An Investigation of TbLpn Methylation in
Trypanosoma brucei

A Senior Honors Thesis

An undergraduate thesis submitted to the Department of Biology and Honors College of the State University of New York College at Brockport in partial fulfillment of the requirements for the Biology Honors Program & the Honors College

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I. Abstract

Human African trypanosomiasis (HAT), more commonly referred to as African Sleeping Sickness, is a disease transmitted through the bite of an infected tsetse fly harboring the human pathogenic parasite, *Trypanosoma brucei*, a hemoflagellate of the taxonomic class Kinetoplastea. The disease impacts primarily populations of sub-Saharan Africa, and exists in two primary stages: the bloodstream and the central nervous system (CNS) stage. In the first stage, *T. brucei* will migrate through the lymph and blood, continually proliferating and causing unspecific signs and symptoms. Over time, the parasite will penetrate the blood-brain barrier and migrate to the central nervous system, where various neurological disturbances can occur, greatly increasing the severity of the disease. It is at this stage that the disease becomes difficult to treat, as antibodies that circulate the body can no longer reach the parasite, and the brain immune response is less efficient than other immune responses within the body. However, early intervention is also difficult, due to the lack of specific signs or symptoms present during the bloodstream stage of the disease. In the event of early diagnosis of HAT, the drugs that are currently available for treatment will likely cause negative side effects. Vaccination is also not possible due to the trypanosome's antigenic variation, which allows the *T. brucei* to successfully elude the host's immune responses through their expression of the variant surface glycoproteins (VSGs). VSGs cover the *Trypanosoma* cell with a frequently-changing "protective coat," allowing the parasite to avoid detection by host antibodies. Five Protein Arginine Methyltransferase enzymes (PRMTs) are also found in *T. brucei,* which suggests that arginine methylation plays a predominant role in the parasite's life cycle. PRMTs have been shown to interact with a trypanosome homologue of lipin, termed TbLpn, which acts as phosphatidate phosphatase (PAP) enzyme, and actively converts phosphatidate to diacylglycerol (DAG) during the synthesis of triacylglycerol (TAG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). PE is particularly important in *T. brucei's* synthesis of

glycosylphosphatidylinositol (GPI), which is utilized to anchor VSGs and give the parasite its ability to evade the immune system through antigenic variation. TbLpn is the only phosphatidic acid phosphatase known to date to exhibit arginine methylation. One of the main objectives of this project was to confirm the importance of TbLpn, and to verify that it is the major enzyme responsible for the dephosphorylation of phosphatidic acid in *T. brucei.* To determine this, a phosphatidic acid phosphatase (PAP) assay was performed, and the results showed that that the wild type TbLpn produced 48.05 (± 26.09) nmol of phosphate per minute per mg of protein, whereas the TbLpn-depleted cells produced only 21.96 (± 14.59) nmol Pi/min/mg. These results confirmed the importance of TbLpn for the dephosphorylation of phosphatidic acid, and its effect on enzymatic activity in *T. brucei*, as the cells with trace amounts of TbLpn displayed significantly less activity. The other main objective of this experiment was to determine the effect of TbLpn methylation by TbPRMT7 on its enzymatic activity. Following an additional PAP assay, the phosphatase activity of wild type TbPRMT7 *T. brucei* cells was compared to that of TbPRMT7-depleted cells, and the effect of methylation on TbPRMT7 PAP activity was deemed insignificant, with minimal difference in measured enzymatic activity. It can therefore be concluded that the methylation of TbPRMT7 is not important for the enzymatic activity of TbLpn. However, it cannot be confirmed from these results that TbPRMT7 methylation plays no role in *Trypanosoma brucei*. Further experiments must be conducted to determine what role arginine methylation has on TbLpn enzymatic activity.

II. Introduction

***Trypanosoma brucei* and African Sleeping Sickness.** Human African trypanosomiasis (HAT), commonly known as African sleeping sickness, is a vector-borne parasitic disease caused by infection with the parasitic protozoan *Trypanosoma brucei*[1]. Human African trypanosomiasis primarily affects poor, remote rural populations in sub-Saharan Africa, where suitable habitats for its vector, the tsetse fly, are present. Transmission of the disease can occur in both children and adults during daily life activities, including farming, hunting, fishing, or washing clothes, through the bite of a tsetse fly[2].

Tsetse flies, of the genus *Glossina,* resemble common house flies, having a similar size that ranges from 8 to 17 mm. Yet, tsetse flies remain easily distinguishable while resting, as their wings are visibly folded so that one wing rests directly on top of the other over their abdomen. Tsetse flies also have a long proboscis which extends directly forward and is attached by a distinct bulb to the bottom of their head[3] (Figure 1). Tsetse flies frequently inhabit rural areas, living in the woodlands and thickets along the East African savannah. In central and West Africa, tsetse flies live in forests and vegetation along streams. Tsetse flies most commonly bite during daylight hours, and both male and female flies can transmit the HAT infection. Tsetse flies originally acquire their infection by taking a blood meal on an infected human being or other mammalian host harboring human pathogenic parasites, often belonging to the genus *Trypanosoma*[1]. However, it should be noted that even in areas where the disease is endemic, only a small percentage of flies are infected. Occasionally, other forms of disease transmission may occur as well. A pregnant woman can pass the HAT infection to her unborn baby, and in theory, HAT can also be transmitted by blood transfusion or sexual contact. However, such cases have rarely been documented[4].

Figure 1: The Tsetse Fly. A close up of a tsetse fly, of the genus *Glossina*, taking a blood meal. Today, living tsetses are almost exclusively found on in sub-Saharan African. Tsetse flies are recognized as a vector of African sleeping sickness, incriminated in the transmission of the pathogenic parasite, *Trypanosoma brucei*[4].

Human African trypanosomiasis can take on two clinical forms, depending on the subspecies of the parasite involved. *Trypanosoma brucei gambiense* is the most common subspecies and is found in 24 countries located in west and central Africa. This form is most prominent, and currently accounts for 97% of reported cases of African sleeping sickness. The second form, *Trypanosoma brucei rhodesiense*, is found in 13 countries in eastern and southern Africa, leaving this form to represent under 3% of reported cases[1].

Upon infection, there are two main stages of Human African trypanosomiasis that occur. In the first stage, the parasite is found in the peripheral circulation, but it has not yet invaded the central nervous system (CNS). Once the parasite crosses the blood-brain barrier and infects the central nervous system, the disease then enters the second stage. Depending on which subspecies the host was infected with, there will then be different rates of disease progression, as the clinical features depend on which form of the parasite (*T. b. rhodesiense* or *T. b. gambiense*) is causing the infection. However, either form of infection will eventually lead to coma and death if left untreated[5].

In the past century, three major Human African trypanosomiasis epidemics have ravaged the African continent. The first outbreak largely affected equatorial Africa, and was the most fatal. The outbreak took place between 1896 and 1906 and left an estimated 800,000 people deceased. The second major epidemic then occurred between 1920 and the late 1940s, and prompted the colonial powers to begin investing in vector control and to undertake active surveillance of the population. This led to the disease being almost eradicated in the early 1960s; however, the collapse of surveillance and control activities led to a progressive re-emergence of the disease, which then reached a peak in the late 1990s[2]. There has since been a decrease in reported infections, as the disease is currently reasonably well-controlled. However, under-reporting is known to mask the true burden of the disease, and in certain locations of Africa the outbreak numbers remain high. In recent years, 7,000 to 10,000 new cases have been reported annually. Over 95% of these cases of human infection are found in Democratic Republic of Congo, Angola, Sudan, Central African Republic, Chad, and Uganda[4]. The prevalence of Human African trypanosomiasis within local African populations is demonstrated in Figure 2.

