Trypanosoma brucei bloodstream forms depend upon uptake of *myo*-inositol for Golgi phosphatidylinositol synthesis and normal cell growth

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Running Title: *myo*-Inositol transport in bloodstream *T. brucei*

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ABSTRACT

myo-Inositol is a building block for all inositol-containing phospholipids in eukaryotes. It can be synthesized *de novo* from glucose-6-phosphate in the cytosol and endoplasmic reticulum. Alternatively, it can be taken up from the environment via Na^+- or H^+-linked *myo*-inositol transporters. While Na^+-coupled *myo*-inositol transporters are found exclusively in the plasma membrane, H^+-linked *myo*-inositol transporters are detected in intracellular organelles. In *Trypanosoma brucei*, the causative agent of human African sleeping sickness, *myo*-inositol metabolism is compartmentalized. *De novo* synthesized *myo*-inositol is used for glycosylphosphatidylinositol production in the endoplasmic reticulum, whereas the *myo*-inositol taken up from the environment is used for bulk phosphatidylinositol synthesis in the Golgi. We now provide evidence that the Golgi localized *T. brucei* H^+-linked *myo*-inositol transporter (TbHMIT) is essential in bloodstream forms. Down-regulation of TbHMIT expression by RNA interference blocked phosphatidylinositol production and inhibited growth of parasites in culture. Characterization of the transporter in a heterologous expression system demonstrated a remarkable selectivity of TbHMIT for *myo*-inositol. It only tolerates a single modification on the inositol ring, such as the removal of a hydroxyl group, or the inversion of stereochemistry at a single hydroxyl group relative to *myo*-inositol.

INTRODUCTION

myo-Inositol is the precursor for all inositol-containing phospholipids, including phosphatidylinositol (PI), phosphatidylinositol (poly)phosphates, inositol phosphorylceramide (IPC) and glycosylphosphatidylinositol (GPI) in all eukaryotes. In mammalian cells, it is taken up from the environment via sodium/*myo*-inositol cotransporters (SMITs) or proton-linked *myo*-inositol transport (HMIT). Human SMIT1 and SMIT2 belong to the sodium/glucose transporter family, SGLT/SLC5, whose members in general mediate uptake of sugars and osmolytes in the gastrointestinal tract and the kidney (1). They are localized in the plasma membrane and, besides *myo*-inositol, also transport xylose and glucose (2, 3). In contrast, the human HMIT belongs to the sugar/polyol transport facilitators family, GLUT/SLC2A (4). While most members of this family are also located in the plasma membrane and regulate sugar homeostasis within the body, subclass III transporters, to which HMIT belongs, are typically localized intracellularly (5, 6). Interestingly, GLUT12/SLC2A12 and HMIT/SLC2A13 have been found to co-localize with Golgi markers (7, 8). Although the substrate specificities of the subclass III GLUT/SLC2A transporters have been studied in model systems, their physiological roles have not been firmly established (5, 6). Notably, HMIT completely lacks sugar transport activity, but instead transports *myo*-inositol with a K_m of approximately 100 µM in a *Xenopus* oocyte expression system (8).

Alternatively to uptake, *myo*-inositol can be synthesized *de novo* in a reaction sequence that is conserved from bacteria to mammals, using glucose-6-phosphate as an initial substrate (9). Endogenously produced as well as imported *myo*-inositol can then be used for inositol phospholipid synthesis in a process that is generally believed to occur in the endoplasmic reticulum (ER). In yeast, plants, protozoa and mammals,

PI synthase has been localized to the ER using cell fractionation and immunolocalization studies (10-13). Reconstitution experiments involving purified PI synthase from *Saccharomyces cerevisiae* showed that it incorporates asymmetrically into model vesicles, suggesting that its active site may face the cytosolic side of the ER in yeast (10). However, the *in vivo* topology of the active site of PI synthase has not been determined experimentally.

Interestingly, recent reports in protozoan parasites indicated that the Golgi represents an additional site for inositol phospholipid synthesis. Direct evidence for the presence of PI synthase in the Golgi was obtained from immunolocalization studies in *T. brucei* bloodstream forms (13), showing that the enzyme has a dual localization in the ER and Golgi. In support of the Golgi being the site of bulk PI synthesis in trypanosomes (13), a subsequent study revealed that *T. brucei* procyclic forms express a plasma membrane- and Golgi-localized proton-linked *myo*-inositol transporter, TbHMIT (14). Down-regulation of TbHMIT inhibited bulk PI formation, but had no effect on GPI synthesis, demonstrating that PI synthesis in *T. brucei* is compartmentalized, with the Golgi representing the site of synthesis of bulk membrane PI utilizing exogenous *myo*-inositol (14), and the ER being the site of PI synthesis for GPI production utilizing *de novo* synthesized *myo*-inositol (15). Transporter-mediated *myo*-inositol uptake has also been characterized in other protozoan parasites, including *Leishmania donovani* (16-19) and *Trypanosoma cruzi* (20, 21). These parasites all cause devastating human diseases, including Leishmaniasis, Chagas disease and human African sleeping sickness. Membrane transporters are of particular importance for these pathogens to acquire essential nutrients from their respective hosts and offer attractive targets for rational drug design and/or the delivery of cytotoxic substrate analogues. The reported dependence

of *T. brucei* procyclic forms in culture on exogenous *myo*-inositol (13, 14) validates HMIT as potential drug target.

In this report, we extend our previous analysis of *myo*-inositol uptake in *T. brucei* procyclic forms to the pharmacologically more relevant bloodstream form. We demonstrate that the expression of TbHMIT is essential for normal growth of *T. brucei* bloodstream parasites in culture and that it is involved in *myo*-inositol transport and PI formation within the Golgi. In addition, we have tested a series of *myo*-inositol stereoisomers and structural analogs and related compounds to characterize the substrate specificity of TbHMIT.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were of analytical grade and purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (Buchs, Switzerland) or ICN Biomedicals (Tägerig, Switzerland). Antibiotics and fetal bovine serum (FBS) were obtained from Invitrogen (Basel, Switzerland). Primers and sequencing services were from Microsynth AG (Balgach, Switzerland). Restriction enzymes were purchased from Thermo Scientific (Wohlen, Switzerland). *myo*-[2-^{3}H(N)]inositol (15-20 Ci/mmol) (*myo*-[^{3}H]inositol) and [^{3}H]ethanolamine (40-60 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, USA), and dCTP-[α-^{32}P] (3000 Ci/mmol) from PerkinElmer Life Sciences (Schwerzenbach, Switzerland).

Trypanosomes and culture conditions – Bloodstream form *T. brucei* derived from MiTat 1.2, co-expressing T7 RNA polymerase and a tetracycline repressor (22), were cultured at 37 °C with 5% CO_2 in HMI-9 (23) containing 10% heat-inactivated FBS and 1 μg/ml G418. *T. brucei* strain 427 procyclic forms were cultured at 27 °C in SDM-79 (24) containing 5% heat-inactivated FBS.

RNAi-mediated gene silencing – Expression of TbHMIT (Tb927.11.5350) was down-regulated in *T. brucei* bloodstream forms by RNAi-mediated gene silencing using a stem loop construct containing a phleomycin resistance gene. The stem-loop was excised from plasmid pAG3020 (14) using BamHI and HindIII and re-ligated into plasmid pMS1720RNAiBSF (25), resulting in plasmid pAG3020-BSF. Plasmid extraction was performed using the Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before transfection of *T. brucei* bloodstream forms, plasmid DNA was linearized with NotI and precipitated with phenol and chloroform.

Generation of hemagglutinin (HA)-tagged TbHMIT – Over-expression of C-terminally 3xHA-tagged TbHMIT was performed using the inducible vector pALC14 as described previously (14), resulting in plasmid pAG3020-BSF2. Before transfection of *T. brucei* bloodstream forms, plasmid DNA was linearized with NotI and isolated with phenol and chloroform.

