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ABSTRACT

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Trypanosoma brucei is an extracellular kineotoplastid parasite that causes human African trypanosomiasis (HAT), also known as sleeping sickness. As trypanosomes undergo vector to host transition, heavy transcriptional adaptation such as metabolic shift to glycolysis and upregulated endocytosis occurs. Specifically, glycolysis in the infectious stage becomes the sole source of energy production; thus, the glucose transport mechanism in *T. brucei* provides one of the most promising therapeutic targets for development of new drugs to treat HAT. Despite an established trypanosome hexose transporter (THT) model for glucose transport across the plasma membrane, there remains gaps in the detailed mechanism of glucose transport especially as it relates to glucose transport across the glycosomal membrane. Using 2-NBDG, a fluorescent glucose analog, we measured glucose uptake rates in the presence of small molecule inhibitors and by using RNA interference (RNAi) to knockdown key proteins to investigate the mechanism of glucose transport in trypanosomes. We have confirmed a direct role of THT in glucose transport of BSF trypanosomes; however, in our investigations, we observed an unexpected ATP-dependence on glucose transport in live trypanosomes, which initiated further study where we focused on the role of endocytosis as an ATP-coupled bulk glucose transport mechanism. Experimental approaches that inhibited endocytosis reduced the observed glucose uptake rate confirming a role for endocytosis-coupled glucose transport in BSF trypanosomes. We provide evidence for an endocytosis-coupled glucose transport mechanism in BSF trypanosomes as an additional and important mechanism that functions in parallel with the established THT model.

Keywords: *Trypanosoma brucei*, sleeping sickness, trypanosome hexose transporter, THT, glucose transport, endocytosi

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1. Introduction

1.1 Kinetoplastids

T. brucei is an extracellular kinetoplastid parasite. Having diverged early in eukaryotic evolution, kinetoplastids are a group of flagellated protists that are characterized by a unique organelle named the kinetoplast that contains many copies of the mitochondrial DNA or kDNA [1],[2],[3]. Kinetoplastids include disease-causing parasitic species such as *Leishmania donovani, Trypanosoma cruzi, and Trypanosoma brucei*[4]. *Leishmania donovani* is an intracellular parasite transmitted by species of sand fly throughout tropical and temperate regions including Africa, China, India, Europe and South America and causes visceral leishmaniasis, also known as the black fever. *Trypanosoma cruzi* is transmitted by the feces of the "kissing bug" in South and Central America and causes Chagas disease. *Trypanosoma brucei*, the organism of our study, is transmitted by tsetse flies in Sub-Saharan Africa and is a causative agent of human African trypanosomiasis, or African sleeping sickness and animal trypanosomiasis, also called nagana in cattle and horses[4]. *T. brucei* is divided into three major subspecies: *T. b. gambiense, T. b. rhodensiense,* and *T. b. brucei*. Human infection occurs via *T. b. gambiense, and T. b. rhodensiense,* whereas *T. b. brucei* infects only non-primate mammals. Notably, infection by *T. b. rhodensiense* causes rapid manifestation of trypanosomiasis symptoms. *T. b. gambiense,* on the other hand, causes a chronic infection that goes unnoticed for a long time in the majority of cases but still accounts for 95% of reported cases [4]. *T. b. brucei*, in contrast to the two human pathogens, is susceptible to Trypanosome Lytic Factor (TLF), a high-density lipoprotein in human blood and possess no pathogenic properties in humans [5]. Thus, with similar genetic make-ups amongst three subspecies, *T. b. brucei* serves as a model organism for studies in many laboratories.

1.2 *T. brucei* lifecycle

T. brucei has a complex lifecycle. As a fly takes a blood meal from an infected individual, bloodstream form (BSF) trypanosomes in the mammalian host transition into a proliferative procyclic form (PF) trypanosomes in the midgut of the fly. PF trypanosomes in the midgut then rapidly divide to become epimastigotes, which eventually arrest their cell cycles and migrate toward the salivary gland of the fly where they become the infective metacyclic trypomastigotes. Starting as the long slender form of BSF upon initial inoculation in the mammalian host, BSF trypanosomes proliferate and transition into dormant stumpy form arrested in G1 phase, which then transition back into the midgut of the tsetse fly for the next full cycle [6]. Because of the drastic environmental changes that trypanosomes face in their life cycle, trypanosomes change and adapt their morphology and their transcriptional regulation to best suit their environment. PF trypanosomes possess a fully functional mitochondrion and utilize proline and other amino acids as well as glucose for their metabolism. Their cell surface is also covered with a dense proline-rich acidic repetitive protein, named procyclin that are linked by a glycosylphosphatidylinositol (GPI) anchor [7]. Once transitioned to the bloodstream, however, trypanosomes lose their functional mitochondrion and convert their energy production solely to glycolysis [8-12]. The procyclin covering the surface of PF cells is also replaced with highly immunogenic VSG molecules that are constantly recycled in BSF cells. BSF cells harbor more than 1000 different copies of VSG in the genome, of which only one variant is expressed at once [13, 14]. By having more than one variant being expressed at once in mixed population, BSF cells negate the existing antibodies that recognize the specific VSG antigen [15].

1.3 *T. brucei* differentiation

Up to 25% of all transcripts are expected to be regulated during the differentiation of trypanosome [16-18]. In addition, the differentiation from BSF to PF is triggered by the environmental change from the mammalian host to the fly. In vitro, however, the differentiation can be triggered by the addition of *cis*-aconitate facilitated by both the *cis*-aconitate transporters and temperature shift from 37°C to 29°C [19, 20]. Notably, PF differentiation from BSF can also be induced by the removal of glucose in the media, of which inhibition of glucose transporters by phloretin was also shown to have similar effect [21, 22].

Trypanosomes express two families of its surface-associated hexose transporters for its glucose uptake: THT1 and THT2 [23, 24]. In PF cells, which do not require glucose for their survival, THT2 is predominantly expressed with very little THT1. Upon introduction to the bloodstream of the mammalian host, where rich glucose is available, THT1 becomes the dominant form of hexose transporter, with downregulated THT2 expression in BSF cells [24].