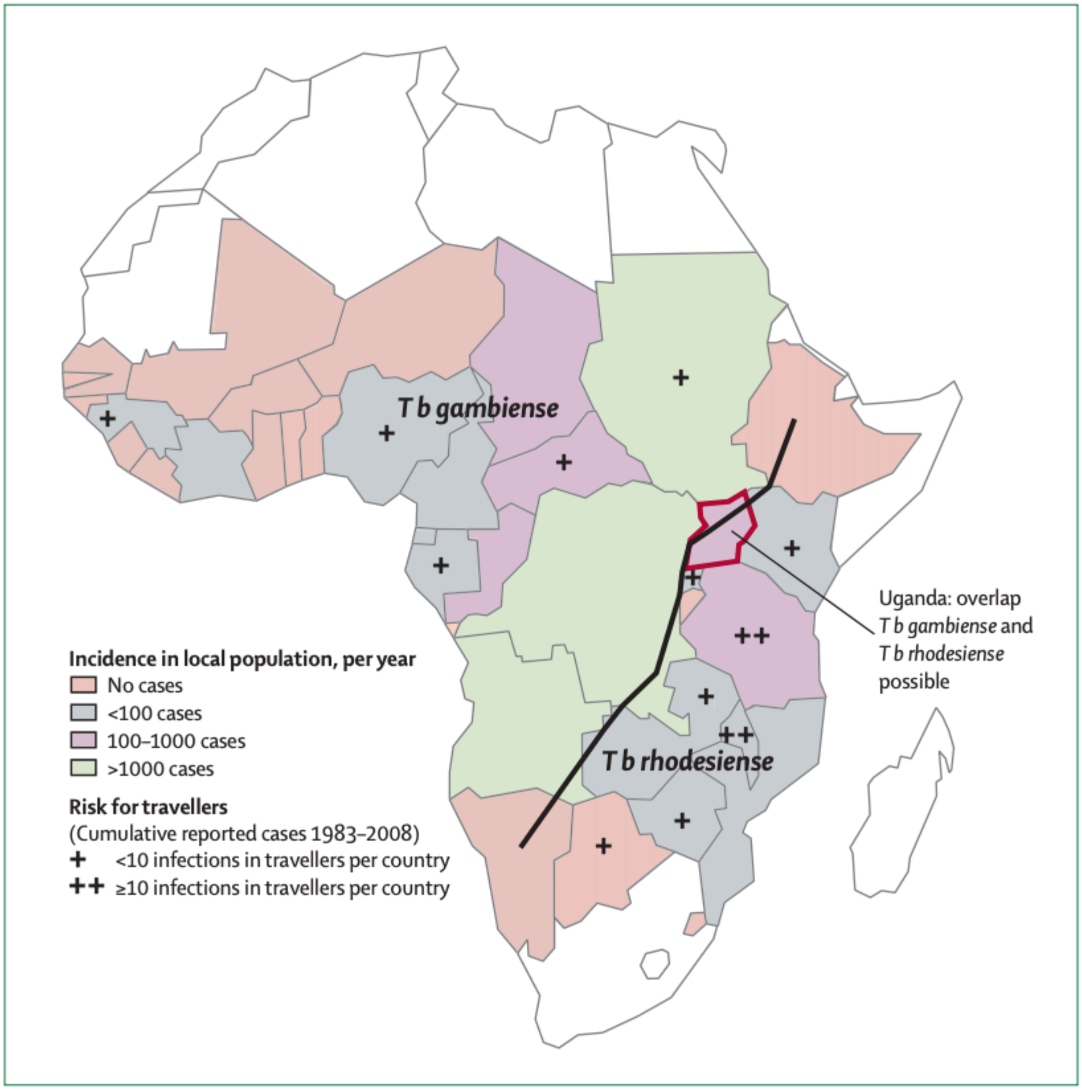

Figure 2: Distribution of human African trypanosomiasis. The prevalence of HAT within various African countries, and the incidence of *T. b. gambiense* or *T. b. rhodesiense* in local population, in number of cases per year. The black line divides the areas in which *T. b. gambiense* prevails, and those in which *T. b. rhodesiense* predominates.

***Trypanosoma brucei* Morphology.** *Trypanosoma brucei* is a unicellular, parasitic protozoan hemoflagellate of the taxonomic class Kinetoplastea, which is comprised of organisms containing an organelle with a large mass of circular DNA, known as a kinetoplast (Figure 3). The trypanosome cell is elongated and has a highly polarized microtubule cytoskeleton, which defines the cell shape and remains intact throughout its cell cycle. The most posterior structure is the mouth of the flagellar pocket, which is the exit point for the flagellum. The flagellum is tethered along the exterior length of the parasite, and the flagellar pocket is the only site of endo- and exo-cytosis. The motility of the trypanosome is dependent upon its flagellum, which has a conventional axonemal structure plus an associated paraflagellar rod. This rod is a semi-rigid structure found in kinetoplastids and euglenoids, and also contributes to parasite motility[6] (Figure 4).

As the trypanosome goes through its life cycle, its morphology repeatedly changes. The parasite forms trypomastigotes in vertebrate hosts and epimastigotes in the insect vector. The trypomastigotes are pleomorphic in size ranging from 16-42µm in length by 1-3µm in width. They occur as elongate slender dividing forms, with long free flagellum, or as stumpy, non-dividing infective (metacyclic) forms with no free flagellum. The epimastigotes are also variable in size ranging from 10-35µm in length by 1-3µm in width[7].

The most obvious morphological difference between the different life cycle stages of the trypanosome is the position of the kinetoplast, relative to the posterior end of the cell. In the mammalian host (bloodstream form), the kinetoplast lies close to the posterior end, and each daughter kinetoplast remains in this region throughout the cell cycle. In the insect host (procyclic form (PF)), the kinetoplast lies midway between the cell nucleus and the posterior end; in epimastigote forms (present in the tsetse salivary gland), the kinetoplast is anterior to the central nucleus. The reasons for these morphological changes during the trypanosome's life cycle remain unknown, although they are clearly required to establish the cell architecture necessary for cell division at each stage. Perhaps an increased length of attached flagellum along the cell body assists motility in bloodstream forms, whereas the longer anterior flagellum aids substrate attachment of the epimastigote stage[6] (Figure 5).

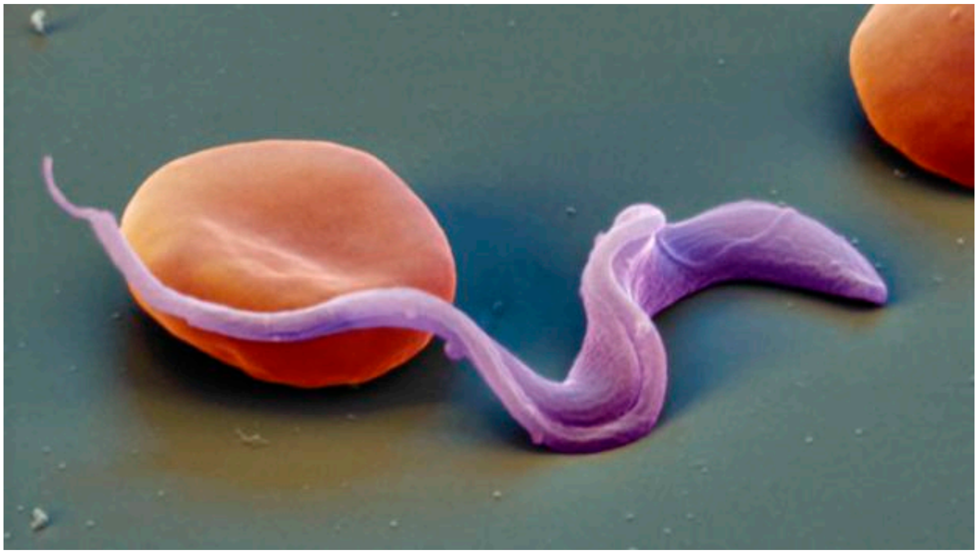

Figure 3: ***Trypanosoma brucei*****.** A *T. brucei* (purple) amongst red blood cells. The parasitic protozoan is responsible for the infection of mammalian host cells with African trypanosomiasis (sleeping sickness). *T. brucei* ranges in length from 15-35 μm[8].

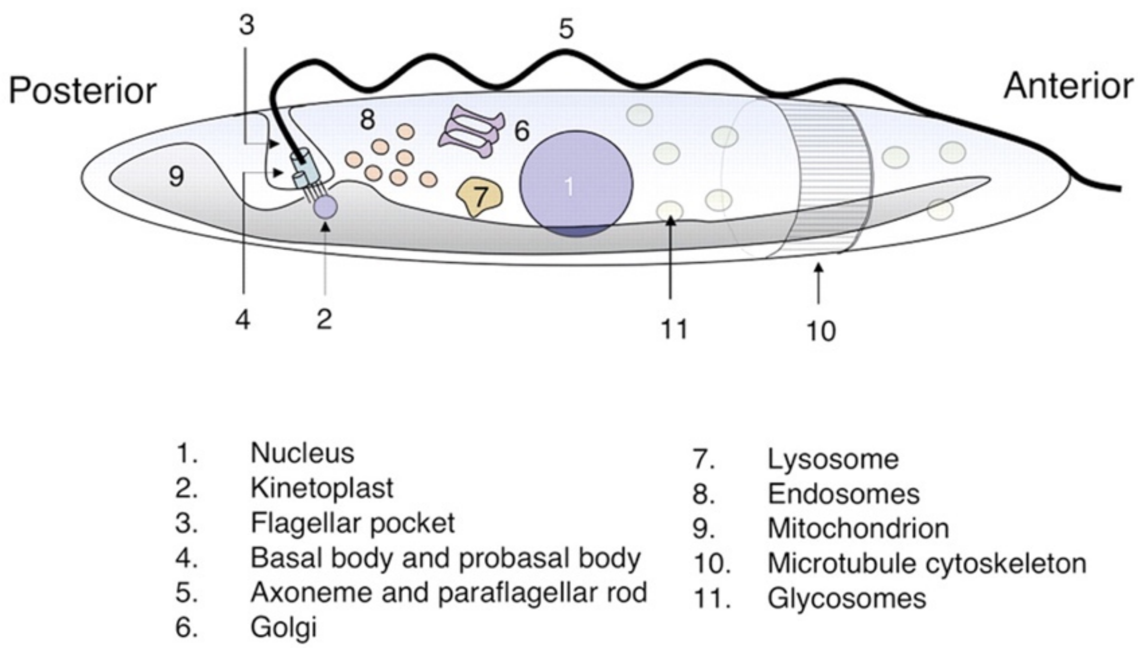

Figure 4: ***Trypanosoma brucei*** **Morphology.** A simplified representation of the position of major structural features within the trypanosome cell. A cutaway section towards the anterior of the cell shows the microtubule cytoskeleton underlying the cell membrane[6].