Stable transfection of trypanosomes and selection of clones – T. brucei bloodstream forms (4-5 × 10^7 cells) were harvested at mid-log phase (0.8-1.1 x 10^6 cells/ml) by centrifugation at 1250 x *g* for 10 min, suspended in 100 µl of buffer (26) (90 mM $NaPO_4$, 5 mM KCl, 0.15 mM $CaCl_2$, 50 mM HEPES pH 7.3) and mixed with 10 µg of linearized plasmid pAG3020-BSF or pAG3020-BSF2. Electroporation was performed in a 0.2 cm pulse cuvette (Bio-Rad Laboratories, Reinach, Switzerland) with a Lonza Nucleofector System (Ruwag Lifescience, Bettlach, Switzerland) using program FI-115. Electroporated cells were immediately inoculated in 10 ml of HMI-9, containing 10% heat-inactivated FBS, and, if required for selection, 1 µg/ml phleomycin (for RNAi) or 0.1 µg/ml puromycin (for over-expression). Clones were obtained by limiting dilutions in 24-well plates in HMI-9, containing 10% heat-inactivated FBS, in the presence of 1 µg/ml phleomycin or 0.1 µg/ml puromycin. Antibiotic-resistant clones were tested for the presence of the introduced gene by PCR. Expression of HA-tagged TbHMIT or induction of RNAi was started by addition of 1 µg/ml tetracycline to parasite cultures.

RNA isolation and Northern blot analysis – Total RNA for Northern blotting was isolated using the Total SV RNA Extraction Kit (Promega, Dübendorf, Switzerland), following the manufacturer's instructions. RNA (10 µg) was separated on formaldehyde-agarose gels (1% agarose, 2% formaldehyde in 3-N-morpholino propane sulfonic acid) and transferred to GeneScreen Plus nylon membranes

(PerkinElmer Life Sciences). ^{32}P-Labeled probes were made by random priming the same PCR products used as inserts in the stem-loop vector using the Prime-a-Gene Labeling System (Promega). Hybridization was performed overnight at 60 °C in hybridization buffer containing 7% (w/v) SDS, 1% (w/v) bovine serum albumin, 0.9 mM EDTA, 0.5 M Na_2HPO_4, pH 7.2, and the membrane was analyzed by autoradiography using BioMax MS film and a TransScreen-HE intensifying screen. Ribosomal RNA was visualized on the same formaldehyde-agarose gel by ethidium bromide staining to control for equal loading.

myo-Inositol uptake assays – T. brucei bloodstream forms (1 x 10^8 cells) at mid-log phase were harvested by centrifugation at 1250 x *g* for 10 min and suspended in phosphate-buffered saline (PBS; 135 mM NaCl, 1.3 mM KCl, 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, pH 7.4) at 27 °C. Uptake of *myo*-[^{3}H]inositol was measured by adding 50 nM of *myo*-[^{3}H]inositol to cells at 37 °C. At various time points, uptake of label was terminated by pelleting 1.5 × 10^7 parasites by centrifugation (1500 x *g*, 5 min, 4 °C) and washing three times in ice-cold PBS. After resuspension of the pellet in 100 µl PBS, radioactivity was measured by scintillation counting using a Packard Tri-Carb 2100TR liquid scintillation analyzer (PerkinElmer Life Sciences). Aliquots of the parasite suspensions before centrifugation were used to determine the total amount of radioactivity in the assay.

Metabolic labeling of trypanosomes, lipid extraction and thin layer chromatography (TLC) – Metabolic labeling of trypanosomes was performed as described before (27). Briefly, *myo*-[^{3}H]inositol was added to bloodstream or procyclic form trypanosomes at mid-log phase, and incubation was continued for 16 h. Cells were harvested by centrifugation at 1750 x *g* for 10 min, washed with ice-cold Tris-buffered saline (10 mM Tris, 144 mM NaCl, pH 7.4) to remove

unincorporated label, and bulk phospholipids were extracted twice with 10 ml chloroform:methanol (CM; 2:1, by vol.). CM fractions were pooled, dried under nitrogen and resuspended in a small volume of CM. Aliquots were treated with 6 µl PI-specific phospholipase C from *Bacillus cereus* (Thermo Scientific, Wohlen, Switzerland) for 60 min, as described elsewhere (28). Lipids were analyzed by TLC on Silica Gel 60 plates (Merck) using solvent system 1 composed of chloroform:methanol:acetic acid:water (25:15:4:2, by vol.) (29). Appropriate lipid standards were run alongside the samples to be analyzed. Radioactivity was detected by scanning the air-dried plate with a radioisotope detector (Berthold Technologies, Regensdorf, Switzerland) and quantified using the Rita Star software provided by the manufacturer. For analysis of GPI precursors, bloodstream form trypanosomes were labeled for 16 h with trace amounts of [^{3}H]ethanolamine (28). After harvesting the cells and extracting bulk lipids as described above, GPI lipids were extracted from the pellet using chloroform:methanol:water (10:10:3, by vol.) and partitioned between water and butan-1-ol. [^{3}H]-labeled GPI lipids in the butan-1-ol rich upper phase were analyzed by TLC using solvent system 2 composed of chloroform:methanol:water (10:10:3, by vol.) (28). Radioactivity was detected as above.

Mass spectrometry and inositol analysis - Total lipids for mass spectrometry analysis were extracted using a modified Bligh and Dyer method (30). Briefly, *T. brucei* bloodstream forms were collected at mid-log phase, washed with PBS, resuspended in 100 µl of fresh PBS and transferred to a glass tube. 375 ml chloroform:methanol (1:2, v/v) was then added and vortexed vigorously for 10-15 min. The sample was made biphasic by the addition of 125 ml chloroform and 125 ml water, vortexed again and centrifuged at 1000 x g at RT for 5 min. The lower phase was dried under nitrogen, and stored at 4 °C. Total lipid extracts were dissolved in 15

ml of choloroform:methanol (1:2, v/v) and 15 ml of acetonitrile:*iso*-propanol:water (6:7:2, by vol.) and analysed with a Absceix 4000 QTrap, a triple quadrupole mass spectrometer equipped with a nanoelectrospray source. Samples were delivered using a Nanomate interface in direct infusion mode (~125 nl/min). Lipid extracts were analyzed in both positive and negative ion modes using a capillary voltage of 1.25 kV. Tandem mass spectrometry (MS/MS) scanning (daughter, precursor and neutral loss scans) was performed using nitrogen as the collision gas with collision energies between 35-90 V. Each spectrum encompasses at least 50 repetitive scans. MS/MS spectra were obtained with collision energies as follows: 35-45V, PC/SM in positive ion mode, parent-ion scanning of m/z 184; 35-55V, PI in negative ion mode, parent-ion scanning of m/z 241; 35-65V, PE in negative ion mode, parent-ion scanning of m/z 196; 20-35V, PS in negative ion mode, neutral loss scanning of m/z 87; and 40-90V. MS/MS daughter ion scanning was performed with collision energies between 35-90V. Assignment of phospholipid species is based upon a combination of survey, daughter, precursor and neutral loss scans, as well previous assignments (31). The identity of phospholipid peaks was verified using the LIPID MAPS: Nature Lipidomics Gateway (www.lipidmaps.org).

For the inositol analysis, bloodstream forms were collected and lipids were extracted as above. An internal standard of D_6 *myo*-inositol was added to samples prior to hydrolysis by strong acid (6M HCl, 110 °C), derivatisation with TMS and analysis by gas chromatography-mass spectrometry, as published elsewhere (32). *myo*-Inositol was quantified and the mean and standard deviations of three separate analyses were determined.