1.4 Glycolysis in *T. brucei*

BSF trypanosomes rely exclusively on glycolysis for its ATP production, making a constant 5mM glucose concentration in the bloodstream a perfect environment to thrive [25]. In contrast, the insect stage of PF trypanosomes is capable of producing ATP through both the glycolysis pathway as well as the respiratory Krebs cycle [26, 27]. Nevertheless, glycolysis in both lifecycle stages of trypanosomes occurs in a unique peroxisome like-organelle called the glycosome [28]. In the glycosome, where more than 90% of the protein content consists of glycolytic enzymes, the first several steps of glycolysis take place to produce 3-phosphoglycerate (3-PGA). Further degradation of 3-PGA to pyruvate then occurs in the cytosol [29, 30]. Under aerobic conditions, the NADH produced in glycolysis is used to reduce dihydroxyacetone

phosphate (DHAP) to glycerol 3-phosphate (Gly-3-P). Gly-3-P is subsequently reoxidized to DHAP by molecular oxygen via glycerol-3-phophate oxidase (GPO) in the mitochondria to generate proton-motive force in the oxidative phosphorylation process. DHAP is then returned back to glycosomes [31]. Under anaerobic condition, on the other hand, Gly-3-P is converted to glycerol in exchange for one ATP molecule in the glycosome. Glycerol is then excreted into the cytosol [32]. Interestingly, phosphoglycerate kinase (PGK), the last enzyme in producing 3-PGA, is localized in the glycosomes in BSF, while PF harbors PGK in the cytosol [33]. In glycosomes, there is no net production of ATP; 2 ATP molecules are produced by PGK and subsequently balanced by hexokinase (HK) and phosphofructokinase (PFK) ATP consumption. In the cytosol, however, the final product of glycosomal glycolysis, 3-PGA, is metabolized into pyruvate via pyruvate kinase (PYK), which results in net one ATP production. As a result, aerobic conditions in BSF produces 2 molecules of ATP per glucose molecule through reoxidation of NADH by trypanosome alternative oxidase and glycerol-3-phosphate dehydrogenase, while anaerobic condition produces one molecule of ATP per glucose molecule in BSF [25].

1.5 *T. brucei* glucose transport

1.5.1 Glucose transport as the rate-limiting step in glycolysis

The importance of glucose for survival of BSF parasites, glucose transport in *T. brucei* has been extensively studied. First, glucose transport in BSF trypanosomes has been proposed to be a non-energy dependent carrier-mediated pathway and not free diffusion [34, 35]. Moreover, the glucose transport step has been further suggested as the rate-limiting step of glycolysis in BSF trypanosomes [36]. The flux control coefficient of glucose transport across the membrane has been identified to be between 0.3 and 0.5 at the normal 5 mM glucose concentration in blood [37]. When

the external glucose concentration is lower than 5 mM, the glycolytic rate does not exceed rate at which the BSF cells take up glucose through its plasma membrane; hence, the glucose transport step is the rate-limiting step only at external glucose concentrations lower than 5 mM. When external glucose concentration is higher than 5mM, the glycolytic rate becomes overtaken by rate at which hexokinase phosphorylates its glucose substrate or the uptake to the glycosomes [31].

1.5.2 Substrate specificity of hexose transporter

Extensive characterization of the structure-activity relationships of glucose transport in trypanosomes has been done [35, 38, 39]. The substrate specificity of BSF transporters demonstrates that hydroxyl groups located on C-3 and C-4 are important, while C-2 and C-6 are more lenient on the transport [38]. Though not as strict in terms of substrate specificity, PF transporters also show preference for the C-3 hydroxyl group [39]. Significant structural differences in trypanosome glucose transporters can be identified by comparison with the mammalian glucose transporter GLUT1. In addition to the substrate recognition ability which allows transport of D-fructose in the trypanosome transporter, pharmacological differences between the transporters demonstrates a 1000-fold stronger inhibition by cytochalasin B and phloretin on GLUT1 compared to parasite counterparts [33]. Despite the unique biology of trypanosomes compared to the mammalian host, no viable drugs targeted against the trypanosome glucose transporter other than cytochalasin B and phloretin have been identified.

1.5.3 Trypanosome hexose transporters (THT)

Two functionally distinct modes of glucose transport are relevant in kinetoplastids: facilitated diffusion and active transport coupled with ion or ATP [40]. Studies done in PF have demonstrated that THT2 has significantly higher affinity for its hexose substrates than THT1.

Furthermore, THT2 activity is identified to be sensitive to proton ionophores such as FCCP at low substrate concentration, suggesting that THT2 may be a proton coupled glucose transport [41]. The effect of proton coupled THT2 transport was, however, not noticed in a higher substrate concentration from a study done later [39]. No effect of proton or ions have been observed in THT1 of BSF cells. In a study done in a reconstituted liposome from BSF cells, glucose transport of THT1 was characterized as an ATP-independent facilitated diffusion with severe inhibition upon cytochalasin B and phloretin treatment [34]. As cytochalasin B and phloretin are well known inhibitors of GLUT1 a facilitated diffusion transporter, the idea that THT1 is a facilitated diffusion carrier has been acknowledged.

1.5.4 Trypanosome hexose transport isoform members

Trypanosome hexose transport (THT) has been universally accepted as the only glucose transporter in *T. brucei*. Sharing a high similarity to Pro-1 glucose transporter from *Leishmania enriettii*, two isoforms of THTs are transmembrane proteins belonging to glucose transporter superfamily exemplified by facilitated diffusion glucose transporter GLUT1 [24]. The notable differential expression of the two different families in each lifecycle stage reflects differences in the availability of environmental glucose in the mammalian host and fly vector. Complicating the assessment of THT function, the *T. brucei* 427 genome harbors five putative copies of each family member (*THT1*-, *THT1E*, *THT1.3*, *THT1.4*, *THT1.5* and *THT2.1*, *THT2.2*, *THT2.3*, *THT2.4*, and *THT2A*) on chromosome 10. Family membership is based on the high predicted sequence identity shared amongst members [42, 43]. In PF parasites, THT1-, THT1.4, THT2.2, and THT2A are the most abundant THT1 and THT2 family member transcripts, respectively. In BSF, on the other hand, THT2.2 and THT2A are the most abundant THT2 family. All THT1 family members are expressed at the similar level in BSF parasites.