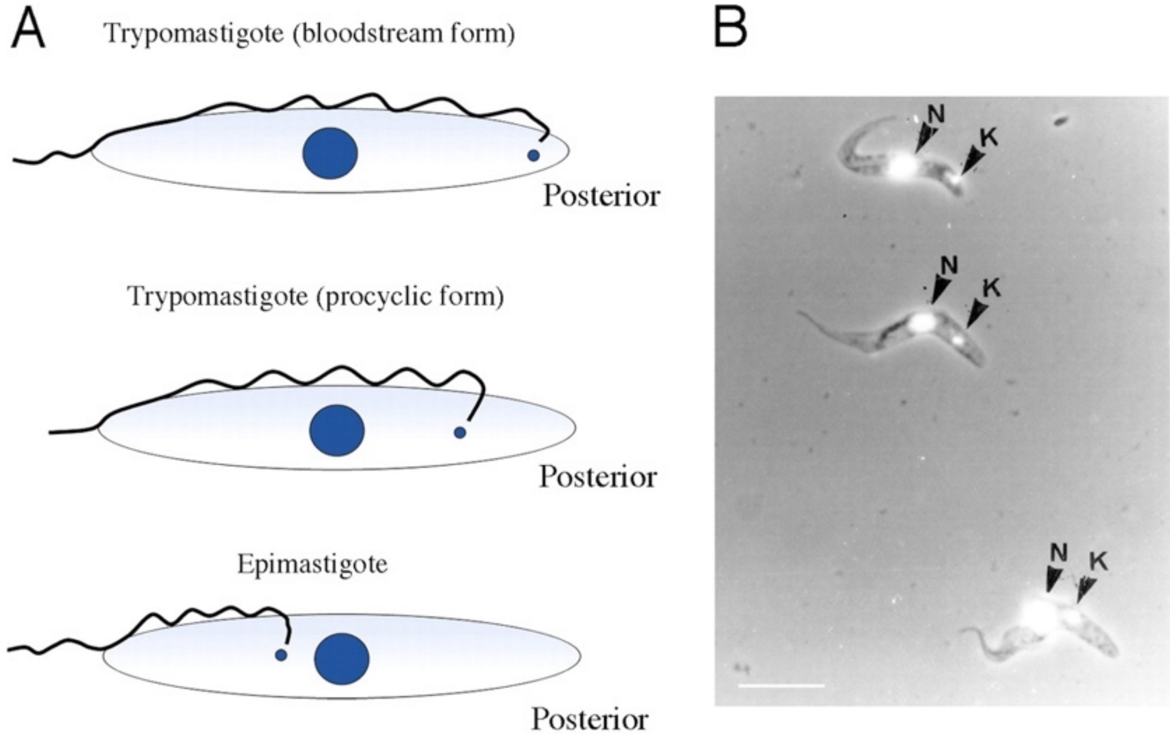

Figure 5: Position of the kinetoplast. The kinetoplast changes position during the trypanosome life cycle. (A) Demonstration of the relative position of the kinetoplast in trypomastigote bloodstream and procyclic forms, as well as in epimastigote forms with respect to the nucleus and posterior end of the trypanosome. (B) Example of a phase contrast image of trypanosome cells undergoing differentiation between their bloodstream and procyclic forms. (The cells have been counter-stained with DAPI to reveal the position of the nucleus (N) and kinetoplast (K))[6].

The Life Cycle of *Trypanosoma brucei.* The trypanosome is transmitted between mammalian hosts by the tsetse fly, in which the parasite initially establishes itself in the midgut after a bloodmeal, but migrates to the salivary glands in preparation for transmission to a new mammalian host[6]. During a blood meal on the host, an infected tsetse fly then injects metacyclic trypomastigotes into skin tissue, allowing the parasites to enter the host's lymphatic system and pass into the bloodstream. Once inside the host, the trypanosomes transform into their bloodstream-form trypomastigotes, and are carried to other sites throughout the body, reaching blood fluids such as lymph and spinal fluid. The trypomastigotes continue to replicate within the host by binary fission[9] (Figure 6).

The entire life cycle of this protozoan parasite is extracellular, so it must adapt its metabolism to the available extracellular nutrients. The two morphologically distinct bloodstream-form (BSF) trypomastigotes characterized in terms of metabolism are either long slender (LS) or short stumpy (SS) trypomastigotes. The LS trypomastigotes are rapidly dividing procyclic forms, which mainly catabolize glucose and proline, and undergo a developmental transition to the non-dividing SS stage[10]. The LS stage is very well adapted for growth within the mammalian host, whereas the SS stage appears pre-adapted for life in the vector – in the absence of being taken up by the vector the trypomastigote will ultimately die. Similarly, the long slender BSF trypomastigotes are unable to undergo transformation to tsetse-infective forms, and will also die in the tsetse midgut. Therefore, this change from LS to SS is necessary for the transition of the trypanosomes from their previous life in a glucose-rich, highly-oxygenated blood environment into the glucose-poor, poorly-oxygenated environment of the tsetse gut[11] (Figure 7).

T. brucei infection of the tsetse fly begins when the bloodstream form trypomastigotes are ingested into the midgut of the tsetse with the short stumpy BSF trypomastigotes. In the vector, the SS trypomastigotes proceed to develop into the procyclic stage; here, there is rapid replication and infection of the midgut. If infection of the midgut is successful, then procyclic trypomastigotes will migrate into the ectoperitrophic space and along the alimentary canal, and then into the proventriculus (PV) space, where the parasites proceed to reinvade the lumen of the midgut. At this stage, the long epimastigotes proceed to asymmetrically divide into long and short epimastigotes, which attach to the epithelium of the salivary glands by their flagella, creating attached epimastigotes (AE). The epimastigotes then go through Epi-Trypo cell division, thus creating the trypomastigote form of the parasite, which is then used to create the infective metacyclic trypomastigote (MT). Then, when the infected tsetse fly feeds on a subsequent vertebrate host, the MT cells get injected at the site of feeding and attempt to infect the vertebrate[12]. Similar to the LS to SS transition in the mammalian host, the epimastigote to metacyclic stage is irreversible. As a result, the metacyclic trypomastigote are unable to change back to a dividing epimastigote form and, in the absence of inoculation into a mammalian host, will subsequently die.

Once inside the host, the parasite can then migrate through the blood-brain barrier (BBB) and invade the central nervous system. The blood-brain barrier (BBB) is a structural and functional barrier that protects the CNS from invasion by blood-borne pathogens, including parasites. However, some parasites, such as *T. brucei*, have the ability to cross the BBB during the course of infection, thus causing severe neurological disturbances in the host that can at times be fatal. The ways in which such parasites possess the ability to cross the barrier, and how the immune system controls the parasites within the brain, remain unclear[13]. Though, it has been found that the parasite is able to continue to survive free in the bloodstream of mammals, as they possess the ability to evade antibody responses through antigenic variation. This entails the sequential expression of antigenically distinct variable surface glycoproteins (VSGs), which are linked to the surface membrane by a glycosylphosphatidylinositol (GPI) anchor[6].

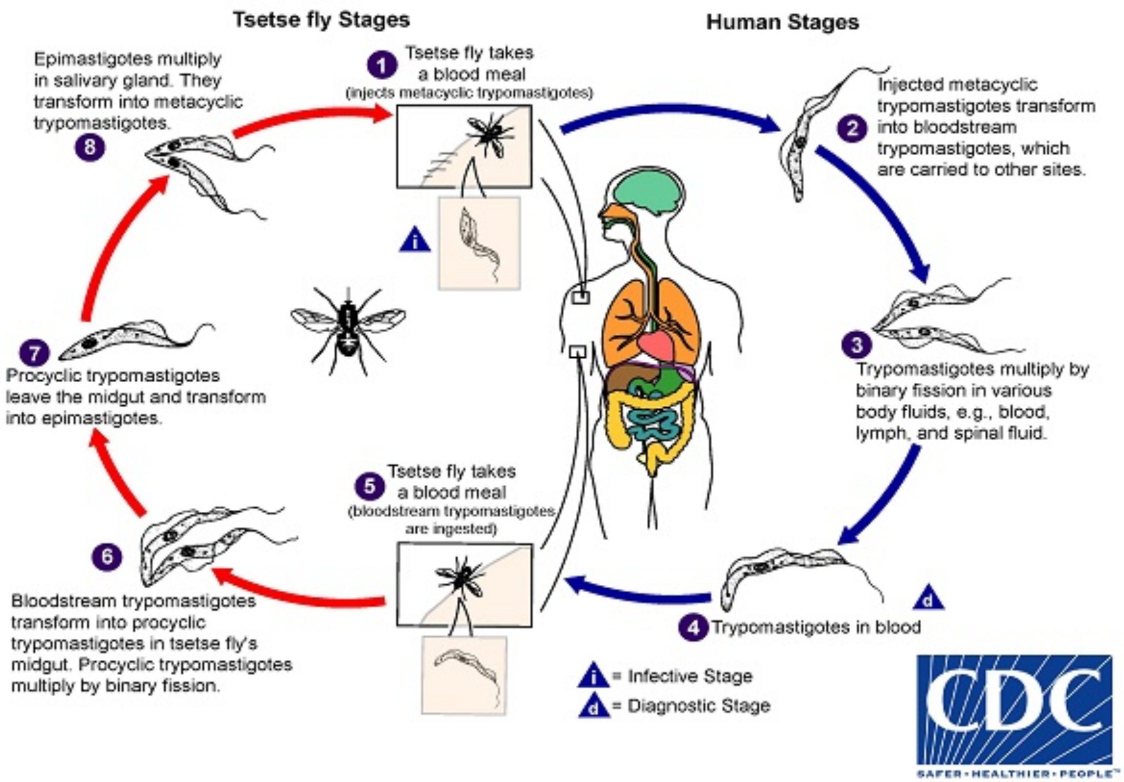

Figure 6: *T. brucei* Life Cycle. *T*. *brucei* spans its life cycle through two different hosts, the tsetse fly and the mammalian. The morphology of *T. brucei* changes as it proceeds through its life cycle. The cycle in the fly takes approximately 3 weeks[9].