Microscopy – For immunolocalization of HA-tagged TbHMIT, trypanosomes were cultured in the presence of tetracycline for 24 h to induce protein expression and

processed as described (14). HA-tagged proteins were detected using monoclonal mouse anti-HA antibody (Covance, Munich, Germany) at a dilution of 1:250 in PBS for 1 h at room temperature. Golgi was visualized by incubating fixed parasites for 1 h at room temperature with rabbit anti-TbGRASP antibody (kindly provided by G. Warren, University of Vienna; used at a dilution of 1:1000). Subsequently, the slides were washed three times with PBS and incubated with the corresponding secondary antibodies, Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) at a dilution of 1:1000 and 1:500, respectively, for 1 h at room temperature. Slides were washed three times with PBS and mounted using Vectashield containing 4′,6′-diamidino-2-phenylindol (DAPI; Vector Laboratories, Burlingame, USA). Fluorescence microscopy was performed on a Leica AF6000 microscope (Leica Microsystems, Heerbrugg, Switzerland), using the software provided by the manufacturer.

Substrate specificity of TbHMIT – *Xenopus laevis* oocytes were prepared, injected with Tb927.11.5350 cRNA and defollicated as described previously (14). Electrophysiological experiments were performed as described before (14). *myo*-Inositol, *epi*-quercitol, *vibo*-quercitol, *proto*-quercitol, *scyllo*-inositol, *muco*-inositol, *allo*-inositol, *epi*-inositol, 1D-*chiro*-inositol, 1L-*chiro*-inositol, 1L-*epi*-2-inosose, phytic acid, L-quebrachitol, D-pinitol, N-00601 ((1R,4S)-6-methoxycyclohexane-1,2,3,4,5-pentol), N-50350 ((1R,3S)-6-methoxycyclohexane-1,2,3,4,5-pentol) and D-*myo*-inositol-3-phosphate at 200 µM were applied for 20 s for each measurement. Potential inhibition of TbHMIT was tested by applying a combination of 200 µM *myo*-inositol and one of the compounds described above at an equal concentration. The resulting signal was compared with that elicited by 200 µM *myo*-inositol alone.

RESULTS AND DISCUSSION

Characterization of T. brucei TbHMIT in bloodstream form parasites – To study if TbHMIT is essential in *T. brucei* bloodstream forms in culture, we generated tetracycline-inducible RNAi cell lines against Tb927.11.5350. Transfection of *T. brucei* bloodstream forms with plasmid pAG3020-BSF and selection by resistance to phleomycin resulted in several clones, one of which (A3) was selected for all subsequent experiments. After 2 days of induction of RNAi by addition of tetracycline to the culture, parasite growth decreased compared to uninduced (control) cells (Fig. 1A). Northern blot analysis showed that after 2 days of RNAi, Tb927.11.5350 mRNA was undetectable (Fig. 1A, inset). The uptake of *myo*-inositol into bloodstream form RNAi parasites was measured by adding trace amounts of *myo*-[^{3}H]inositol to trypanosomes cultured for 2 days in the absence or presence of tetracycline and measuring radioactivity in the cell pellets after centrifugation. The results show that uptake of *myo*-[^{3}H]inositol into control trypanosomes increased linearly over 90 min (Fig. 1B). A similar time-dependent linear increase in cell-associated radioactivity was also observed for RNAi parasites after down-regulation of TbHMIT, however, uptake of *myo*-[^{3}H]inositol was reduced to approximately half of that in control cells. To demonstrate that the *myo*-[^{3}H]inositol that was taken up was being metabolized into inositol-containing phospholipids, bloodstream forms cultured in the absence or presence of tetracycline were incubated for 16 h in the presence of *myo*-[^{3}H]inositol followed by analysis of radiolabeled lipids by TLC and radioactivity scanning. In the absence of tetracycline, a single [^{3}H]-labeled lipid class was detected (Fig. 1C top panel, and Fig. S1A), which was identified as [^{3}H]PI based on its co-migration with a commercial PI standard and complete susceptibility to PI-specific phospholipase C (Fig. S1B). In parasites after RNAi-mediated down-

regulation of TbHMIT, formation of [^{3}H]PI was reduced by >85% (Fig. 1C, middle panel). No formation of [^{3}H]inositol phosphorylceramide ([^{3}H]IPC), which is readily labeled in procyclic forms (Fig. 1C; bottom panel; see also (14)), was observed in un-induced bloodstream forms. This observation is consistent with previous reports showing that IPC synthesis in *T. brucei* bloodstream forms is largely absent (31, 33). In addition, we analyzed the formation of GPI precursor lipids by labeling bloodstream form parasites cultured in the absence or presence of tetracycline with [^{3}H]ethanolamine, which gets incorporated into GPIs (28). As shown in Fig. 1D, formation of the major ^{3}H-labeled GPI precursors, P2 and P3, was readily observed in parasites after depletion of TbHMIT. This result demonstrates that, as previously shown in procyclic forms (14), GPI synthesis is not affected by down-regulation of TbHMIT.

The effect of TbHMIT RNAi on parasite lipid composition was investigated by extracting the lipids and analyzing them by ES-MS. In negative ion mode a range of peaks was observed in the un-induced RNAi cells, corresponding to the phospholipid profile of wild-type parasites (Fig. 2A; see (31) for comparison). These include the major plasmalogen (alk-enyl acyl) phosphatidylethanolamine (PE) species at 727 m/z, and PI molecular species at 865, 887 and 913 m/z, corresponding to 36:0, 38:3, and 40:4 PI. Upon induction of the RNAi cells for 48 hours with tetracycline (Fig. 2B), the intensity of all PI molecular species clearly diminished compared to un-induced cells, while the plasmalogen PE is still the dominant species (compare Fig. 2A with Fig. 2B). To confirm the decrease in the PI molecular species, parent ion scans of 241 m/z (collision-induced inositol-1,2-cyclic phosphate fragment) were recorded (Fig. S2). In extracts of un-induced cells all major PI species are clearly detected (Fig. S2A). In contrast, in extracts from parasites after 48 h of tetracycline induction the

intensities of these fragments are drastically decreased (Fig. S2B). In addition, the amounts of inositol-containing phospholipIds were quantified by measuring the *myo*-inositol contents in lipid extracts from control and induced cells and normalizing to cell numbers. The results show that RNAi cells after down-regulation of TbHMIT only had 79.5 ± 2.5% of *myo*-inositol-containing lipids compared to non-induced cells (100 ± 4%; mean values ± standard deviations from three independent experiments).

In the induced cells, apart from the reduction in PI species, the intensities of two phospholipid species at 762 and 795 m/z had increased (Fig. 2B). These two species were subjected to fragmentation (Fig. S3A and Fig. S3B, respectively) and identified as PS 34:0 and PG 38:5, respectively. The only other obvious change in the phospholipids was observed in positive ion mode (Fig. S4), which shows the choline-phosphate containing species of phosphatidylcholine (PC) and sphingomyelin (SM). The species at 794 m/z, representing PC 40:4, is clearly decreased in cells after TbHMIT RNAi compared to un-induced cells (compare Fig. S4A with Fig. S4B). The cells are obviously trying to compensate for a lack of PI, but the reasons for these specific changes are unknown, other than to maintain the correct membrane fluidity for normal cellular functions.

Localization of TbHMIT in T. brucei bloodstream forms – Immunofluorescence microscopy revealed that ectopically expressed HA-tagged TbHMIT in *T. brucei* bloodstream forms localized to a distinct intracellular structure located between the nucleus and the kinetoplast (Fig. 3). The signal completely co-localized with TbGRASP (Fig. 3), a marker for the Golgi in *T. brucei* (34). These findings are in line with a previous report showing that TbHMIT localizes to the Golgi in *T. brucei* procyclic forms (14).

