofluorescence analysis (IFA) of the TbFab1-HA cell line, costained for the lysosomal marker TbCatL, indicated that TbFab1-HA localizes in the post-nuclear region, consistent with a role in endocytic trafficking. A mixture of profiles was observed ranging from prominent to weak colocalization with the lysosome (Fig. 4A, left to right). This peri-lysosomal localization suggests that TbFab1 may also be present on late endosomes, which are closely associated with the lysosome in *T. brucei* [14]. To address this we Ty epitope-tagged one chromosomal allele of TbRab7, a late endosomal marker, in the TbFab1-HA cell line. Costaining for the HA and Ty epitopes consistently showed overlapping localization (Fig. 4B). Collectively these results indicate that TbFab1 localizes to the late endo/lysosomal membranes.

Fluorescent biosensors consisting of protein domains that bind specific PIPs fused to GFP have been used to localize PIPs in many cell types [49]. The ENTH domain of yeast Ent3p has been shown to specifically bind PI(3,5)P₂ in vitro [36]. We expressed this biosensor domain with a 3xHA epitope tag ectopically in *T. brucei* and used IFA to localize the reporter. Immunoblots with anti-HA indicated expression of tagged sensor of the expected size (~26 kDa,

not shown). Costaining with antibodies to TbCatL revealed a more restricted localization in the lysosome than seen for TbFab1-HA (Fig. 4C). Together, these results support a role for TbFab1 in the late endosome/lysosome, but suggest that its product, PI(3,5)P₂, accumulates most prominently at the lysosome.

3.3 TbFab1 is dispensable for endocytic and biosynthetic lysosomal trafficking The localization of TbFab1 and the profound growth defect we observed when TbFab1 is knocked down suggests that TbFab1 may function in endo-lysosomal trafficking. To address this, we used a set of standard assays to test various routes to the lysosome. First, we measured the ability of cells to take up Alexa488-tomato lectin (TL) as a surrogate for receptor-mediated endocytosis. TL binds to N-linked carbohydrate epitopes in the flagellar pocket and is efficiently taken up and delivered to the lysosome [14, 50]. Control and TbFab1 knockdown cells were incubated with TL at 4°C, to measure binding to the flagellar pocket, or 37°C, to measure endocytosis, followed by analysis by flow cytometry (Fig. 5A). In both cases, mean cell associated fluorescence was equivalent in the two populations, indicating that TbFab1 is not required for endocytosis. Next, we tested whether TbFab1 is required for delivery of endocytosed material to the lysosome. Cells were incubated with FITC-TL for 30 minutes at 4°C, then washed and shifted to 37°C to allow endocytosis (Fig. 5B). Because FITC fluorescence is pH sensitive the fluorescence signal decreases as TL traverses the increasingly acidic endosomal compartments to the terminal lysosome [51]. No difference was seen in the rate or extent of signal loss indicating that transport of receptor-bound cargo to the lysosome was not affected by TbFab1 depletion. The equivalent decline in FITC fluorescence also suggests that lysosomes of TbFab1-knockdown cells have a normal internal pH.

Trafficking of biosynthetic cargo to the lysosome can be measured by using pulse-chase analysis to monitor the processing of the endogenous soluble lysosomal thiol protease TbCatL

[14, 52]. TbCatL is synthesized as 53 and 50 kDa proproteins (“I” and “X,” respectively) that are processed to a 44 kDa mature form (“M”) upon arrival in the lysosome. Knockdown had no affect on the rate of TbCatL processing (Fig. 5C), indicating that TbFab1 is dispensable for biosynthetic trafficking to the lysosome.

3.4 TbFab1 is required for turnover of ISG65 ISG65 (invariant surface glycoprotein 65 kDa) [53, 54] is a type I membrane glycoprotein with lysine residues in its cytoplasmic tail that can be ubiquitinated to mark the protein for degradation in the lysosome [21-23]. Ubiquitin-targeted degradation is mediated by the ESCRT machinery in other systems, and such has been argued for ISG65 turnover in trypanosomes [20]. However, work from our laboratory has called this into question, suggesting rather that the ESCRT machinery is actually involved in rescue of ISG65 from lysosomal degradation [15]. Given these different interpretations, and the fact that PI(3,5)P₂ has been implicated in ESCRT function in other systems [26, 27, 55], we asked what role TbFab1 might play in regulating ISG65 turnover.