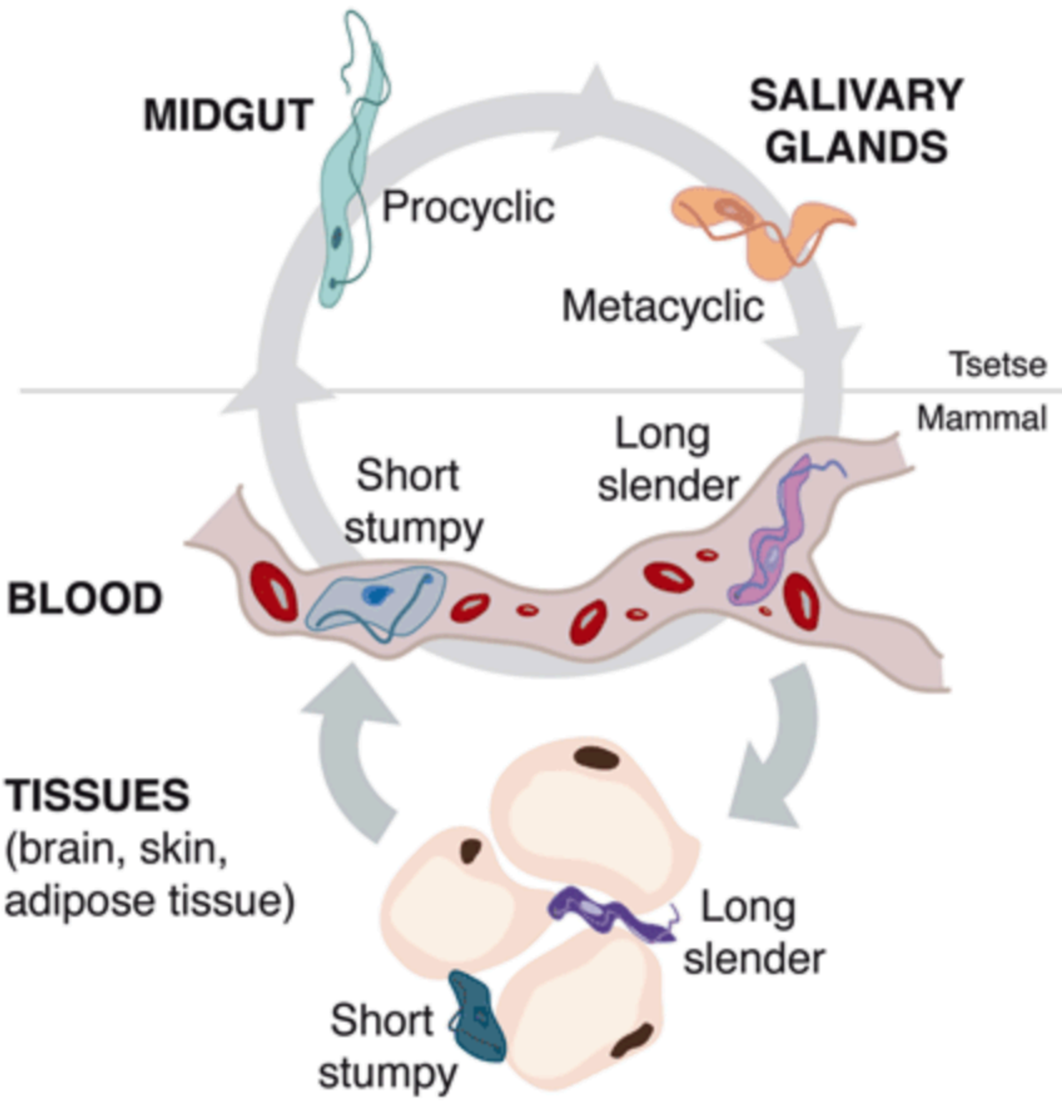

Figure 7: Stages of Metabolism within a *T. brucei* Life Cycle. *Trypanosoma brucei*, an extracellular protozoan parasite, encounters very different extracellular environments during its life cycle within the mammalian host and tsetse fly vector. In order to meet these challenges, there are significant alterations in metabolism made, including the shift from the bloodstream long slender form (LS), and short stumpy form (SS)[10].

Variant Surface Glycoprotein (VSG). *T. brucei* are extracellular blood parasites whose cell surface is covered with a dense layer of a single glycoprotein, termed variant surface glycoprotein (VSG). *T. brucei* are able to successfully elude the adaptive immune response produced by a host through their expression of the antigenic variant surface glycoprotein. By covering the cell with a frequently-changing "protective coat" represented by the VSG, the parasite is able to avoid detection by host antibodies[14]. This evasion of the immune system by expressing immunologically unrelated VSG variants is known as antigenic variation[15].

The VSG role as a protective cell coat is activated in the tsetse fly salivary gland, prior to infecting the human host, and functions during the bloodstream mammalian infectious cycle. It is then inactivated once again upon return to the tsetse fly midgut. Additionally, although their genome contains approximately 1500 VSG genes, bloodstream parasites, such as *T. brucei*, express only one type of VSG coat at a time. Under the pressure to express a different VSG, a repressive gradient extends from the chromosomal end, shutting down the active VSG expression site. The silencing of the VSG expression site then prompts trypanosomes to continue on from the proliferative bloodstream-stage, to the non-proliferative form, as a pre-adaptation for life in the insect (Figure 8). It is the remodeling of chromatin that triggers the silencing, in order to initiate the change of trypanosome performance from proliferative to non-proliferative. The VSG-switch and coat exchange begins with dilution during cell division, with the cells dividing approximately every 6 hours, and turnover being much slower. It is during this time that the coat is cleansed of any antibodies, which is aided by the vigorous motility mediated by the flagellum and endocytosis at the flagellar pocket. Therefore, the role of VSG at the human host–parasite interface is extremely active; it has now been acknowledged that VSG function responses may be responsible for the exhaustion of the immune system, the survival of the parasite and the transmission to the vector[14].

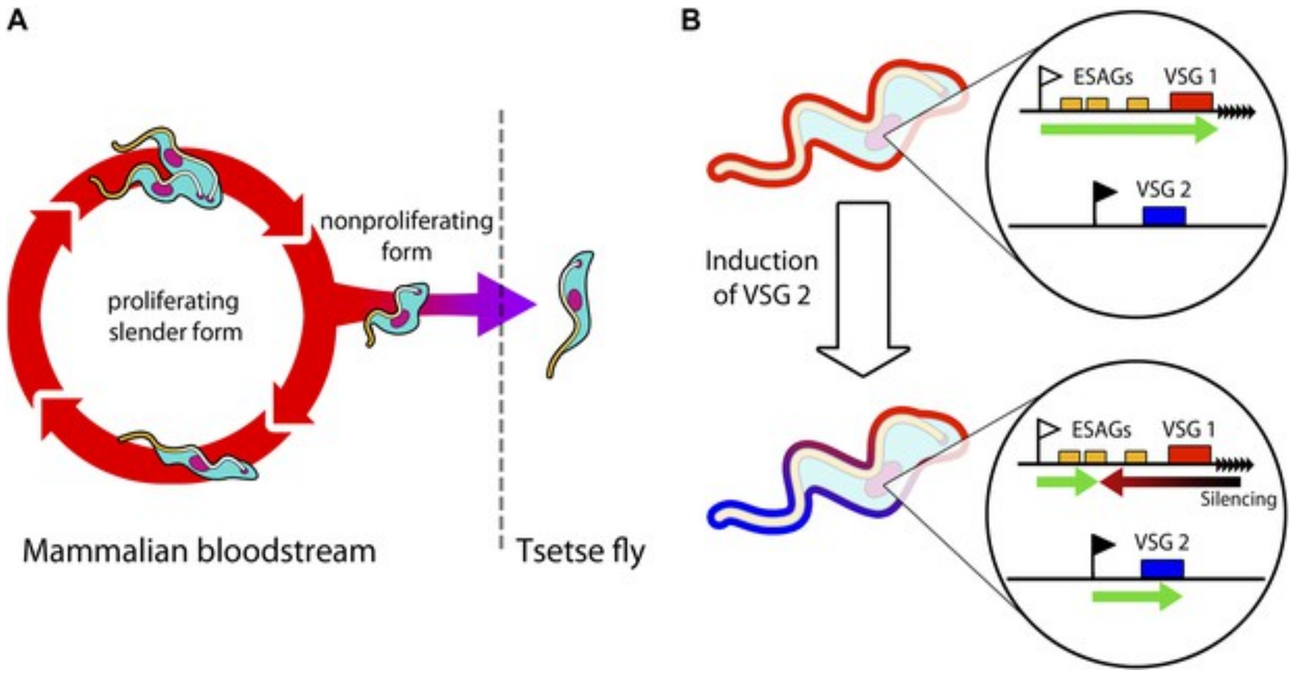

Figure 8: Variant Surface Glycoprotein Expression. Trypanosomes proliferating in the mammalian bloodstream are capable of silencing VSG and changing into a non-proliferative form that can then be transferred into a tsetse fly. *T. brucei* express only one VSG coat at a time. In order to avoid detection by host antibodies, trypanosomes repeatedly switch to new VSG coats[16].

Signs and Symptoms. Once a human experiences the painful bite of an infected tsetse fly, the appearance of a chancre, which is an ulcer where the parasites congregate, will develop and then disappear within 2 or 3 weeks[14]. Inside the host, the parasite will migrate to the lymph and blood, continually proliferating and causing unspecific signs and symptoms of illness. Although, cases have been reported of few individuals who received the infection via a tsetse fly bite, and did not show any immediate signs of contagion. Over time, the parasite may cross the blood-brain barrier and migrate to the central nervous system, where various neurological disturbances can occur, increasing the severity of the disease[17] (Figure 9).

There are two sub-species of *Trypanosoma brucei* that can be attributed to the cause of HAT infection. *Trypanosoma brucei gambiense* is the most prominent form of infection and is commonly found in west and central Africa, accounting for nearly 97% of reported cases of sleeping sickness[1]. *T. b. gambiense* is characterized by a chronic, slowly progressive infectious course with an estimated average duration of approximately 3 years, divided between both stages[2]. The other form of infection, caused by *T. b. rhodesiense*, is usually more acute and progresses rapidly, lasting only a few weeks to several months[17].

Human African trypanosomiasis disease is divided into two stages. The initial stage is the haemolymphatic stage, which occurs shortly after infection, and is when the trypanosomes invade the host and proliferate within the subcutaneous tissues, blood and lymph. Consequently, the infection causes symptoms such as chronic and intermittent fever and headaches; however, these symptoms often go untreated, leaving the disease infrequently diagnosed at this stage[14]. The second stage, known as the meningoencephalitic stage, is more severe, and appears slowly over time. It is during this stage that the *T. brucei* penetrate through the blood-brain barrier, and attack the CNS[2]. Generally, the progression into the meningoencephalitic stage occurs after an average of 300–500 days in HAT caused by *T. b. gambiense*; HAT CNS invasion in *T. b. rhodesiense* is much more rapid, and estimated to take place after only 3 weeks to 2 months after infection[17].