The Golgi localization of TbHMIT in *T. brucei* bloodstream forms is noteworthy. It is believed that PI synthesis occurs on the cytosolic side of the ER (10, 35). It should be noted, however, that in several studies in plants (11), yeast (36), protozoa (13) and mammalian cells (12), PI synthase was found to localize not only in the ER, but also in close proximity to or in the Golgi. As has been demonstrated in *T. brucei* (13, 14), PI synthesis in the ER and Golgi may serve different purposes. PI production in the ER is required for GPI synthesis, while PI produced in the Golgi provides bulk PI for membrane formation. Based on the localization of TbHMIT in the Golgi, these results suggest that the last step in PI synthesis, i.e. the attachment of *myo*-inositol to CDP-diacylglycerol (CDP-DAG), may occur in the lumen of the Golgi. Interestingly, a recent report showed that *T. brucei* CDP-DAG synthase localizes to the ER/Golgi (37), which would be consistent with PI synthesis taking place in the lumen of the ER and Golgi. In addition, membrane topology prediction programs indicate that the active site of *T. brucei* PI synthase is on the luminal side of the ER. Based on these observations we propose the following model for compartmentalization of PI synthesis in *T. brucei* (Fig. 4). In procyclic forms, *myo*-inositol is taken up from the environment via plasma membrane localized TbHMIT; in bloodstream forms, *myo*-inositol uptake likely occurs via a different transporter. Cytosolic *myo*-inositol is then transported into the Golgi via TbHMIT, where it is used for bulk PI and, subsequently, IPC synthesis. In contrast, *de novo* synthesis of *myo*-inositol starts with the cytosolic conversion of glucose-6-phosphate to inositol-3-phosphate, which in turn is transported into the ER via an unknown transporter. After dephosphorylation, newly synthesized *myo*-inositol is used for PI formation by PI synthase. Subsequently, PI is translocated from the luminal to the cytosolic face of the ER, where it is used to initiate GPI synthesis. Recently, a similar mechanism has also been proposed to occur

352 in the intra-erythrocytic stage of the malaria parasite, *Plasmodium falciparum* (38). In
353 addition, it is worth mentioning that the two PI synthase isoforms described in
354 *Arabidopsis thaliana* show different substrate specificities (11), suggesting that two
355 pools of PI may exist and that these may enter alternative routes of metabolism.
356 Furthermore, a Golgi localization of PI synthase (11-12, 36) and HMIT (8) has also
357 been documented in other cells. Thus, we propose that PI formation and metabolism
358 may be similarly compartmentalized in other eukaryotes as well.

359 Our data using exogenously added *myo*-[^{3}H]inositol showed that after depletion
360 of TbHMIT by RNAi, uptake of *myo*-[^{3}H]inositol into bloodstream form parasites still
361 occurred, albeit at a clearly reduced level (Fig. 1B), yet the formation of [^{3}H]PI was
362 blocked (Fig. 1C). Together with the observed localization of TbHMIT in the Golgi,
363 these data suggest that *myo*-[^{3}H]inositol may be taken up in *T. brucei* bloodstream
364 forms via a different transporter, located in the plasma membrane, and subsequently
365 remains metabolically inactive because of the lack of the Golgi-localized TbHMIT,
366 preventing entry of (cytosolic) *myo*-[^{3}H]inositol into the Golgi for [^{3}H]PI production.
367 These results differ slightly from our previous findings in *T. brucei* procyclic forms
368 (14), where RNAi against TbHMIT not only blocked [^{3}H]PI formation but also *myo*-
369 [^{3}H]inositol uptake into parasites. Interestingly, in procyclic forms TbHMIT is not
370 only present in the Golgi but can also be detected in the plasma membrane, mediating
371 *myo*-[^{3}H]inositol uptake into the cell. We are currently addressing these differences
372 between bloodstream and procyclic forms with further experiments.

373 Collectively, these results demonstrate that expression of TbHMIT is essential for
374 normal growth of *T. brucei* bloodstream forms in culture and that it is involved in
375 *myo*-inositol transport into the Golgi for PI formation within the lumen of the Golgi.

Substrate Specificity of TbHMIT – Perfusion of TbHMIT-expressing *Xenopus laevis* oocytes with 200 µM *myo*-inositol resulted in inward currents of 20 ± 1 nA (mean value ± standard deviations using 13 oocytes from two independent batches). The same concentration of *myo*-inositol did not elicit any currents in water-injected control oocytes. These results are consistent with a previous report (14). To identify other potential substrates, oocytes expressing TbHMIT were exposed to a series of commercially available compounds that are structurally related to *myo*-inositol (Fig. 5). The currents elicited by these compounds (each applied at a concentration of 200 µM) were compared to those obtained with *myo*-inositol (Fig. 6). Interestingly, we found that two quercitol isomers, *epi*- and *vibo*-quercitol, elicited currents comparable to those induced by *myo*-inositol (76 ± 9% and 83 ±3%, respectively, of the *myo*-inositol current). In contrast, <5% of the current obtained with *myo*-inositol was elicited by another quercitol isomer, *proto*-quercitol (Fig. 6). Quercitols comprise a group of 6-C-containing polyols, which compared to the group of inositol isomers lack one hydroxyl group (Fig. 5) and in the case of *proto*-quercitol, contain a C1 epimerisation. In addition, small currents (15-25% of the *myo*-inositol current) were also obtained with *scyllo*-inositol, *epi*-inositol, 1L-*chiro*-inositol and phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate). In contrast, the currents induced by *muco*-inositol, *allo*-inositol, 1D-*chiro*-inositol, 1L-*epi*-2-inosose (2L-2,3,4,6/5-pentahydroxycyclohexanone), L-quebrachitol (2-O-methyl-L-*chiro*-inositol), D-pinitol (3-O-methyl-D-*chiro*-inositol), N-00601 ((1R,4S)-6-methoxycyclohexane-1,2,3,4,5-pentol), N-50350 ((1R,3S)-6-methoxycyclohexane-1,2,3,4,5-pentol) and D-inositol-3-phosphate were <5% of the control *myo*-inositol current (Fig. 6).

Together, these results provide an insight into the selectivity of the transporter in terms of functional groups and stereoselectivity. Among the different analogs tested,

deleting a single hydroxyl group at the 1 (as in (-)-*vibo*-quercitol) or 6 position (as in (+)-*epi*-quercitol) only induced a small decrease in current activity. Inversion of stereochemistry at a single hydroxyl group relative to *myo*-inositol was also partly tolerated, leaving 10-25% of current activity as in *scyllo*-inositol, *epi*-inositol, 1L-*chiro*-inositol and 1D-*chiro*-inositol. However a double modification of inositol in terms of inversion of stereochemistry and substitution (deoxy- or methyl ether) essentially suppressed all current activity, as in quebrachitol, (+)-*proto*-quercitol, *muco*-inositol, *allo*-inositol and D-pinitol. Note that 1L-*epi*-2-inosose, which features a carbonyl group at position 4 of inositol, showed no transporter activity, and that simple carbohydrates such as glucose, galactose and mannose, which are also structurally related to inositol, were not accepted by the transporter (see (14)). In addition, all compounds were analysed for their potential to inhibit TbHMIT-mediated *myo*-inositol transport in *Xenopus* oocytes. Co-application with *myo*-inositol showed that none of compounds affected the *myo*-inositol-elicited currents, demonstrating that at the concentrations tested they did not act as inhibitors of TbHMIT. Finally, to establish the apparent affinities of TbHMIT for transport of *epi*- and *vibo*-quercitol, *Xenopus* oocytes were exposed to increasing concentrations of the compounds and currents were recorded. The results showed that the EC_{50} values for *epi*- and *vibo*-quercitol (121 µM and 104 µM, respectively; mean values from 2 independent experiments) were in the same range as that for *myo*-inositol (61 µM; see (14)).