A cycloheximide-chase protocol [15] was used to measure the turnover of ISG65 in control and TbFab1-knockdown cells (Fig. 6). In control cells, ~60% of initial steady state ISG65 was degraded during the 4-hour chase period, and this degradation was almost completely blocked by treatment of cells with the thiol protease inhibitor FMK024, confirming turnover in the lysosome. Silencing of TbFab1 completely eliminated ISG65 turnover, demonstrating that TbFab1 is required for efficient targeting to the lysosome for degradation.

3.5 TbFig4 is required for ISG65 turnover In yeast, strains with Fig4 mutations typically have the same phenotype as those with Fab1 mutations, likely because the entire Fab1:Vac14:Fig4 complex is required for Fab1 activity [41]. To address whether this might also be the case in *T. brucei*, we expressed a tet-inducible TbFig4 RNAi construct in BSF cells. Cell growth was

normal for the first 24 hrs of silencing and thereafter was significantly retarded (Fig. 7A). *TbFig4* mRNA levels were reduced ~50% at 24 hrs (qRT-PCR, % of control 53.7±5.6, mean±S.D., n=3) and ISG65 turnover was completely abolished (Fig. 7B). The phenocopying of *TbFab1* and *TbFig4* silencing are consistent with function in the same pathway and the existence of a complex as seen in other systems.

4. Discussion

Recent studies have advanced our understanding of the role of the late endosome in lysosomal trafficking in *T. brucei*, revealing significant contrasts between trypanosomes and other model systems. However, detailed knowledge is still lacking in trypanosomes, prompting our investigation of PI(3,5)P₂, a potent signaling molecule with many roles in late endo/lysosomal trafficking. Biochemical analyses indicate that PI(4,5)P₂ is the major PIP₂ species in trypanosomes, but PI(3,5)P₂ is a small but detectable portion of the total. These results agree with similar measurements in yeast and in animal cells in which PI(3,5)P₂ is present at as little as 1% the level of PI(4,5)P₂ [37, 43-45]. A previous study also identified PI(4,5)P₂ as the major PIP₂ species in trypanosomes using methodology that may not have had the sensitivity required to detect low levels of PI(3,5)P₂ [56]. Consequently, this is the first formal demonstration of PI(3,5)P₂ in trypanosomes. Hypotonic stress upregulates PI(3,5)P₂ levels in yeast, and this proved to be a useful strategy in validating our HPLC assay. It is presently unknown whether stress conditions or developmental changes may similarly lead to upregulation of PI(3,5)P₂ in *T. brucei*.

Simple homology searches identified clear orthologues of the ternary complex that regulates PI(3,5)P₂ metabolism: *Fab1* (kinase), *Fig4* (phosphatase), *Vac14* (scaffold). RNAi silencing of *TbFab1* resulted in a rapid and persistent cessation of growth followed by cell death correlating with a significant reduction in the steady state level of PI(3,5)P₂. Based on this result we conclude that *TbFab1* is correctly identified as a PI3P-5 kinase. C-terminal chromosomal

epitope tagging was used to characterize the TbFab1 protein. A full-length species (156 kDa) was detected, but far and away the strongest signal was a 63 kDa species. Trans-splicing datasets indicate alternate use of an internal splice acceptor site that would be consistent with a truncated TbFab1 of this size [46]. Interestingly, this truncation results in loss of three of four homology domains in the TbFab1 orf (see Fig 1), most notably the FYVE PI3P binding domain. Although the C-terminal catalytic domain remains, it is difficult to imagine this species playing a major role in PI(3,5)P₂ metabolism in the absence of substrate recognition and membrane binding. Rather, we assume that the full-length protein is the sole functional isoform. Immunolocalization studies indicate variable colocalization of TbFab1 with the lysosome (p67), and consistently strong overlapping localization with the late endosome (TbRab7). Interestingly, when PI(3,5)P₂ was probed using an ENTH biosensor it was found to localize strongly with the lysosome (TbCatL). These results suggest that the primary site of PI(3,5)P₂ synthesis is on late endosomal, and to a lesser extent lysosomal, membranes, but that the end product resides mostly in the lysosome. This is similar to the situation with PI(4,5)P₂, where the kinase (TbPIPKA) localizes anterior to the mouth region of the flagellar pocket, but the product localizes quite distinctly to the flagellar pocket proper [56].