Sleep disorders often dominate as a leading symptom during the meningoencephalitic stage, giving the disease its common name "sleeping sickness." Previously conducted somnographic studies have confirmed that the disease causes dysregulation of the circadian rhythm of the sleep/wake cycle and fragmentation of sleeping pattern, rather than simply inversion of sleep. In the most severe cases, the disappearance of the circadian rhythm of prolactin, renin, growth hormone, and cortisol secretion is also seen. This results in severe neurological symptoms including tremors, fasciculations, general motor weakness, possible paralysis of a limb, and dyskinesia[2]. Other symptoms appear once the disease reaches its terminal stage, including disturbances in consciousness, dementia with incoherence, double incontinence and seizures, which are all consequences of CNS demyelination and atrophy. If the infection is not stopped within a few months, the result is typically death[14].

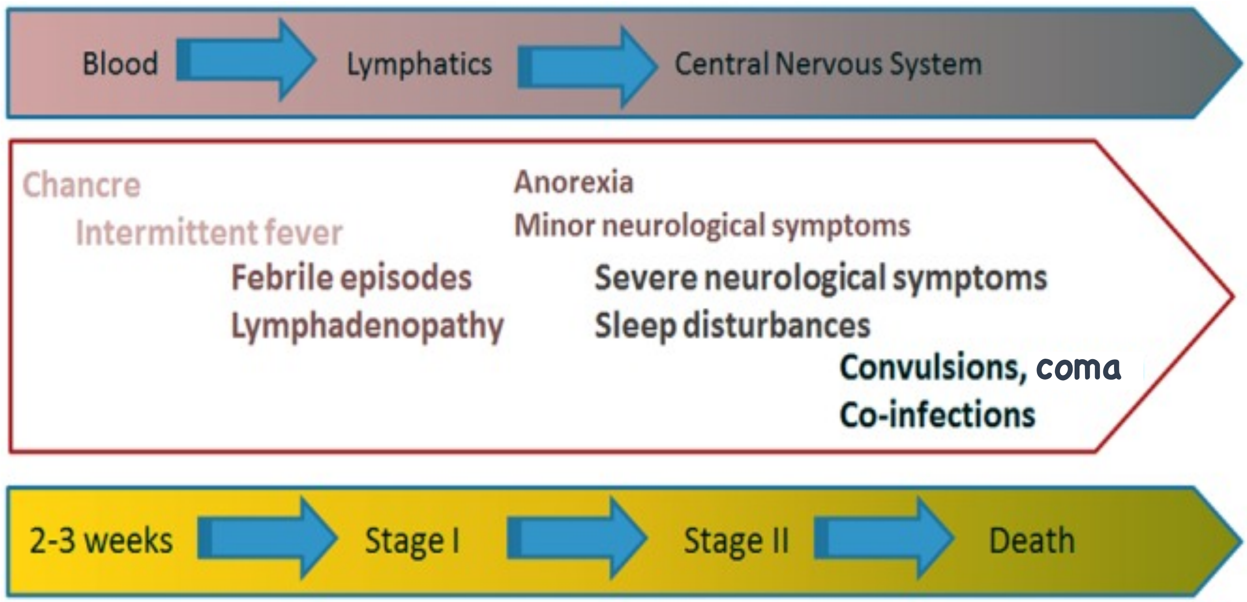

Figure 9: The Progression of Human African trypanosomiasis. Infection begins when a tsetse fly carrying the parasite *T. brucei* bites a mammalian host. The infection then progressively worsens, moving through stages as it travels through the host's system. The diagram displays the body systemic pathway taken by the parasite, as well as the corresponding stages, and some possible respective symptoms[14].

Treatment. Diagnosis of Human African trypanosomiasis must be made as early as possible in order to avoid the progression of the infection to the neurological stage, to elude complicated and risky treatment procedures. The earlier the disease is identified, the better the prospect outcome[1]. Unfortunately, an early diagnosis of HAT is difficult to make due to the lack of specific signs or symptoms present during the first stage of the disease, and because of the lack of sensitivity to the current available treatments[17]. Additionally, little progress has been made in terms of treatment of HAT in the recent decades[18].

Human African trypanosomiasis is notoriously difficult to treat due to the high toxicity and complex administration of the drugs that are required for treatment[17]. The drugs that are currently used to treat HAT are now dated, and scarcely available. Often times, parasite resistance is also encountered. Additionally, because HAT occurs in different stages of varying severity, the course of treatment depends on the current stage of the disease. Therefore, accurate diagnosis and staging of the *T. brucei* is critical, and is determined primarily by laboratory examinations[2].

The treatment of HAT is stage specific. The drugs most commonly used for treatment of HAT in the first stage are pentamidine and suramin. Both drugs were developed more than half a century ago, but have been well tolerated by patients, with hypotension and hypoglycemia as the most common reported side-effects. Pentamidine is used for the treatment of *T. b. gambiense* in the first stage, whereas suramin is used strictly for first stage *T. b. rhodesiense* HAT. Adverse reactions and severe side-effects to the drugs have also been reported following treatment, including cases of anaphylactic shocks, severe cutaneous reactions, and renal failure[18].

The drugs that are most commonly used to treat second-stage HAT are melarsoprol and eflornithine. A fifth drug, nifurtimox, is sometimes used in combination under special authorizations[17]. The organo-arsenical compound melarsoprol is the most widely used drug for treatment of second-stage HAT caused by *T. b. gambiense*, but it can be used to treat both forms of the disease. Melarsoprol is often the drug of choice in resource-poor countries where the eflornithine is not available or affordable[2]. However, due to its derivation from arsenic, melarsoprol has many undesirable side effects, the most extreme being reactive encephalopathy, which can be fatal[1]. The second drug used for second-stage HAT treatment is Eflornithine (DL-alpha-difluoromethylornithin), which is the only new molecule that has been registered for treatment of this disease within the last half a century. Treatment with eflornithine has been successful when used for *T. b. gambiense* infections, but rarely for *T. b. rhodesiense* HAT, as this form tends to be more resistant. Eflornithine has been better tolerated by patients than melarsoprol, but can cause side effects such as diarrhea, convulsions, and hallucinations. The final second-stage drug, Nifurtimox, is a cheap, orally administered drug commonly administered to treat American trypanosomiasis, which is also known as Chagas disease. Nifurtimox has demonstrated contradictory results in the treatment of *T. b. gambiense* HAT, and the type and frequency of adverse reactions are poorly documented. However, it may be possible that Nifurtimox could become an effective therapeutic alternative, perhaps in combination with other drugs, at least for *gambiense* HAT[18].

There are currently two new drugs in development that may have potential as effective treatments for HAT in the future. DB 289 is a diamidine derivative that has recently shown noteworthy activity against African trypanosomes *in vitro*, as well as in various animal models in the first stage of HAT. A combination treatment of megazol with suramin is also being tested, and has been observed as effective in a long-term experimental mouse model in late-stage HAT. However, further development of megazol is required. Several years and substantial resources will be necessary in order to develop a compound that is fully up to registration[18].

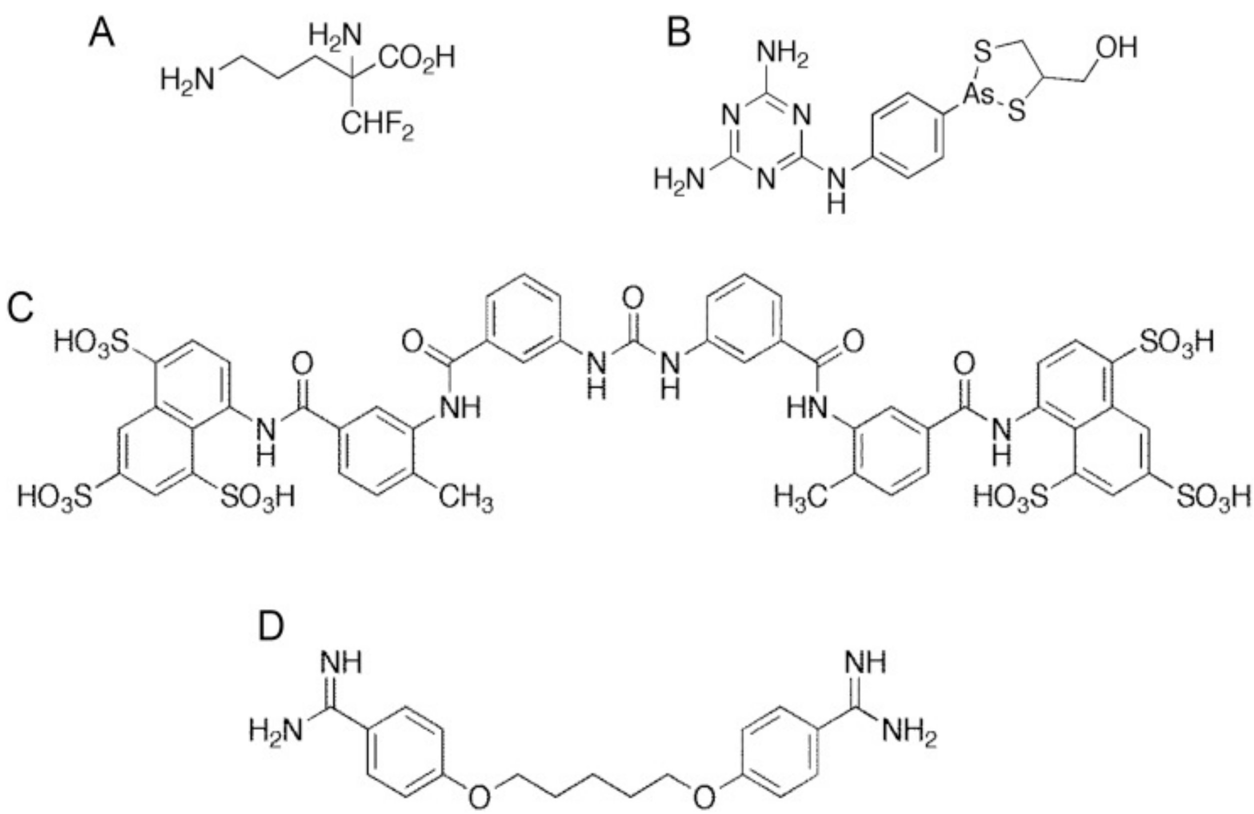

Figure 10: Chemical Structures of drugs used to treat HAT.
(A) eflornithine; (B) melarsoprol; (C) suramin and (D) pentamidine[19].