Together, these data show that TbHMIT is remarkably selective for *myo*-inositol. It tolerates a single modification on the inositol ring only, such as the removal of a hydroxyl group at the 1- or 6-position or the inversion of stereochemistry at a single hydroxyl group relative to *myo*-inositol, but no additional modifications. Interestingly,

426 TbHMIT (14) and its orthologs from *T. cruzi* (20) and *Leishmania* parasites (18, 39)
427 show no transport activity for monosaccharides. This is in marked contrast to all other
428 members of the GLUT/SLC2A family, including the intracellularly located members
429 of the subclass III, which transport many different monosaccharides (1, 5, 6). In
430 addition, HMIT's transport specificity is also different from that of the SMITs, which
431 transport both *myo*-inositol and monosaccharides (2, 3).
432

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ABBREVIATIONS

ER, endoplasmic reticulum; ES-MS, electrospray-mass spectrometry; GPI, glycosylphosphatidylinositol; HA, hemagglutinin; HMIT, proton-linked myo-inositol transporter; IPC, inositol phosphorylceramide; MS/MS, tandem mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SMIT, sodium/*myo*-inositol cotransporters; TLC, thin layer chromatography

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FIGURE LEGENDS

Figure 1. Essentiality of TbHMIT in *T. brucei* bloodstream forms. (A) Growth of *T. brucei* bloodstream form RNAi parasites. Expression of TbHMIT in *T. brucei* bloodstream forms was down-regulated by tetracycline-inducible RNAi, and growth of trypanosomes was monitored for 5 days. Data points represent cumulative cell numbers of RNAi cells incubated in the absence (filled symbols) or presence (open symbols) of tetracycline and correspond to mean values ± standard deviations from three experiments using clone A3. The *inset* shows a Northern blot analysis of total RNA extracted from trypanosomes after 2 days of incubation in the absence (-) or presence (+) of tetracycline (Tet) and probed with [^{32}P]-labeled oligonucleotides used as inserts for the respective stem-loop vector (*top panel*); rRNA was stained with ethidium bromide and used as loading control (*bottom panel*). Two other RNAi clones showed similar growth defects and reductions in TbHMIT mRNA levels upon treatment with tetracycline. (B) *myo*-inositol uptake by *T. brucei* bloodstream forms. After 2 days of incubation in the absence (filled symbols) or presence (open symbols) of tetracycline, RNAi parasites (clone A3) were washed and subsequently incubated with trace amounts of *myo*-[^{3}H]inositol (50 nM, final concentration). After indicated times, parasites were washed, and the amount of radioactivity in the cell pellet was measured. Uptake of *myo*-[^{3}H]inositol at each time point was calculated and plotted as a function of incubation time. Data points represent mean values ± standard deviations of triplicate determinations from three independent experiments. (C) Analysis of *myo*-[^{3}H]inositol-labeled lipids. *T. brucei* bloodstream form TbHMIT RNAi cells (clone A3) were incubated in the absence or presence of tetracycline for 3 days. During the last 16 h of incubation, parasites (2 x 10^{8} cells) were labeled with 25 µCi of *myo*-[^{3}H]inositol. [^{3}H]-labeled lipids were extracted from parasites incubated

596 in the absence (top panel) or presence (middle panel) of tetracycline to down-regulate
597 TbHMIT, separated by one-dimensional TLC using solvent system 1, and visualized
598 by scanning the plate (14). Extracts from equal cell equivalents were applied. The
599 bottom panel represents extracts from *T. brucei* procyclic forms run on the same TLC
600 plate for comparison, indicating the migration of [^{3}H]inositol phosphorylceramide
601 ([^{3}H]IPC) and [^{3}H]phosphatidylinositol ([^{3}H]PI). The vertical lines indicate the site of
602 sample application (left line) and the migration of the solvent front (right line),
603 respectively. (D) Analysis of [^{3}H]ethanolamine-labeled GPI lipids. Trypanosomes
604 were cultured in the absence (top panel) or presence (middle panel) of tetracycline for
605 2 days to down-regulate TbHMIT, and then labeled during 16 h with
606 [^{3}H]ethanolamine. [^{3}H]GPI lipids were extracted and analyzed by TLC using solvent
607 system 2. The major GPI lipids, designated P2 and P3, were identified based on
608 published R_f values (28) and the migration of [^{3}H]-labeled PP1, i.e. the major GPI
609 precursor lipid in *T. brucei* procyclic forms, run on the same plate (bottom panel). The
610 migration of residual [^{3}H]PE is also indicated. The vertical lines indicate the site of
611 sample application (left line) and the migration of the solvent front (right line),
612 respectively.

613 **Figure 2. Phospholipid analysis of *T. brucei* bloodstream form TbHMIT RNAi**
614 **cells.** Lipid extracts from parasites incubated in the absence (A) or presence (B) of
615 tetracycline for 48 hours were analyzed by negative ion ES-MS survey scans (600-
616 1000 m/z (mass/charge)). The major phospholipid classes are annotated: PI,
617 phosphatidylinositol; PE, phosphatidylethanolamine; EPC, ethanolamine
618 phosphorylceramide. The arrows in (B) refer to the phospholipid species that increase
619 or decrease after RNAi against TbHMIT compared to non-induced cells (A).

Figure 3. Localization of TbHMIT in *T. brucei* bloodstream forms. Trypanosomes cultured in the presence of tetracycline for 24 h to induce expression of HA-tagged TbHMIT were washed, allowed to settle onto microscope slides, fixed with paraformaldehyde, and permeabilized with Triton X-100. TbHMIT was detected using anti-HA antibody (first panel) whereas the Golgi was stained with anti-GRASP antibody (second panel). The third panel shows an overlay of panels A and B, with DNA stained with DAPI. The corresponding differential interference contrast (DIC) micrograph is shown in the fourth panel.

Figure 4. Schematic of compartmentalized *myo*-inositol metabolism in *T. brucei*. For details see main text. Glc-6-P, glucose-6-phosphate; GPI, glycosylphosphatidylinositol; Ins, inositol; Ins-3-P, inositol-3-phosphate; IPC, inositolphosphoryl ceramide; PI, phosphatidylinositol; TbHMIT, *T. brucei* H^+-coupled *myo*-inositol transporter; GlcNAc-PI, N-acetylglucosaminyl phosphatidylinositol; GlcN-PI, glucosamine phosphatidylinositol; TbPIS, *T. brucei* PI synthase; TbSLS1, *T. brucei* sphingolipid synthase 1; TbINO1, *T. brucei* 1-D-*myo*-inositol-3-phosphate synthase; TbIMPase, *T. brucei* inositol monophosphatase.

Figure 5. Structures of the various inositols and analogs tested in this study. The numbering refers to the parent *myo*-inositol numbering; the positions and substitutions with a change relative to *myo*-inositol are indicated with the carbon number in blue and highlighted in red color.

Figure 6. Substrate specificity of TbHMIT. *Xenopus* oocytes expressing TbHMIT were exposed to 200 µM *myo*-inositol and a series of structurally related compounds (all at 200 µM) and the currents were recorded. The bars indicate currents relative to the response elicited by *myo*-inositol (mean values ± standard deviations from at least

644 3 determinations). N-00601: (1R,4S)-6-methoxycyclohexane-1,2,3,4,5-pentol; N-

645 50350: (1R,3S)-6-methoxycyclohexane-1,2,3,4,5-pentol.