Surprisingly, given all the known functions of PI(3,5)P₂ in other systems, knockdown of TbFab1 had little effect on basic lysosomal trafficking. Uptake of endocytic cargo including tomato lectin and transferrin (not shown) was unaffected. This contrasts with depletion of two other PIP's, PI3P (TbVps34 knockdown) and PI(4,5)P₂ (TbPIPKA knockdown), both of which result in defective endocytosis [56, 57], and further supports the specific assignment of TbFab1 activity to PI(3,5)P₂. In addition, lysosomal delivery, measured as decline in FITC fluorescence as cargo moved to more acidic compartments, was unimpaired by TbFab1 depletion. This is consistent with results from yeast, but different from observations in *Drosophila* and human cells, where soluble cargo is readily taken up but fails to traffic to the lysosome [58, 59]. This finding also suggests that lysosomal pH is not affected by TbFab1 knockdown, as the final fluorescence

intensity of control and knockdown cells was equivalent. Early studies of Fab1/PIKfyve-deficient cells found elevated vacuolar/lysosomal pH [26, 32]. However, recent work using more quantitative methods found that normal internal pH was maintained in the degradative organelles of PIKfyve-inhibited mammalian or Fab1Δ yeast cells [60], consistent with our observations in *T. brucei*. Finally, TbFab1 ablation had no effect on biosynthetic trafficking to the lysosome as assessed by the kinetics of TbCatL maturation, similar to results with Cathepsin D in mammalian cells using conditional expression of a dominant negative PIKfyve mutant [59].

The major phenotypic effect of TbFab1 knockdown is complete inhibition of lysosomal degradation of ISG65, and this effect is recapitulated by knockdown of TbFig4. One might expect that depletion of the PI(3,5)P₂ phosphatase would have the opposite effect, but in yeast Fig4p is an essential member of the PI(3,5)P₂-regulating ternary complex and is required for Fab1p activity [41]. Recent observations in mice suggest the same holds true in mammalian systems [61]. Trypanosomes have orthologues of all the components of the PI(3,5)P₂ complex, and it seems likely that a similar phenomenon is in play - knockdown of TbFig4 negatively impacts TbFab1 activity and hence the counter-intuitive phenocopying of TbFab1 knockdown. Collectively these results suggest that fine tuning of PI(3,5)P₂ levels is critical for efficient regulation of ISG65 turnover.

The likely locus for PI(3,5)P₂ action is the LE/MVB compartment, which is marked by TbRab7 and the ESCRT components TbVps23, TbVps28, and TbVps4 [14, 15, 20], and where TbFab1 prominently localizes (this work). Our previous studies indicate that both TbRab7 and TbVps4 act to retard ISG65 turnover, e.g., knockdown of either accelerates degradation, and our favored model is that these agents facilitate retrieval/recycling from the LE/MVB to early compartments of the endosomal system [15]. Whether this is dependent on ubiquitinylation is not clear. Although the ESCRT machinery is typically thought to mediate lysosomal delivery, a role in recycling is not unprecedented [24]. Within this hypothetical framework there are at least

two obvious (simplistic) ways in which PI(3,5)P₂ might function in ISG65 trafficking. First, PI(3,5)P₂ could be a negative regulator of Rab7/ESCRT-dependent rescue. In this scheme depletion of PI(3,5)P₂ leads to enhanced recycling of ISG65 from the LE/MVB. Alternatively, PI(3,5)P₂ could be a positive regulator of a distinct Rab7/ESCRT-independent pathway for LE/MVB-to-lysosome trafficking. In this scenario PI(3,5)P₂ depletion would block delivery of ISG65 to the lysosome. Such a pathway, should it exist, would likely be distinct from normal biosynthetic pathways to the lysosome since TbFab1 ablation has no effect on normal transport of TbCatL.