1.5.5 Expression and cellular distribution of THT

Differential regulation of the transporters reflects the characteristics of the two different transporters and the environment they are adapted to in regards to glucose. Glucose transporters from *Leishmania mexicana,* a kinetoplastid parasite related to the African trypanosome, are localized to both the flagellum and the pellicular plasma membrane [44, 45]. To assess the localization of representative THT1 and 2 family members in T. *brucei*, THT1E and THT2A were expressed with a C-terminal eYFP fusion and fluorescence and imaged using a confocal microscopy. (Figure 1) Images of cytosolic eYFP and THT1 and THT2 fusion at the coronal plane cross section demonstrate that both THT1 and THT2 fusions are primarily surface-associated, while cytosolic eYFP has punctuation illuminating the whole cytosol. The representative fusion THT proteins reveal that THT is indeed localized on the plasma membrane. The finding demonstrates that THT proteins are transmembrane proteins alike the expected structure and topology of an established facilitated diffusion carrier. These findings parallel those reported from the Tryptag database (Tb927.10.8440, 8450, 8510, 8520).

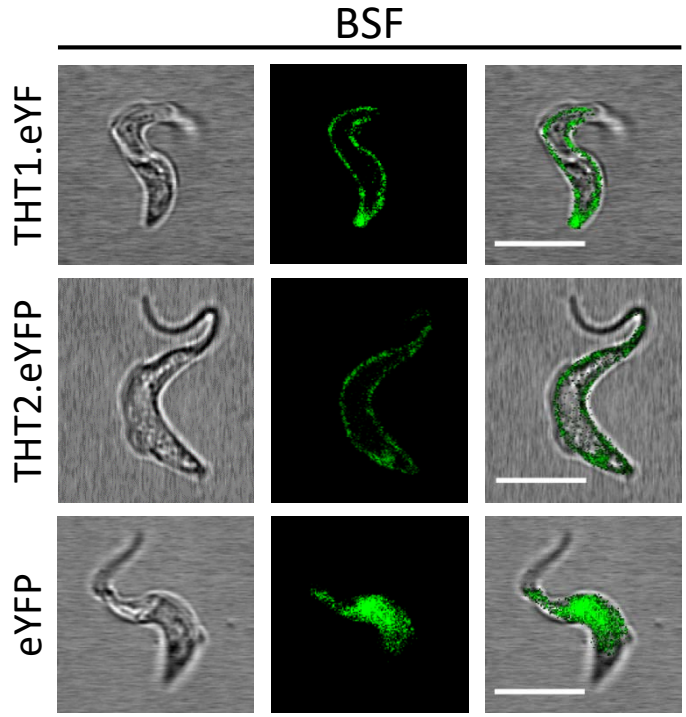

Figure 1. Confocal cross section image of THT eYFP fusions and cytosolic eYFP in BSF cells. Images of BSF cells expressing THT1E.eYFP, THT2A.eYFP, and cytosolic eYFP were fixed with 4% paraformaldehyde PBS solution and imaged on a confocal microscope. Vertical cross section images reveal localization of THT1E.eYFP and THT2A.eYFP fusions are surface associated, while eYFP is localized mainly in the cytosol.

1.6 General role of endocytosis in *T. brucei*

Endocytosis, the ability of cells to internalize extracellular material, is a fundamental property of any eukaryotic cell and crucial for many cellular processes such as nutrient uptake, cell-cell communication, and cellular defense. Subsequently, endocytosis is also essential in trypanosomes [46]. BSF trypanosomes, in fact, have one of the highest endocytosis rates known in all eukaryotes [47, 48]. As an extracellular parasite, the BSF lifecycle stage is under constant pressure from the host's immune system elicited by the immunogenic VSG. As a way to evade the host's immune system, the infectious BSF parasites continuously and rapidly recycle the antibody-VSG complexes through endocytosis and degrade endocytosed VSG-antibody complexes in

lysosomes [49, 50]. *T. brucei* mediates its endocytosis through a clathrin-dependent pathway originating in the flagellar pocket, a small invagination of the plasma membrane in the posterior of the cell. The endocytic rate is equivalent to the internalization of the entire flagellar pocket every 1-2 minutes [47, 48]. With trypanosomes swimming in the anterior direction, the sweeping movement caused by the hydrodynamic flow of the extracellular fluid further enhances localized endocytosis occurring within the flagellar pocket [13]. Clathrin knockdown trypanosomes exhibit no detectable endocytosis, indicating that trypanosomes primarily employ clathrin-mediated endocytosis [46]. Furthermore, inhibition of endocytosis through clathrin knockdown resulted in cell death suggesting the crucial role endocytosis plays in *T. brucei's* survival. In addition, phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], a molecule that plays a crucial function in eukaryotic endocytosis, has also been identified to play a similarly important role in trypanosome endocytosis. Thus, PIP kinase, which is responsible for generating $PI(4,5)P_2$, is an important regulator of endocytosis. Knockdown of PIP kinase leads to depletion of $PI(4,5)P_2$ and enlargement of flagellar pocket resulted from impaired endocytosis [51]. Interestingly, the mammalian adaptor protein 2 (AP-2), which interacts with $PI(4,5)P_2$ and facilitates cargo selection and clathrin pit formation in the mammalian counterparts, is not present in the *T. brucei* genome; instead, the trypanosome homologs AP-1 and AP-3 are speculated to replace AP-2 activity in trypanosomes [52, 53]. Taken together, endocytosis is an essential cellular function in trypanosomes.

1.7 Monitoring metabolites in *T. brucei*

Glucose measurements have been previously made using constitutively expressed Forster Resonance energy transfer (FRET) biosensors in both lifecycle stages of trypanosomes [54]. Using the FRET biosensor, accurate glucose concentrations in both the cytosol and the glycosome were

determined. Furthermore, a molecular pH sensor has been targeted to the glycosome and accurately calibrated to measure the slight acidification of the glycosome upon glucose depletion [55]. The link between pH and glucose implicates a complex glucose regulation. Thus, by non-destructively monitoring important metabolites such as glucose, pH, and ATP in the cytosol and various organelles, a detailed mechanism of glucose regulation in *T. brucei* can be deduced. Our lab has expressed a FRET biosensor in *T. brucei* that is sensitive to ATP cytosolic changes in both BSF and PF trypanosomes. In addition, various biosensors capable of measuring pH, ATP/ADP ratio, and oxidation level are currently being developed to be expressed in trypanosomes. Such biosensors could be used to measure changes in important metabolites and enhance the understanding of glucose transport system in trypanosomes.