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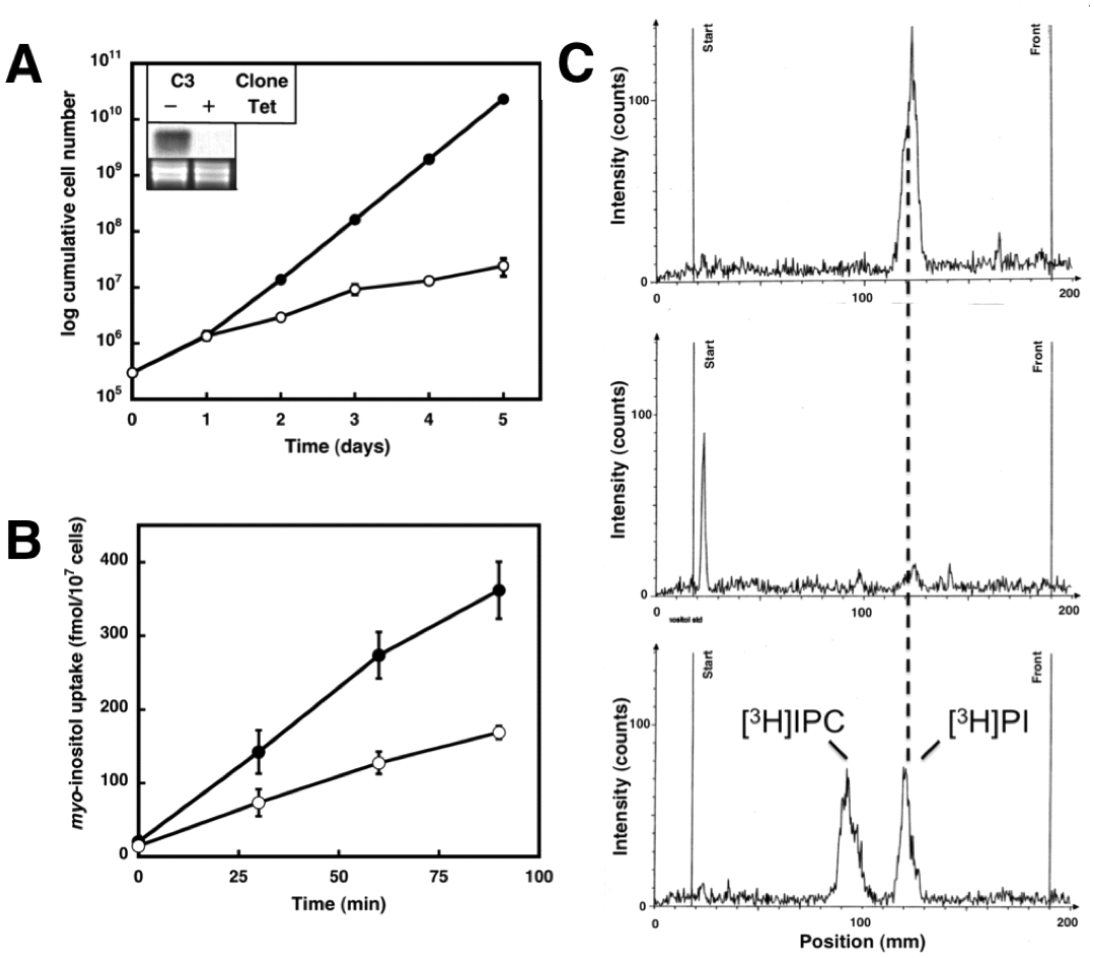

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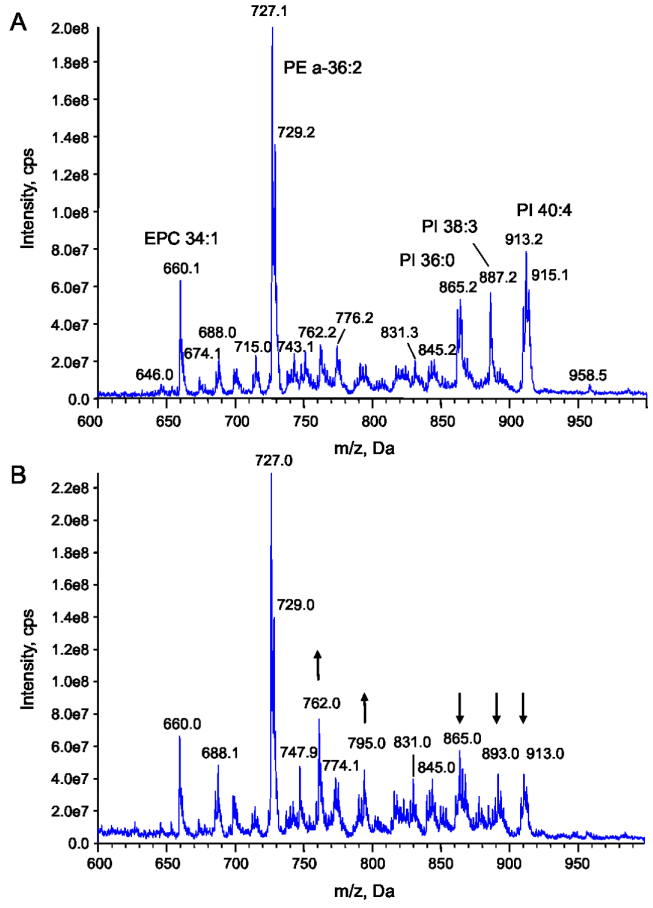

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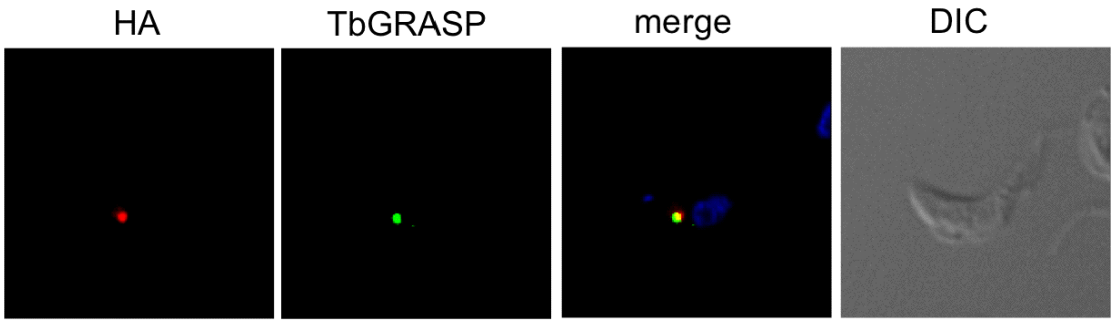

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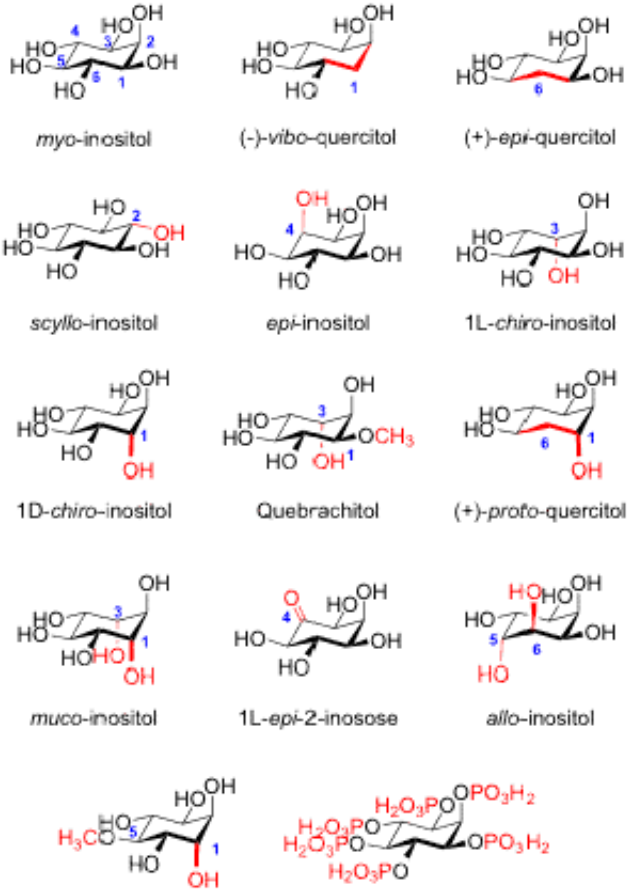
myo-inositol
(-)-vibo-quercitol
(+)-epi-quercitol
scyllo-inositol
epi-inositol
1L-chiro-inositol
1D-chiro-inositol
Quebrachitol
(+)-proto-quercitol
muco-inositol
1L-epi-2-inosose
allo-inositol
D-pinitol
phytic acid