Protein Arginine Methylation. The posttranslational methylation of arginine residues that occurs on several proteins of mammals and yeast, to name a few, affects numerous cellular processes, including transcription, RNA processing, DNA replication and repair, and signal transduction. Generally, protein arginine methylation is responsible for changes in protein function, typically caused by influencing protein-protein interactions, or protein-nucleic acid interactions. By adding a methyl group to an arginine, the side chain shape is altered, and thus results in an increase of hydrophobicity and steric hindrance. Consequently, arginine methyl proteins are often distorted in their abilities to bind protein partners or nucleic acids[20].

Protein arginine methyltransferases (PRMTs) are the enzymes responsible for the catalysis of such methylation reactions. PRMTs are classified into four types, termed PRMT-I to IV, on the basis of the terminal arginine modification produced as a result of each type. PRMT types I, II, and III, catalyze the first step of an arginine methyl transfer reaction, where a single methyl group from the methyl donor S-adenosylmethionine (AdoMet) is moved to the terminal nitrogen group of an arginine residue in a protein. This ultimately yields a monomethylarginine (MMA). Type I enzymes then add a second methyl group to the same nitrogen group, yielding asymmetric dimethyl arginine (ADMA). On the other hand, type II PRMTs add a second methyl group to the adjacent nitrogen atom, forming a symmetrical dimethyl arginine (SDMA). Type III PRMTs can only catalyze the production of MMA. Finally, PRMT type IVs are only capable of catalyzing the transfer of a single methyl group to the internal nitrogen of arginine (Figure 11)[20].

In *Trypanosoma brucei*, the transcription of protein-coding genes is essentially unregulated. Instead, it is mRNA binding proteins that predominantly regulate gene expression in *T. brucei*, which impacts processes such as transcriptional stability and translational efficiency. Because protein arginine methylation is a posttranslational modification process that targets RNA binding proteins, *T. brucei* is consequently largely impacted. Alterations in the levels of proteins will then effect various aspects of *T. brucei* biology, including cell division, DNA replication and repair, and RNA synthesis and processing, as well as translation and vesicular transport[21].

To date, five PRMTs have been found to be encoded in the *T. brucei* genome – TbPRMT-1-3-5-6-7. These PRMT-encoding genes in trypanosomes are highly conserved, and suggest that arginine methylation plays a predominant role in the parasite's life cycle. TbPRMT1 has been found to exhibit type I PRMT activity *in vivo*; once it experiences a knockdown through RNA interference (RNAi), a reduction in asymmetric dimethyl-arginine results, indicating that TbPRMT1 is responsible for the majority of ADMA formation. This is in contrast to *in vitro* studies, where TbPRMT1 appears to prefer glycine-arginine-rich (GAR)-domain substrates. TbPRMT3 is also a type I PRMT enzyme, which lacks a zinc finger and differs in substrate specificity when compared to its human homologue. This ultimately suggests that PRMT3 may have functions independent of methyltransferase activity. In contrast, TbPRMT5 is a type II PRMT homologue responsible for exhibiting a broad substrate range that includes both GAR and non-GAR domains. TbPRMT6 is also a type I enzyme; it has been discovered that TbPRMT6 lacks the ability to methylate GAR domain-containing targets, and can undergo auto-methylation with unknown consequences. However, current RNAi studies of TbPRMT6 suggest that it plays a vital role in the *T. brucei* life cycle. Finally, the trypanosome TbPRMT7 enzyme, a type III enzyme, is the most unique of all, and is one of the most active PRMTs discovered thus far. This suggests that TbPRMT7 is likely involved in various cellular processes[20].

The presence of these varying types of PRMTs found in *Trypanosoma* validates the importance of protein arginine methylation, and its distinct contributions made to the *T. brucei* life cycle. The complexity of the trypanosome's life cycle transitions, which require a considerable amount of changes to be made in both gene regulation and morphology, demonstrate that the regulation of protein function through arginine methylation may be increasingly important as the parasite moves through the environment, its vectors, and their mammalian hosts. Of the five documented *T. brucei* PRMTs, only TbPRMT1-5-6 remain unchanged at the RNA level during the development phase of its life cycle, as well as during the transition from the log to stationary phase. TbPRMT3 and 7 experience decreased RNA levels, of approximately 60%, during the transition from log growth to stationary growth in the insect vector midgut procyclic form. This is then followed by a 30% increase of RNA in TbPRMT7 during differentiation from the stumpy mammalian bloodstream form to the procyclic cell form[20].

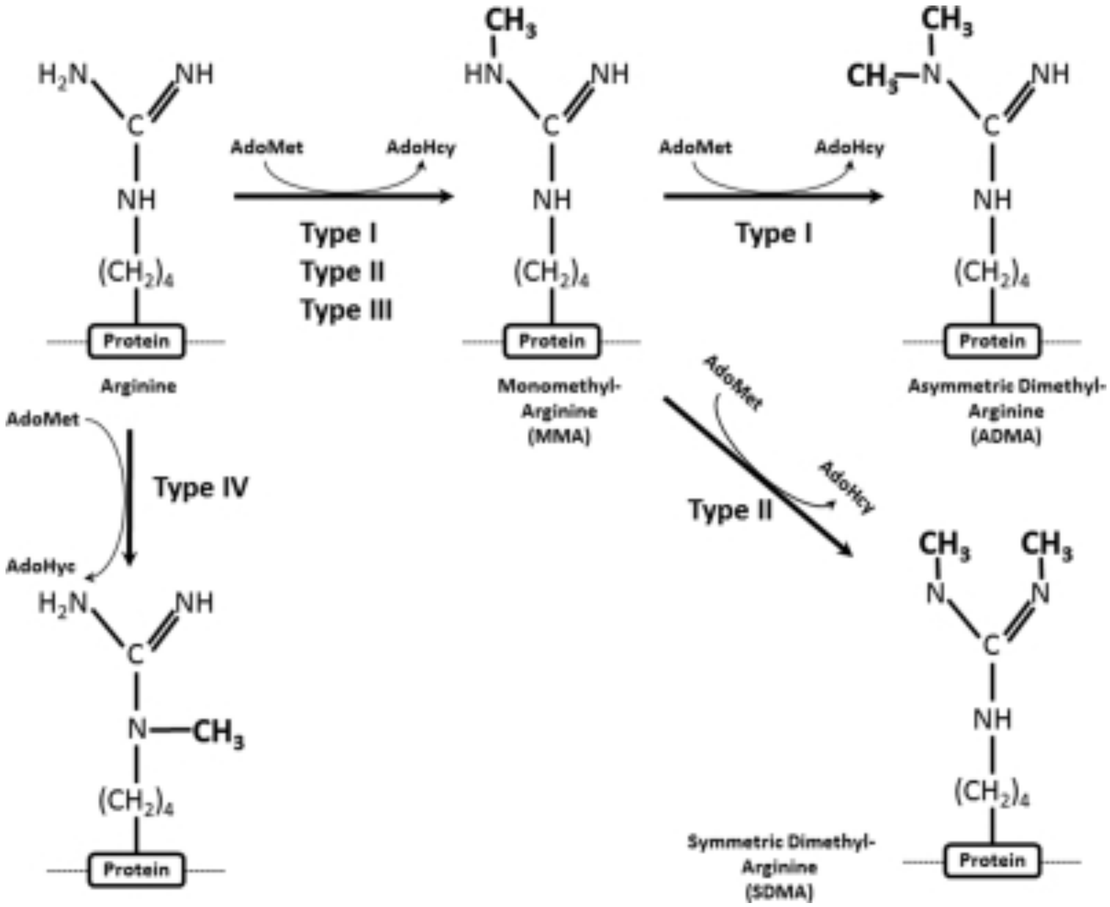

Figure 11: Protein Arginine Methylation Reactions. There are four types of PRMTs utilized in arginine methylation, each of which catalyze the transfer of a methyl group to nitrogen. The types of PRMTs are classified by the way the terminal arginine is modified; type I PRMTs synthesize ADMA by adding a second methyl group to the same terminal nitrogen, type II PRMTs transfer an additional methyl group to the adjacent terminal nitrogen, yielding SDMA, and Type IV PRMTs catalyze the transfer of a single methyl group to the internal nitrogen. Type III PRMTs can only catalyze the transfer of a methyl group to the terminal nitrogen, which yields a monomethylarginine (MMA), and S-adenosyl homocysteine (AdoHcy) as a by-product[20].

Lipin Proteins and TbLpn. Lipins are a family of proteins that have previously been shown to play an important role in phospholipid biosynthesis, gene regulation, and adipocyte development. In prior studies, TbPRMT1 was shown to interact with a trypanosome homologue of lipin, which was termed TbLpn[22]. Additionally, it has been found that all lipin proteins exhibit two regions of high sequence conservation, of which are located in the N and C-terminals of proteins, and are commonly known as the N-LIP and C-LIP domains[23]. Research shows that TbLpn possesses no homology to any other known proteins outside of these two domains, and suggests that TbLpn is a Kinetoplastid-specific protein[22].