2. Experiments - endocytosis coupled glucose transport in T. brucei

2.1 Results

2.1.1 Effect of glucose transporter inhibitors on 2-NBDG uptake

To demonstrate the ability to quantitatively measure altered rates of glucose uptake, cells were incubated with the fluorescent glucose analog 2-NBDG (300μM) and the rate of glucose uptake was scored by measuring the intracellular 2-NBDG fluorescence using flow cytometry following removal of the remaining extracellular 2-NBDG. As a proof of concept, phloretin and cytochalasin B, the only known glucose uptake inhibitors in trypanosomes, were used as positive controls for glucose uptake inhibition. In both lifecycle stages, incubation with phloretin and

cytochalasin B (1mM and 100uM respectively) in HMI9 media resulted in significant decrease in 2-NBDG uptake response as expected (Figure 2).

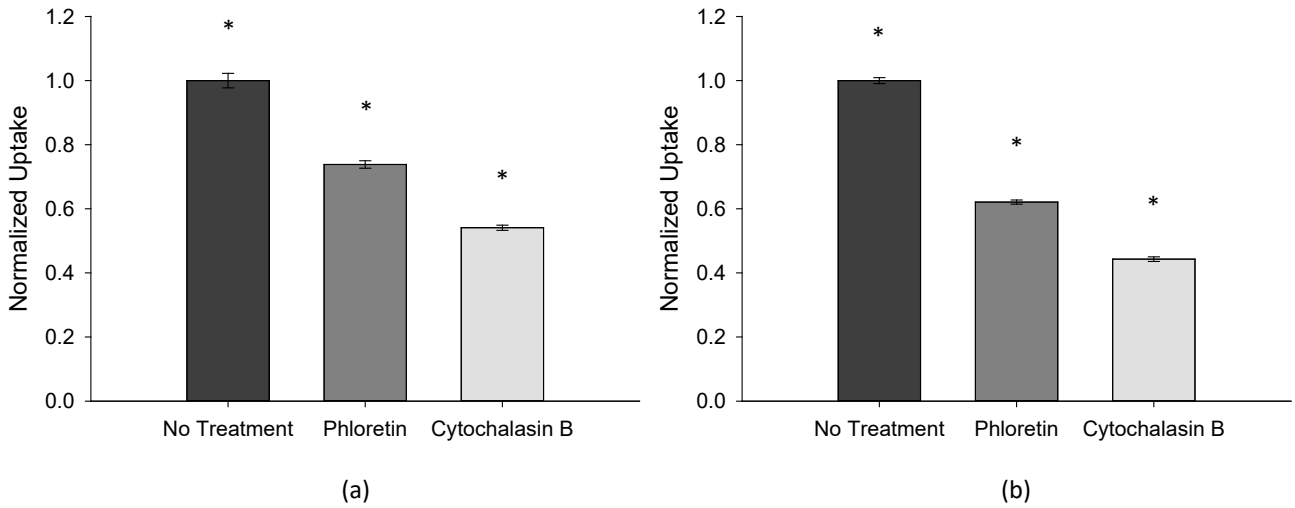

Figure 2. 2-NBDG uptakes in phloretin and cytochalasin B treated BSF and PCF cells. (a) Phloretin and cytochalasin B, known glucose transport inhibitors in *T. brucei,* were used to inhibit 2-NBDG uptake in BSF cells. 2-NBDG uptake was decreased by ~30% and ~50% in phloretin and cytochalasin B (1mM and 100uM) treated cells, respectively. (b) Phloretin and cytochalasin B (1mM and 100uM) also inhibit 2-NBDG uptake in PCF. 2-NBDG uptake was decreased by ~40% and ~50% in PCF cells. Error bars represent the standard deviation for n = 3 replicates. $^{*}p< .05$.

2.1.2 Impact of silencing the THT on transcript abundance and glucose uptake

To demonstrate the glucose transport ability of the THT carriers, both isoforms of THT were silenced using tetracyclin inducible RNAi system in BSF. Silencing THT1 and THT2 individually in BSF parasites led to a significant (50%) transcript abundance reduction in each isoform. Interestingly, silencing THT2 alone in BSF parasites led to a marked 2-fold increase in THT1 transcript suggesting compensatory roles THT1 and THT2 have on each other. THT1, with a low affinity (K_m ~1mM), does the bulk of glucose transport in BSF parasites in glucose rich environment, compared to THT2 with high affinity (K_m ~50 μM) [39, 56]. Inadequate expression level of THT2 in BSF, thus, may force the organism to overexpress the lower affinity form

transporter to overcompensate for the low glucose environment it may encounter. Additionally, simultaneously silencing both THT1 and THT2 yielded in similar reduction of each transcript abundance as to when each isoforms were silenced individually. Surprisingly, RNAi-based silencing of THT1, THT2 or both transporters simultaneously resulted in no detectable impact on growth of BSF parasites. No growth defect in BSF upon silencing both THTs was unexpected, given the absolute reliance of the parasite on glucose for ATP production. Despite the incomplete knockout of the genes, given the essential role of THT as the proposed sole mode of glucose transport, the absolute lack of growth defect observed upon silencing of THT is suggestive of an additional mode of glucose transport mechanism present in BSF parasites.

Furthermore, 2-NBDG uptake scores were measured in BSF cells with both transporters knockdown. Silencing both transporters in BSF parasites resulted in a reduction of 2-NBDG uptake, with a maximum reduction of ~60% after four days of induction (Figure 3). As expected for the established hexose transporter, the reduction in 2-NBDG uptake rate undoubtedly provides evidence for levels of THT's involvement in glucose transport. Nonetheless, assessment of the expected rate of transport inhibition from the RNAi silencing is difficult due to its incomplete

geneknockout. Furthermore, evidence of no growth defect in transporter silenced trypanosomes indicates a need for an additional glucose transport pathway that bypasses the transporters.