In either scenario a complete understanding of how PI(3,5)P₂ mediates LE/MVB pathways in trypanosomes will depend on identifying the relevant effector proteins it recruits. One possibility is TbEpsinR [62], the sole ENTH domain protein evident in the *T. brucei* genome, which shares significant homology with yeast Ent3p, a known PI(3,5)P₂ effector with a role in sorting cargo into ILVs [36]. However, TbEpsin localizes to early endosomes and the flagellar pocket, not the LE/MVB, where it functions in clathrin-dependent endocytosis. This localization is dependent on synthesis of PI4P (PI4KIIIβ knockdown), and presumably its derivative PI(4,5)P₂ [62]. Furthermore, as noted above, TbPIPKA, the kinase responsible for PI(4,5)P₂ synthesis, localizes to the flagellar pocket mouth [56]. Taken together these data argue against a role for TbEpsinR in trafficking pathways specific to the LE/MVB.

A more promising effector protein in ISG65 sorting pathway(s) may be the ESCRT III protein Vps24, which has been shown to bind PI(3,5)P₂ in mammalian cells [63]. TbVps24 (Tb927.11.10000) has not been studied empirically, but as a putative ESCRT III component it would be expected to play a role in vesicle scission into the lumen of late endosomes. A mechanism could be envisioned whereby negative regulation of ESCRT-dependent ISG65 rescue is mediated by PI(3,5)P₂ binding to TbVsp24. Obviously, there are even more speculative and complex scenarios that could be elaborated to account for the unusual behavior of ISG65 in the LE/MVB compartment, and more work will be required to bring clarity, but

collectively our findings lay a solid experimental foundation for future investigations. Such work should be informative, both to the specifics of late endosomal trafficking in trypanosomes, and in a broader sense to more complex eukaryotic systems.

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Figure legends

Figure 1. *T. brucei* Fab1 and Fig4 Domain structures of the *T. brucei* and *S. cerevisiae* Fab1 (Tb927.11.1460, AHY75806) and Fig4 (Tb927.11.5490, P42837) orthologues. Diagrams are to scale and sizes (amino acids residues) are in parentheses. The positions in *TbFab1* of the alternate internal trans-splice site (asterisk) and of the introduced HA tag are indicated. Domains are: FYVE (PI3P binding); CCT (homology to chaperone Cpn60/TCP-1, Vac14 binding); CCR (conserved cysteine rich, Vac14 binding); Kinase (catalytic site); Sac (phosphoinositide phosphatase) [42].

Figure 2. PI(3,5)P₂ is dependent on *TbFab1* expression **A.** *T. brucei* and osmotically shocked *S. cerevisiae* were metabolically labeled overnight with [³H]-myo-inositol and PIPs were extracted, saponified, and resolved by anion exchange HPLC. A full chromatograph is presented on the left, and an expansion of the gPIP₂ region is on the right. Characteristic peaks corresponding to gPIP, gPI(3,5)P₂ and PI(4,5)P₂ are indicated. Data are presented as the fraction of the total loaded sample. **B.** Growth of *TbFab1* RNAi cells was evaluated by seeding at 10⁵/mL in media with or without tetracycline. Cell density was determined every 24 hours by hemocytometer and cultures were adjusted to starting density daily. Mean and standard deviation of 3 biological replicates is displayed. Data is representative of 3 independent experiments. **C.** *TbFab1* RNAi cells were labeled overnight with [³H]myo-inositol with or without tetracycline induction and extracted gPIPs were resolved by HPLC (left) as in Panel A. Relative PI(3,5)P₂ levels were quantified (right) by normalizing to the PI(4,5)P₂ level in each sample (mean ± SD, *n*=3, *p*<0.02 by unpaired *t*-test). Total loaded radiolabeled samples varied experimentally, but was typically 20,000-30,000 cpm for both control and silenced cells

Figure 3. Expression of HA epitope tagged *TbFab1* Parental (Par) and *Fab1:HA* cells (10⁷ cell equivalents/lane) were subjected to immunoblotting with anti-HA antibody (left). Mobilities of the dominant short (asterisk, 63 kDa) and full length (arrowhead, 156 kDa) isoforms are indicated (asterisk). A separate blot was performed with anti-Hsp70 as a loading control (right). Mobilities of molecular mass markers (kDa) are indicated.