Lipin proteins act as phosphatidate phosphatase (PAP) enzymes, which actively convert phosphatidate to diacylglycerol (DAG) during the synthesis of triacylglycerol (TAG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE)[23]. Production of TAG remains important for organisms, as it plays a key role in the maintenance of energy homeostasis. TAG is a major energy storage molecule, and its accumulation in adipose tissue allows animals to survive during food deprivation. Furthermore, activity of the PAP enzyme requires use of a DxDxT motif, which is located in the C-LIP domain. The C-LIP domain contains two key protein functional motifs, one of which is the haloacid dehalogenase phosphatase motif (DxDxT); the second nuclear receptor interaction motif is known as LXXIL. LXXIL has a transcriptional coactivator function, which is required for lipin interaction with nuclear receptors, and functions as transcriptional regulator. On the contrary, the function of the N-LIP domain remains unknown. Several studies suggest that this domain may influence catalytic activity, or nuclear localization[24].

The TbLpn protein is 806 amino acids in length and has molecular mass of approximately 86.7 kDa. Previous experiments have suggested that TbLpn can be detected in both the cytosol and nucleus of PF *T. brucei cells*. It has been suggested that, in the nucleus specifically, TbLpn might be heavily modified by post-translational modifications, such as arginine methylation and phosphorylation. *In vivo,* TbPRMT1 physically associates with TbLpn, suggesting that TbLpn may serve as a substrate for methylation by TbPRMT1, and could directly modulate TbLpn enzymatic activity or protein-protein interactions. The arginine residues interact with TbLpn, and are found to be located within preferred motifs for methylation. Additionally, because arginine residues can serve as substrates for PRMTs, they are usually found within glycine/arginine rich (GAR) domains, resulting in arginine residues throughout TbLpn undergoing methylation. The observation that TbLpn displays phosphatidic acid phosphatase activity *in vitro* indicates that TbLpn possibly plays a role in the phospholipid synthesis. Therefore, both *in vitro* and *in vivo*, TbLpn interacts with TbPRMT1. TbLpn, containing methylated arginine residues, is the only phosphatidic acid phosphatase to date to exhibit such a post-translational modification. Thus, TbLpn's ability to dephosphorylate phosphatidic acid then supports the likelihood of its involvement in phospholipid biosynthesis in trypanosomes[22].

Objectives. The overall goal is to determine the roles of TbLpn methylation on its enzymatic activity and subcellular localization. There is, thus far, no report that arginine methylation modulates the enzymatic activity of lipins or any other enzyme. However, arginine methylation has been previously shown to control the subcellular localization of several proteins in yeast and mammalian cells. This makes the observation very interesting and full of potential. In order to determine the possible methylarginine roles, we measured the phosphatidic acid phosphatase activity in *T. brucei* cells depleted for TbPRMT7. In addition, we have initiated site-directed mutagenesis of putative methylated arginine. Predicted methylarginines will be individually converted to lysines using a plasmid containing the wild type TbLpn, and as a result, this plasmid will be able to be tagged and expressed in *T. brucei*.

III. Materials and Methods

Wild type procyclic form *T. brucei* cells and procyclic form *T. brucei* cells depleted for TbLpn by RNA interference (RNAi) were obtained as described[20].

Preparation of Trypanosome Whole Cell (WC) Extracts. Buffer A was prepared using 150 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 20mM Hepes buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), adjusted to a pH of 7.9. *Trypanosoma brucei* cells were spun down using a centrifuge at 3750 RPM for 10 minutes, and then washed in 20 mL of Buffer A. Following the wash, the cells were re-spun at 3750 RPM for an additional 10 minutes, and resuspended in 500 uL of Buffer A. Ten uL of 10% NP-40 (nonyl phenoxypolyethoxylethanol) was added and passed through a 26-gauge needle 3 times.

Phosphatidic Acid Phosphatase Assay. A series of Phosphatidic Acid Phosphatase (PAP) assays were performed; first to verify that TbLpn is the major enzyme responsible for the dephosphorylation of phosphatidic acid in *T. brucei*, and second to determine the effect of TbPRMT7 methylation on TbLpn enzymatic activity. The first assay was completed in 10 tubes, prepared as shown:

Component	1	2	3	4	5	6	7	8	9	10
10X Buffer (ul)	10	10	10	10	10	10	10	10	10	10
DiC8 PA (ul)	2	2	2	2	2	2	0	0	0	0
Wild Type cell extract (ul)	0	0	2	2	0	0	2	2	0	0
TbLpn-depleted cell extract (ul)	0	0	0	0	2	2	0	0	2	2
H_2O (ul)	88	88	86	86	86	86	88	88	88	88

The 10X PAP Buffer was prepared using 0.5M Tris buffer (2-Amino-2-hydroxymethyl-1,3-propanediol, pH 7.5), and 10mM $MgCl_2$. A 10 uL aliquot was distributed to 10, 2 mL tubes, with 2 uL of 1-2-dioctanyol-sm-glycero-3-phosphate (Dic8PA) added to tubes 1-6. To account for the phosphate that was already present in the whole cell extracts, the substrate DiC8 PA was omitted from reactions 7-10. The reactions were then initiated with the addition of 2 uL of TbLpn whole cell extract, and added H_2O to a total volume of 100 uL. The reaction was allowed to proceed for 30 minutes at 30°C. To terminate the reaction, 200 uL of Malachite Green-Molybdate reagent (34 mM ammonium molybdate, 2.16 mM Malachite Green) was added to each reaction tube, and was mixed by inversion. An additional incubation period followed, at room temperature for 30 minutes, to allow for color development. Subsequently, a supplemental 300 uL of H_2O was added to each reaction tube to dilute the solutions, prior to measurement of the absorbance at 660 nm. The amount of phosphate released was then determined using a standard curve, with PAP activity expressed as nmoles of phosphate released per minute per mg of protein.

Effect of Methylation by TbPRMT7 on TbLpn PAP Activity. It has previously been shown that TbLpn is methylated by TbPRMT7 *in vitro*. To determine the effect of those methylation events on TbLpn PAP activity, we measured the PAP activity using whole cell extract from wild type PF *T. brucei*, and PF *T. brucei* depleted for TbPRMT7. The second PAP assay was prepared as shown:

Component	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
10X Buffer (ul)	10	10	10	10	10	10	10	10	10	10
DiC8 PA (ul)	2	2	2	2	2	2	0	0	0	0
Wild Type cell extract (ul)	0	0	10	10	0	0	10	10	0	0
TbPRMT7 - depleted cell extract (ul)	0	0	0	0	10	10	0	0	10	10
H_2O (ul)	88	88	78	78	78	78	80	80	80	80

The same procedure as listed previously for the Phosphatidic Acid Phosphatase assay was used to determine the effects of methylation on TbLpn enzymatic activity. This specific reaction was performed twice, and the average absorbance was used to determine PAP activity.

IV. Results

PAP Activity in TbLpn-RNAi Cells. A Phosphatidic Acid Phosphatase assay was performed to confirm that TbLpn is the major enzyme with PAP activity, and responsible for the dephosphorylation of phosphatidic acid in *T. brucei*. The reactions in tubes 1 and 2 served as negative controls, with no TbLpn RNAi cells, to serve as a blank for the experiment. The reactions in tubes 3 and 4 were then used to determine the enzymatic activity in the wild type *T. brucei* cells. Tubes 5 and 6 contained cells with a reduced amount of TbLpn, to determine the enzymatic activity of TbLpn-depleted cells. By comparison, the importance of TbLpn could then be examined. Reaction tubes 1 through 6 contained DiC8 PA, of which TbLpn utilizes to remove a phosphate and create diacylglycerol, a necessary substrate in the creation of triacylglycerol, phosphatidylethanolamine and phosphatidylcholine. Tubes 7 through 10, DiC8 PA was omitted to account for the phosphate that was already present in the whole cell extracts. The results show that the wild type TbLpn produces 48.05 (± 26.09) nanomoles of phosphate released per minute per milligram of protein, and the TbLpn-depleted cells produce 21.96 (± 14.59) nmol Pi/min/mg.

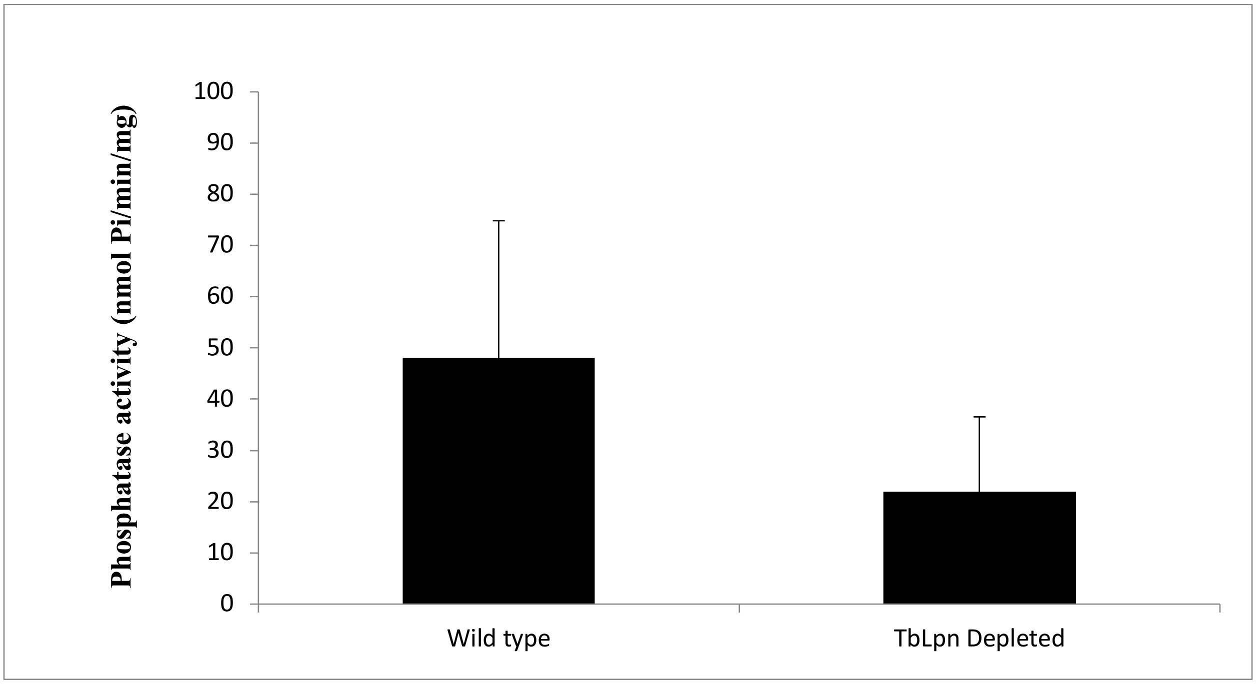

Figure 12: Phosphatidic Acid Phosphatase Activity in TbLpn-RNAi Cells. The amount of phosphate released as a result of TbLpn dephosphorylating phosphatidic acid in *T. brucei* was quantified and shown in nmoles of phosphate released per minute per mg of protein. The enzymatic activity of wild type TbLpn *T. brucei* cells is shown in comparison to TbLpn-depleted cells, allowing verification that TbLpn is the major enzyme responsible for the dephosphorylation.