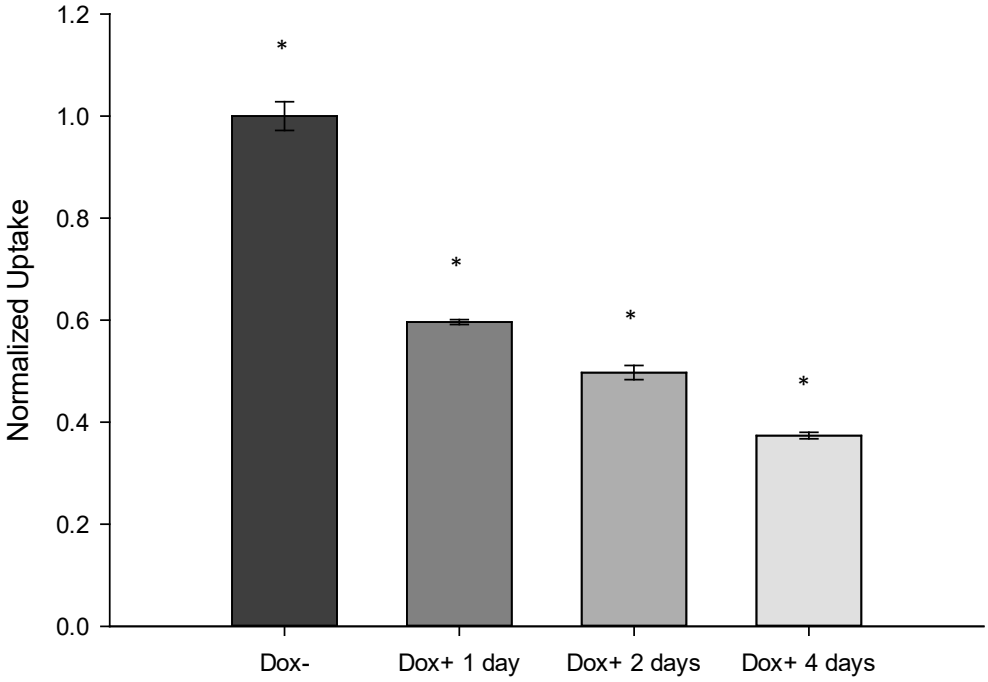

Figure 3. 2-NBDG uptake rates in double THT1 and THT2 knockdown BSF cells. BSF cells were pulsed with 2-NBDG in HMI9 media for 30 minutes and analyzed on flow cytometer. Induction of RNAi to silence THT expression resulted in significant reduction in 2-NBDG uptake with maximum of ~60% reduction in cells induced for 4 days. Decreased 2-NBDG uptake in THT silenced cells provide evidence for THT's glucose transport ability in BSF trypanosomes. Error bars represent the standard deviation for n = 3 replicates. $^{*}p< .05$.

2.1.3 Effect of ATP depletion on glucose uptake

Having observed no growth defect in THT knockdown BSF parasites, detailed study of glucose transport mechanism was conducted in search of an additional glucose transport bypassing the THT carriers. Previous reports have concluded that no co-transported ions or energy requirements (i.e. ATP-dependence) are necessary for glucose uptake in reconstituted membranes from BSF parasites [34]. We began to confirm this part of the existing model of glucose transport

in live cell BSF parasites by assessing the effects of ATP depletion in BSF parasites using 2-NBDG uptake. ATP reduction was achieved by a 30-minute starvation in glucose-free PBS, whereas normal ATP levels were maintained in PBS containing 10mM glucose; the ATP reduction was confirmed by the use of BSF cells expressing the AT1.03^{YEMK} FRET biosensor sensitive to changes in ATP [57]. Cells incubated in glucose-free PBS displayed a decreasing FRET ratio plateauing at ~20 minutes, signifying the reduction of cytosolic ATP level. Cells incubated in PBS containing 10mM glucose, in contrast, displayed a stably high FRET ratio up to more than an hour (Figure 4). Both ATP reduced and normal cells were then washed 2 times in glucose-free PBS and pulsed with 2-NBDG for one minute. In ATP reduced cells, 2-NBDG uptake rate was reduced by 40% in BSF parasites, directly contradicting the previous study done in a reconstituted membrane system in the absence of ATP (Figure 4) [34].

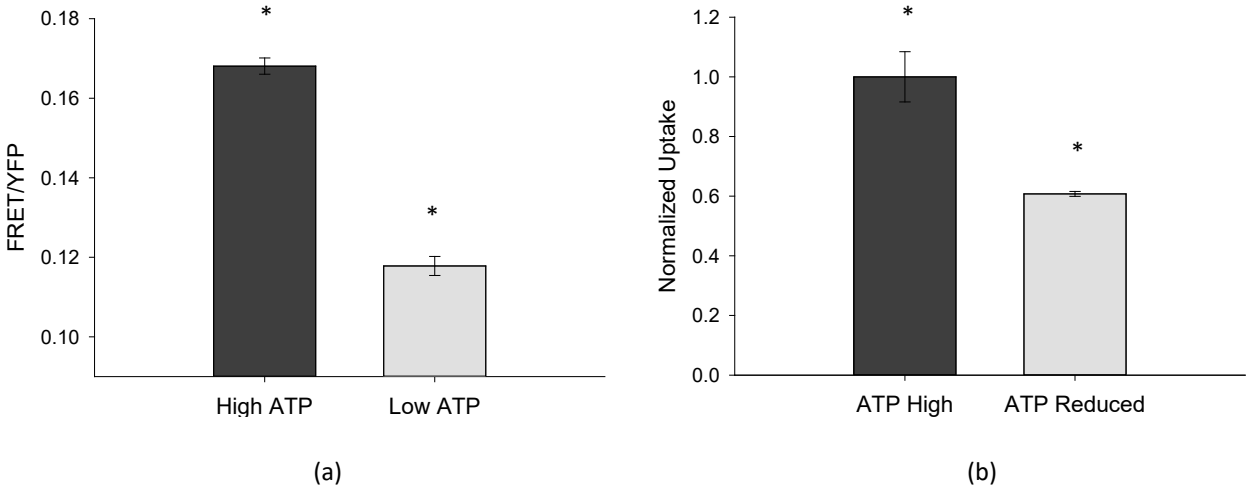

Figure 4. 2-NBDG uptake in ATP depleted BSF cells and ATP level measured by AT1.03^{YEMK} FRET biosensor. (a) Method of ATP reduction was confirmed using AT1.03^{YEMK} FRET biosensor. BSF cells expressing the biosensor were washed into PBS containing 10mM glucose (high ATP) and glucose-free PBS (low ATP), and their FRET ratios (FRET/YFP) were monitored for an hour. Stably high FRET ratio was observed in cells incubating in 10mM glucose (high ATP). FRET ratio decreased in cells incubating in glucose-free PBS (low ATP), eventually plateauing after ~20 minutes. (b) 2-NBDG uptake was measured in ATP reduced BSF cells. Low