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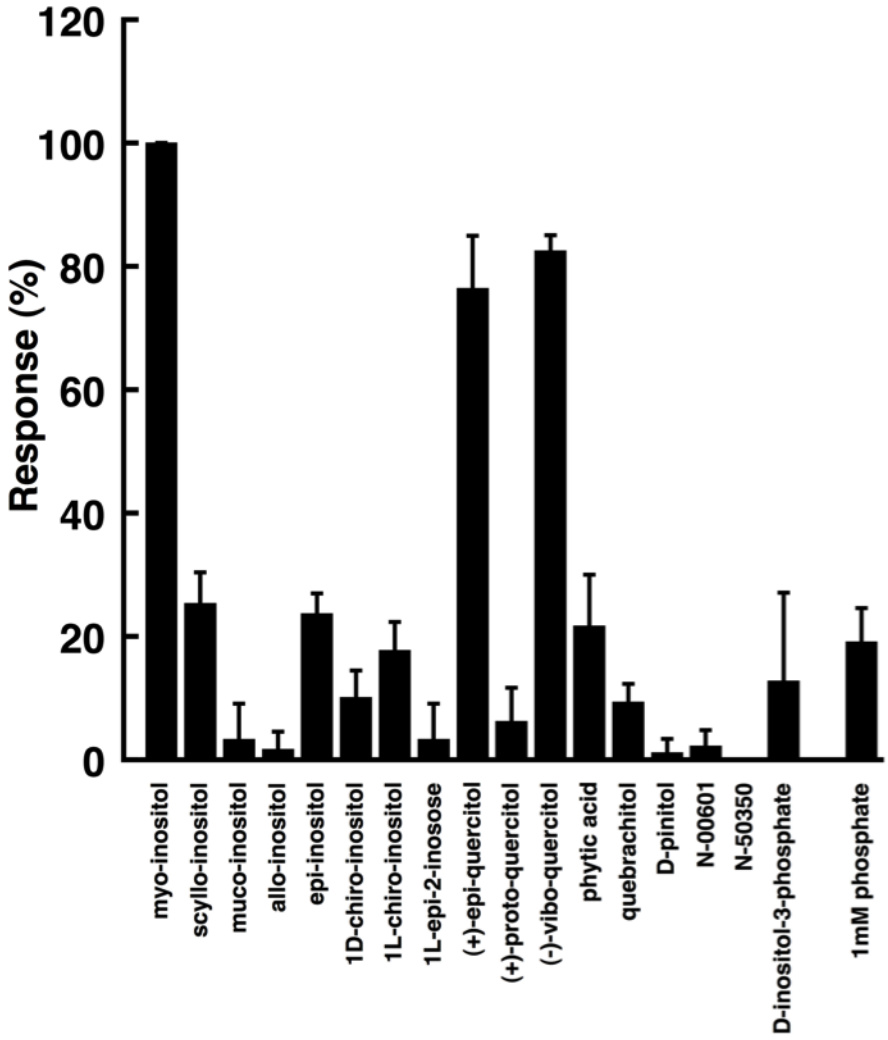

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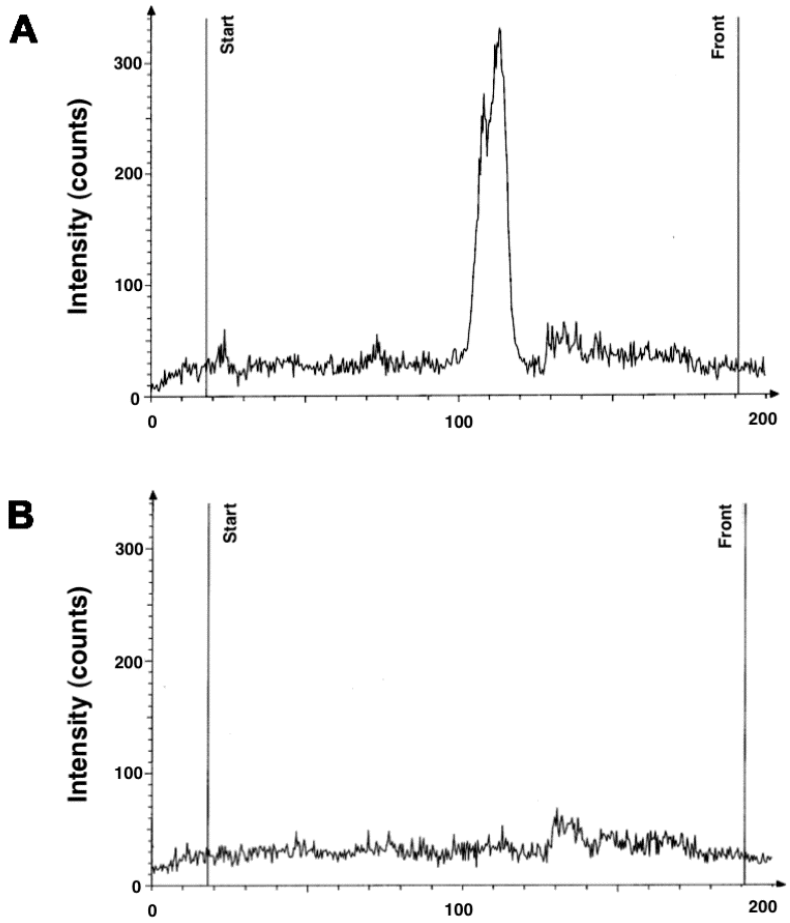
655 **SUPPLEMENTARY FIGURES**

656



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659 **Figure S1. Susceptibility of [^{3}H]-labeled lipids to PI-PLC.** [^{3}H]-labeled lipids were
660 extracted from *T. brucei* bloodstream forms, incubated in the absence (upper panel) or
661 presence (lower panel) of PI-PLC, and separated by one-dimensional TLC using
662 solvent system 1. Radioactivity was detected by scanning the plate.

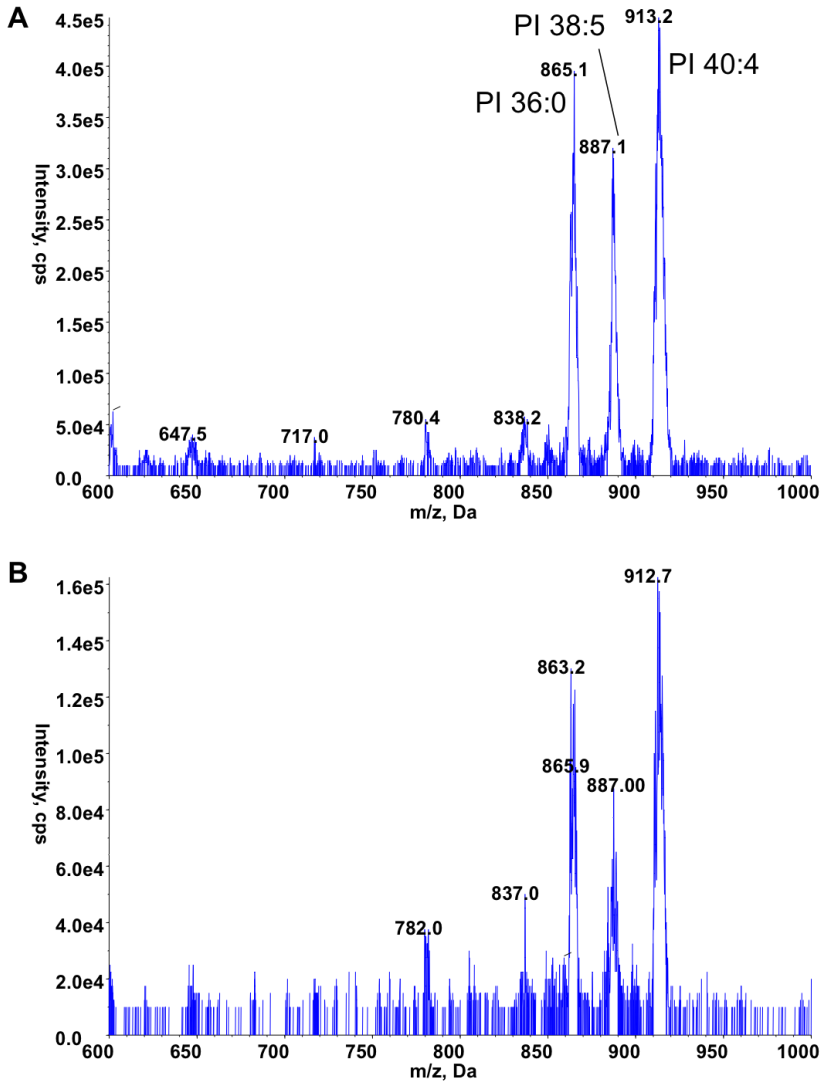

Figure S2. Lipidomic analysis of inositol containing phospholipids from *T. brucei* TbHMIT RNAi knock-down cells by ES-MS-MS. Lipid extracts from bloodstream form parasites incubated in the absence (A) or presence (B) of tetracycline for 48 hours were analyzed by negative ion ES-MS-MS utilising parent ion scans of 241 (600-1000m/z).