PAP Activity in TbPRMT7-RNAi Cells. Two additional PAP assays were performed to determine the effect of arginine methylation on TbLpn PAP activity, using whole cell extract from wild type PF *T. brucei* and PF *T. brucei* depleted for TbPRMT7. Reaction tubes 1 and 2 again served as negative controls, containing no wild type TbPRMT7 RNAi cells, to serve as a blank. The reactions in tubes 3 and 4 contained cells with the wild type amount of TbPRMT7, and tubes 5 and 6 contained cells with a depleted amount of TbPRMT7, which results in undermethylated TbLpn. The relationship of these reactions can then be analyzed to determine the significance of methylation of TbLpn by TbPRMT7. Similar to the PAP assay performed on TbLpn-RNAi cells, tubes 7 through 10 contained no DiC8 PA, which was omitted to account for the phosphate already present. The results show that cell extracts containing wild type levels of TbPRMT7 produce an average of 17.73 (± 2.45) nmoles Pi/min/mg of protein, while the TbPRMT7-depleted cell extracts produce 12.825 (± 3.43) nmoles Pi/min/mg.

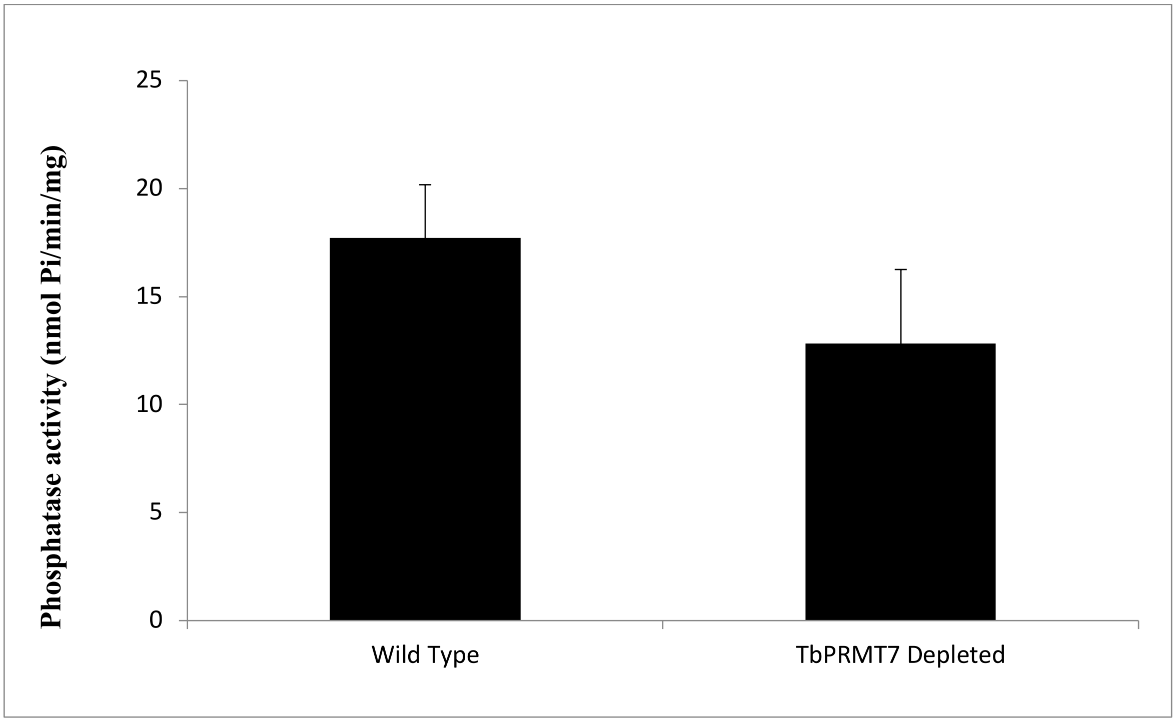

Figure 13: Phosphatidic Acid Phosphatase Activity in TbPRMT7-RNAi Cells. The phosphatase activity of wild type TbPRMT7 *T. brucei* cells is shown in comparison to TbPRMT7-depleted cells, in nmoles of phosphate released per minute per mg of protein. The relationship was utilized to determine the effect of TbPRMT7 methylation on TbLpn PAP activity, which appears insignificant.

V. Discussion

Lipin proteins, including TbLpn, function as phosphatidate phosphatase enzymes which actively convert phosphatidate to diacylglycerol during the synthesis of triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine. The production of TAG is important for the maintenance of energy homeostasis in organisms, and PE in particular plays a role in the creation of glycosylphosphatidylinositol (GPI). GPI production is vital for *T. brucei*, as it allows for the anchoring of variant surface glycoproteins, which then permits *T. brucei* to evade the host's immune system through antigenic variation. TbLpn specifically, containing methylated arginine residues and the ability to dephosphorylate phosphatidic acid, is likely involved in phospholipid biosynthesis in trypanosomes; research has previously shown that TbLpn can be heavily modified by post-translational modifications, and could impact enzymatic activity.

The first objective of this experiment was to confirm that TbLpn is the major enzyme responsible for the dephosphorylation of phosphatidic acid in *T. brucei*, which was done by comparing the nmoles of phosphate released per minute per mg of protein between two extracts; one containing wild type cells with the normal amount of TbLpn, and a second with cells that contained a down-regulated amount of TbLpn (TbLpn-depleted). A PAP assay was performed in order to quantify the amount of phosphate that was released; once TbLpn dephosphorylates the phosphatidic phosphate to create diacylglycerol, a phosphate is released, and the added Malachite Green-Molybdate reagent can then bind to the released phosphate. Following the binding of the reagent, the absorbency can be measured, and the amount of phosphate released can be calculated. The results to follow confirmed that the wild type TbLpn produced greater phosphatase activity than the TbLpn depleted cells, which therefore verifies the importance of TbLpn for the dephosphorylation of phosphatidic acid, and its effect on enzymatic activity in *T. brucei*.

Protein arginine methyltransferases (PRMTs) are the enzymes responsible for the catalysis of methylation reactions, and are classified into four types. The presence of these varying types of PRMTs found in *Trypanosoma* indicate the importance of protein arginine methylation, and the contributions made to the *T. brucei* life cycle. It has been found that *in vivo,* TbPRMT1 physically associates with TbLpn, suggesting that TbLpn may serve as a substrate for methylation by TbPRMT1, and could directly modulate TbLpn enzymatic activity or protein-protein interactions. It has also previously been shown that TbLpn is methylated by TbPRMT7 *in vitro*. In this experiment specifically, the objective was to measure the effect of arginine methylation by TbPRMT7on TbLpn PAP activity, to determine its possible importance. Following the PAP assay, the phosphatase activity of wild type TbPRMT7 *T. brucei* cells was compared to that of TbPRMT7-depleted cells. Upon comparison, the effect of methylation on TbPRMT7 PAP activity was deemed insignificant. There was minimal difference in measured enzymatic activity between the *T. brucei* cells containing the full amount of TbPRMT7, versus the cells that were depleted, and expressed very little TbPRMT7. If TbPRMT7 played a prominent role in PAP enzymatic activity, a larger difference in activity would be expected. It can therefore be concluded that the methylation of TbLpn by TbPRMT7 is not important for the enzymatic activity of TbLpn. However, it cannot be confirmed from these results that TbPRMT7 methylation plays no role in *Trypanosoma brucei*. Because the TbPRMT7-depleted cells contain a minimal level of TbPRMT7, it is possible that this small amount is sufficient to methylate TbLpn. On the other hand, it is also possible that the arginines methylated by TbPRMT7 are not necessary for TbLpn PAP activity.

TbLpn, containing methylated arginine residues, is the only phosphatidic acid phosphatase to date to exhibit such a post-translational modification. This posttranslational methylation of arginine residues affects numerous cellular processes, including transcription, RNA processing, DNA replication and repair, and signal transduction. TbLpn activity appears to be vital to *T. brucei* survival, and TbLpn's ability to dephosphorylate phosphatidic acid supports the likelihood of its involvement in phospholipid biosynthesis in trypanosomes. The trypanosome's life cycle transitions are very complex, and require a considerable amount of changes to be made in both gene regulation and morphology; this ultimately demonstrates the importance of protein function regulation through arginine methylation within the parasite, as it moves through the environment, its vectors, and its mammalian hosts. Because of this, it is increasingly important to understand how TbLpn is affected by arginine methylation, and the resulting effects on cellular localization and enzymatic activity. Further experiments must be conducted to determine what role arginine methylation has on TbLpn enzymatic activity.

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