ATP was achieved by starving BSF parasites in glucose-free PBS for 30 minutes, while high ATP control was maintained in PBS containing 10mM glucose. Followed by a quick wash in glucose-free PBS twice, cells were pulsed with 2-NBDG for 1 minute and analyzed on the flow cytometer. 2-NBDG uptake in ATP reduced cells were significantly reduced in comparison to the ATP high control, suggesting ATP coupled glucose transport in BSF cells. 2-NBDG uptake is normalized to the high control. Error bars represent the standard deviation for n = 3 replicates. $^{*}p< .05$.

2.1.4 ATP coupled nutrient transport bypassing the THT

The contradiction between our evidence supporting ATP-coupled glucose transport and the previous findings may be explained by the distinction between a live cell and a reconstituted membrane. Based on the assumption that glucose transport is facilitated by an ATP-coupled cellular function bypassing the passive THT, the effect of endocytosis on glucose transport was studied. In T. *brucei*, few specific endocytosis inhibitors have been identified, demonstrating a direct relationship of endocytosis and glucose transport difficult. Nevertheless, we scored 2-NBDG uptake rates in BSF cells under various conditions that have been previously established to result in altered endocytosis rates. First, we used addition of sucrose as a means of controlling the endocytosis rate; artificially increasing osmolality via media alteration has shown to create abnormal formation of microcages to inhibit endocytosis by rendering clathrin unavailable for assembly into normal coated pits [58]. 2-NBDG as well as conjugated dextran uptake rates were measured in PBS solutions containing different concentrations of sucrose [58]. As expected, uptake of conjugated dextran, a general marker for fluid-uptake during endocytosis was significantly reduced as sucrose concentration increased (Figure 5). Interestingly, uptake of 2-NBDG similarly decreased with increasing sucrose concentration (Figure 5). If endocytosis were involved in glucose transport pathway of trypanosomes, the effect of endocytosis inhibition would result in hindered glucose uptake rate. Thus, the finding that inhibition of endocytosis via

hypertonic sucrose also decreased the 2-NBDG uptake demonstrates the involvement of endocytosis in glucose transport.

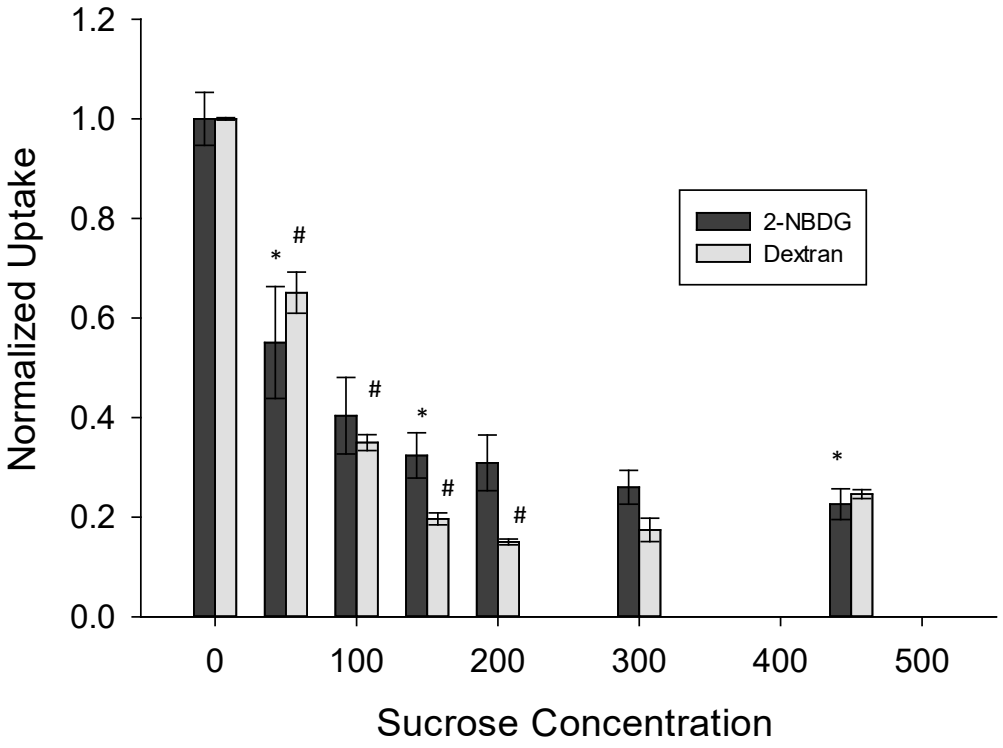

Figure 5. 2-NBDG and dextran-CascadeBlue uptake rates of BSF cells in hypertonic sucrose buffers. Hypertonic sucrose buffers were used to increase osmolality and inhibit endocytosis. Cells were incubated in buffers containing different concentration of sucrose and pulsed with 2-NBDG and dextran for 1 minute and 5 minutes, respectively. Both 2-NBDG and dextran uptakes were significantly reduced in increasing sucrose concentration with the same trend, suggesting that inhibition of endocytosis results in decreased glucose transport rate. Error bars represent the standard deviation for n = 3 replicates. $^{*}p< .05$. $^{\#}p<.05$.