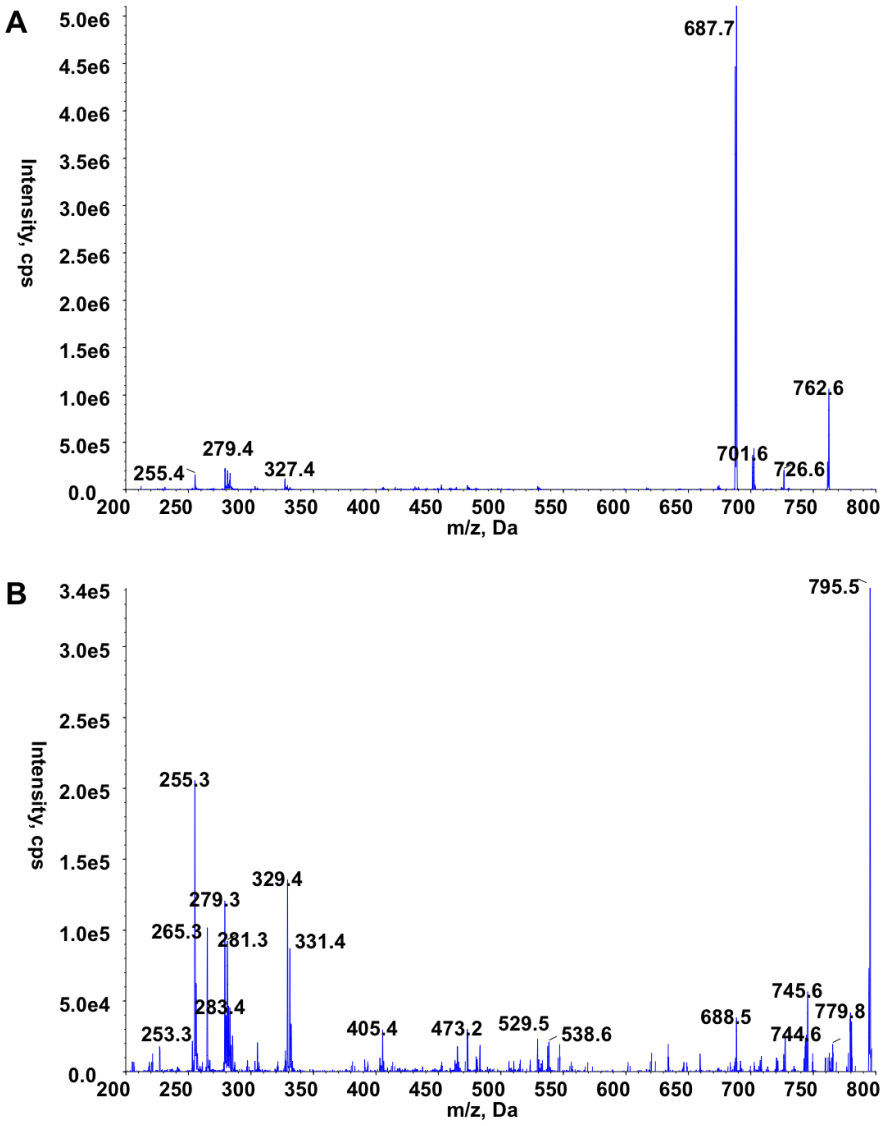

Figure S3. **Fragmentation of phospholipid species m/z 763 (A) and m/z 795 (B) from *T. brucei* TbHMIT RNAi knock-down cells by ES-MS-MS.** Fragmentation reveals that (A) is mainly phosphatidylserine (34:0) and (B) is mainly phosphatidylglycerol (38:5).

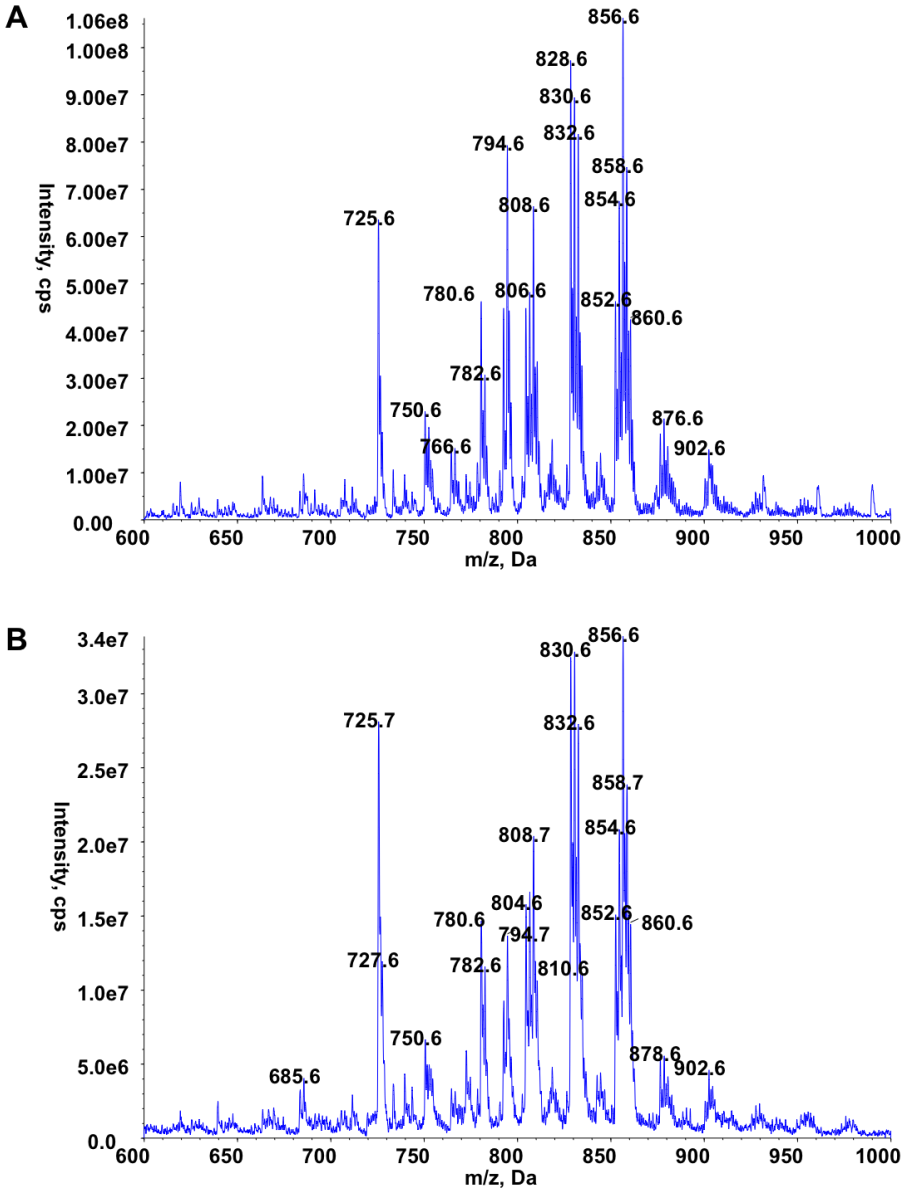

Figure S4. Positive ion ES-MS lipidomic analysis of *T. brucei* TbHMIT RNAi knock-down cells. Lipid extracts from bloodstream form parasites incubated in the absence (A) or presence (B) of tetracycline for 48 hours were analyzed by positive ion ES-MS survey scans (600-1000m/z).