Figure 4. *TbFab1* and PI(3,5)P₂ localize to the lysosome and late endosome IFA was performed with fixed permeabilized cells as described in Materials and Methods. Cells were stained with DAPI to reveal nuclei (n) and kinetoplasts (k) as indicated (left panels only). **A.** *TbFab1*-HA cells costained with antibodies against HA (green) and endogenous *TbCatL* (red), a lysosomal marker. **B.** *TbFab1*-HA cells co-expressing Ty-Rab7, a late endosome marker, stained with antibodies against HA (green) and Ty (red). **C.** Cells expressing ENTH-HA, a PI(3,5)P₂ biosensor, stained with antibodies against HA (green) and *TbCatL* (red). Each set contains three independent triple channel merges with magnified insets of single red, single green and merged red/green channel images of the post-nuclear region.

Figure 5. Effect of *TbFab1* silencing on lysosomal trafficking *TbFab1* RNAi cells were cultured with or without tetracycline for 18 hours prior to all assays. **A.** Cells were incubated (30 min) with A488-TL at 4°C (binding) or 37°C (uptake) and analyzed by flow cytometry. Data are presented as mean fluorescent intensity (MFI, mean ± SD, *n*=3, representative of 3 independent experiments). **B.** Cells were incubated (30 min) with FITC-TL at 4°C, washed, and then shifted to 37°C to allow endocytosis and trafficking to the lysosome. Analysis was by flow cytometry and the data are normalized to T₀ (mean ± SD, *n*=3, representative of 3 independent experiments). **C.** Cells were pulse-chase radiolabeled and at the indicated times *TbCatL* was immunoprecipitated from cell lysates and fractionated by SDS-PAGE. A representative phosphorimage is presented with precursor (I, X) and mature (M) polypeptides indicated

(bottom). Loss of precursors and appearance of mature form were quantified and are presented graphically (top, mean $\pm$ SD, $n=2$).

Figure 6. TbFab1 controls ISG65 turnover TbFab1 silencing was induced with tetracycline for 18 hours and then for an additional 4 hours with and without cycloheximide (100 μ g/ml) as indicated. Duplicate cultures were treated with FMK024 (20 μ M) to inhibit degradation by lysosomal thiol proteases. Samples were prepared for immunoblotting with anti-ISG65 at T₀ and T₄ of the second incubation. The membrane was stripped and reprobed with anti-BiP as a loading control. Representative blots are presented (top). ISG65 signals were quantified and normalized to BiP and are presented as the fraction of T₀ for each data set (bottom, $n = 3$).

Figure 7. TbFig4 A. Growth of TbFig4 RNAi cells was evaluated by seeding at 10⁵/mL in media with or without tetracycline. Cell density was determined every 24 hours by hemocytometer and cultures were adjusted to starting density daily. Mean and standard deviation of 3 biological replicates is displayed. **B.** TbFig4 silencing was induced with tetracycline for 18 hours and then for an additional 4 hours with and without cycloheximide (100 μ g/ml) as indicated. Duplicate cultures were treated with FMK024 (20 μ M) to inhibit degradation by lysosomal thiol proteases. Samples were prepared for immunoblotting with anti-ISG65 at T₀ and T₄ of the second incubation. The membrane was stripped and reprobed with anti-BiP as a loading control. Representative blots are presented (left). ISG65 signals were quantified and normalized to BiP and are presented as the fraction of T₀ for each data set (right, $n = 3$).

A. PIP Synthesis

B. Growth

C. PIP Abundance

A. CatL vs TbFab1-HA

B. Ty-Rab7 vs TbFab1-HA

C. CatL vs ENTH-HA

A. Growth

B. ISG65 Turnover