2.1.5 Impact of silencing endocytosis in glucose uptake

To measure the direct effect of endocytosis in glucose uptake, a genetic approach was used in which the endocytic pathway was inhibited by silencing two genes in BSF parasites: clathrin and PIP kinase. Both clathrin and PIP kinase have been previously silenced and demonstrated to have essential roles in endocytosis and survival, of which clathrin silencing exhibited “big-eye”

phenotype with the enlarged flagellum pocket where the endocytosis exclusively occurs [46, 51]. Silencing either clathrin and PIP kinase resulted in drastic reduction (~50%) of 2-NBDG uptake in the infectious lifecycle stage, providing and solidifying direct evidence for the involvement of endocytosis in glucose transport mechanism. Due to the morphological changes and severe growth defect in silencing these essential genes, viability of both non-induced and induced parasites were tested in parallel using propidium iodide. This cell viability test revealed that morphological changes observed upon silencing of either clathrin and PIP kinase do not correlate to loss of cell viability. Interestingly, a small and distinct population with drastically decreased 2-NBDG uptake was observed in uninduced parasites, possibly representing leaky expression of RNAi system. As trypanosomes are effected by the leaky expression of silencing miRNA and siRNA in uninduced state, the small population with decreased glucose uptake demonstrates the direct effect of inhibiting endocytosis on glucose uptake, independently of the cellular toxicity of antibiotic tetraycyclin. The effect of dextran uptake in endocytosis knockdown cells is currently being investigated.

2.2 Discussion

Glucose metabolism is crucial in the infectious bloodstream stage of *T. brucei*. While glucose level within the human blood plasma is maintained around ~4.7 mM, glucose level in other tissues can have vastly varying concentrations [59, 60]. Moreover, responses from host to the infection can further alter the available glucose in different physiological locations. For instance, in the second stage of human African trypanosomiasis when the parasites have crossed the blood-brain barrier, glucose concentration inside the brain tissue can reach as low as 1 mM [61]. Additionally, the finding that parasites establish persistent infections in the skin, fatty tissue, and

even testes of rodent models [62-64], areas where glucose availabilities may vastly differ, suggests that the parasites encounter a diverse environment especially in respect to glucose availability.

Trypanosome infection has been reported to decrease host blood glucose levels [65]; furthermore, hypoglycorrhachia, or reduced glucose levels in cerebrospinal fluid, has also been observed in human African trypanosomiasis patients and related parasitic infection in animal models [66, 67]. Since the BSF parasites rely solely on glycolysis for its supply of ATP, the parasites need an adaptive system that accommodates its need for higher glucose transport rate when in a low glucose environment, such as fatty tissue or cerebral fluid. These findings point to the parasite's need for a dynamic system to optimize glucose uptake under different environmental conditions, rather than a passive facilitated diffusion model, which cannot increase uptake rate under lower glucose concentration conditions. Thus, the THT model, which has long been proposed as the glucose transport method in T. *brucei,* alone is not the best way to provide an explanation for the rugged adaptability of trypanosomes as they are exposed to diverse environments.

Since the discovery of the genes, THT has been the universally accepted glucose transporter in T. *brucei*. In fact, alongside endocytosis, THT expression is one of major transcriptional changes trypanosomes undergo when transitioning from insect vector to the host [16-18]. To demonstrate the glucose transport ability of THT in live BSF trypanosomes, both isoforms of THT were knockdown using RNAi system. As expected for a well-established carrier protein, silencing of both THT carriers led to a reduction in glucose transport ability. Surprisingly, despite a significant reduction in glucose uptake, no detectable growth defect was observed in THT knockdown BSF cells. If THTs are the sole mode of glucose transport in trypanosomes, silencing such important genes would prevail a significant hindrance on the growth of parasites. Although

interpreting the expected amount of glucose transport reduction proves hard without complete knockout of the genes, the lack of a lethal phenotype in THT knockdown parasites led us to re-evaluate the established glucose transport mechanism in *T. brucei*.

In search of an additional mode of glucose transport mechanism bypassing the THT carriers, we have found evidence supporting energy-driven glucose transport in BSF, which directly contradicts the previous experiments done in a cell-free reconstitution system lacking ATP, Na+ or pH gradients [34]. As the proposed facilitated diffusion carrier protein, THT should not directly require an energy source for its function. Contrary to our findings done in live trypanosomes, the previous findings that show THT as an ATP independent facilitated diffusion transporter were done in a reconstituted membrane system. Our findings in live trypanosomes suggest that an additional mode of glucose transport exists in live trypanosomes that bypasses the ATP-independent THT present on the plasma membrane. Based on the idea that endocytosis is an ATP coupled bulk transport process that can be inhibited by the depletion of ATP, role of endocytosis in glucose transport was explored. Many factors involved in endocytosis are oftentimes difficult to manipulate due to their intertwined roles in many other cellular functions. Furthermore, with few known inhibitor of endocytosis, 2-NBDG uptake rate under hypertonic sucrose condition was scored. Interestingly, hypertonic sucrose buffers significantly decreased 2-NBDG uptake rate in BSF parasites. The effect of hypertonic sucrose on endocytosis was further examined and confirmed using conjugated dextran. Although no specific endocytic machinery is targeted hypertonic sucrose, the similar trend between 2-NBDG and dextran supports our claim in endocytosis's involvement in glucose transport. An ATP-dependent glucose transport pathway in conjunction with evidence for decreased 2-NBDG uptake in endocytosis inhibited trypanosomes

is strongly suggestive that endocytosis acts as an additional glucose transport pathway in parallel with the THT.

To stay away from speculations, a direct measure of inhibiting endocytosis was used to further strength our argument. Two genes directly involved in endocytosis, clathrin and PIP kinase, were silenced and studied [46, 51]. Further supporting our hypothesis of glucose transport through endocytosis, silencing endocytosis through both genes resulted in severe reduction of 2-NBDG uptake. Silencing the essential endocytosis genes manifested morphological changes; however, cell viability test revealed intactness of endocytosis compromised parasites. Furthermore, a small population of significantly reduced 2-NBDG uptake was observed in uninduced control which can be explained by the leaky expression RNAi and strengthen the argument that 2-NBDG uptake reduces in endocytosis compromised trypanosomes. Significant reduction in 2-NBDG transport in clathrin and PIP kinase knockdown provides strong evidence for the direct involvement of endocytosis machinery in glucose transport pathway.

Our study has shown the involvement of endocytosis in glucose transport of T. *brucei*. However, without a proper transporter or a protein identified, a mechanistic model of proposed glucose transport pathway cannot be deduced. More detailed studies need to be done to identify how exactly endocytosis is involved in glucose transport; whether endocytosed glucose gets pumped out into the cytosol from the clathrin coated vesicles via a protein pump or trafficked directly to the glycosome. Moreover, the extent of glucose transport contribution from each THT and endocytosis is yet to be determined.

2.3 Materials and Methods

2.3.1 Reagents and parasite cultures

Adenosine triphosphate (ATP), and 2-deoxyglucose were purchased from Sigma (St. Louis, MO). 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), Phloretin, and cytochalasin B were purchased from Cayman Chemicals (Ann Arbor, MI).

Bloodstream form cells (BSF) 9013, a derivative of strain Lister 427, were cultured in HMI9 media [68] consisting of 10% Serum Plus™ medium supplement (Sigma) and 10% dialyzed fetal bovine serum (Corning). Procyclic form cells (PF) 2913 parasites (also a Lister 427 derivative) were cultured in SDM79 media [69] composed of 15% heat-inactivated fetal bovine serum (Corning). Parasites were routinely maintained under conditions that were optimal for logarithmic growth ($1x10^6$ – $5x10^6$). Expression of RNAi system was induced by the addition of doxycyclin at 1μg/ml into the media.

2.3.2 THT gene expression analysis

To assess mRNA expression of THT, log phase BSF and PF cells growing in HMI9 and SDM79 respectively were centrifuged (1000 x g, 5 min) and gently washed three times in phosphate buffered saline (PBS). RNA isolation of harvested cells was performed using Aurum Total RNA Mini Kit (Bio-Rad).

Real-time quantitative PCR was performed using a Verso 1-step RT-qPCR Kit (ThermoFisher) in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Ct values of transcripts were used to solve relative expression by the comparative Ct ($2^{-\Delta\Delta CT}$) method using the expression of the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene as a control as

described [70]. Transcripts of other house-keeping genes, including the 60S rRNA, TERT, and 18S rRNA genes, were used as internal controls. Dissociation curves were assessed for each reaction and efficiency tests performed for all primers to guarantee specific and equally efficient amplification.

2.3.3 RNAi lines generation

To generate RNAi lines, RT-PCR of corresponding genes were done with primers binding on the genome, THT here, CLH there, PIPKA here. The copy DNA from RT-PCF was cloned into the pZJM RNAi plasmid and were subsequently transfected and incorporated in the genome.

2.3.4 Confocal microscopy

BSF cells expressing THT1E-eYFP, THT2A-eYFP and eYFP in log phase were harvested by centrifugation (1000 rpm, 5 min), washed with PBS 3 times, and resuspended in 1:1 PBS and fix (4% formaldehyde in PBS). Cells were then transferred to slides, allowed to settle for 30 minutes, incubated with VECTASHIELD antifade mounting medium, and visualized on a with enough z-stacks to visualize whole cells.

2.3.5 Uptake assays

To quantitatively measure glucose uptake over time, a fluorescent glucose analogy, 2-NBDG was used. For 2-NBDG uptake assays, BSF and PF cells were washed in HMI9 and SDM79 media respectively 3 times followed by a 15 min incubation at the desired temperature. Labeling was initiated by addition of 2-NBDG (300μM) with or without experimental treatments. To stop the uptake, 1ml of ice-cold PBS containing glucose (300 mM) was added, followed by extensive washing with ice-cold PBS. Cells were then resuspended in PBS supplemented with 10 mM

glucose and propidium iodide (1µg/ml), and analyzed on Attune™ NxT or CytoFlex flow cytometer at 10,000 cells.

Fluid phase endocytosis rate was measured using fluorescent dextran conjugate. Cells were harvested and washed in desired buffer followed by the addition of dextran-CascadeBlue (5mg/ml) in appropriate treatment conditions. To halt the reaction, 1ml of ice-cold PBS was added, followed by extensive washing with cold PBS solution. Cells were then resuspended in PBS supplemented with 10mM glucose and propidium iodide (1µg/ml) and analyzed on Attune™ NxT or CytoFlex flow cytometer.

2.3.6 FFET biosensor – intracellular ATP measurement

To non-destructively measure cytosolic ATP concentrations, constitutively expressed AT1.03^{YEMK} [57] was cloned in expression vector pXS6 and transfected in BSF 90-13 T. *brucei* 427 strain. Expression in these vectors is driven from the *T. brucei* rRNA promoter, typically yielding robust expression [18]. Constructs were transfected into BSF parasites using the Amaxa Human T Cell Nucleofector Kit (Lonza, Basel, Switzerland) as described [19]. The biosensor consists of an CFP/mVenus FRET pair flanking an ATP binding domain of Bacillus subtilis F0F1 ATP synthase subunit. The ratio of the fluorescence from a single fluorophore to the FRET ratio that results from ATP-induced conformation change of the ATP synthase subunit allows quantification of ATP.

For quantitative calculation of intracellular ATP concentration, cells were washed in PBS 3 times and transferred to PBS solution containing 10mM glucose for a high ATP control and 10mM sodium azide as a low ATP control. FRET/YFP ratios of cells at the maximum and minimum ATP concentrations in PBS were measured using CytoFlex flow cytometer.

2.4 Conclusion and Future Direction

In conclusion, data provided above demonstrate endocytosis as an additional glucose transport pathway in BSF trypanosomes. Our findings suggest that THT may not be as crucial as previous thought. Furthermore, with evidence supporting an additional ATP-coupled glucose transport system that bypasses the THT in live trypanosomes, the role of endocytosis, an ATP coupled bulk transport in glucose transport was subsequently investigated. Inhibition of endocytosis via various means such as hypertonic media and genetic silencing has shown decreased glucose analog uptake rates solidifies endocytosis's role in glucose transport. The findings presented above, however, is in its preliminary stage and require further study. A few things need to be identified in order to provide a mechanistic model of the proposed glucose transport pathway. Particularly, exact mechanism of endocytosis in glucose transport needs to be identified. Although trypanosomes primarily employ clathrin-mediated endocytosis, our findings suggest that endocytosis coupled glucose transport may occur via fluid-phase endocytosis not affected by a receptor mediated endocytosis inhibitor. Whether a specific receptor or the sheer volume of extracellular medium endocytosed, the mechanism in which glucose is taken up from the flagellar pocket need a further clarification. In addition, a proper glucose transporter needs to be identified in the endocytic compartments. As endocytic compartments are continually recycled back and forth from the flagellar pocket to the endosome/TGN, the endocytosed glucose need a pathway to the appropriate locations through either a transporter that pumps out glucose into the cytosol or alterative traffic pathway into the glycosomes.

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