Characterization of TbLpn in
Trypanosoma brucei

by

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I. Abstract

Trypanosoma brucei, is a flagellated, unicellular, parasitic protozoan transmitted by the tsetse fly. It is the source of African sleeping sickness in humans. African sleeping sickness has two different stages, the bloodstream and central nervous system stages, each characterized by different symptoms. Problems with treatment result from severe side effects of the drugs used to treat African sleeping sickness. No vaccine is available due to high antigenic variation. *T. brucei* exists as two forms. The procyclic fly form relies on oxidative phosphorylation, expresses procyclin as its surface protein, and is morphologically long and slender. In contrast, the mammalian bloodstream form expresses the surface protein VSG, and is characterized as short and stumpy. In *T. brucei*, gene regulation is controlled primarily at the post-transcriptional level, thus RNA binding proteins play a role in gene regulation. Some RNA binding proteins serve as substrates for enzymes known as protein arginine methyltransferases (PRMTs). These enzymes specifically methylate arginine residues on proteins. A yeast two hybrid approach was used to identify proteins interacting with TbPRMT1 in *T. brucei*. Among the proteins shown to interact with TbPRMT1, one is a homolog of yeast and mammalian lipin proteins. This protein, which is termed Tblpn, has 2 conserved domains characteristic of lipin proteins. In addition, 2 aspartic acid residues were conserved *in T. brucei*. Lipin is involved in adipocyte development in mice. A mutation of lipin causes decreased adipocyte development associated with fatty liver dystrophy. Overexpression of the protein results in obesity in mice. Lipin also plays an important role in fatty acid synthesis and signaling in yeast, but possibly relates to the development of important phospholipids in *T. brucei*, specifically phosphatidylethanolamine and phosphatidylcholine. The objectives of my project were to determine where TbLpn is localized in the cell, to determine

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whether TbLpn interacts with TbPRMT1 *in vivo*, and finally to determine if TbLpn is methylated *in vivo*.

II. Introduction

A.) *T. brucei* and African Sleeping Sickness. *Trypanosoma brucei* is a unicellular, flagellated, parasitic protozoan, responsible for African sleeping sickness in humans. *T. brucei* is also responsible for Nagana in livestock, a similar form of disease to the human African Sleeping Sickness. *T. brucei* are motile and possess a kinetoplast with extra nuclear DNA enclosed in a mitochondrial envelope associated with the flagellum (Figure 1). *T. brucei* range in length from 15-35 μm (1). There are two sub-species of *T. brucei*, *T brucei gambiense,* dominant in Western Africa, and *T.brucei rhodesiense*, which is more prevalent in East and Southern Africa as well as east of the Rift Valley (2). The tsetse fly, of the genus *Glossina*, serves as the vector.

B.) *T. brucei* vector and animal reservoir. The disease caused by *T. brucei*, African Sleeping Sickness, is transmitted by the tsetse fly (Figure 2). The tsetse fly resembles the house fly, with a length of 8-17 mm (3). The tsetse fly prefers to feed on game animals and domestic stock. Human infection occurs when people come into contact with the zoonotic cycle. The people most infected are poachers, hunters and tourists (1).

Figure 1: ***Trypanosoma brucei.*** *Trypanosoma brucei*, is a unicellular, flagellated, parasitic

protozoan, responsible for African sleeping sickness in humans. *T. brucei* range in length from 15-35 µm (1). There are two sub-species of *T. brucei*, *T brucei gambiense,* dominant in Western Africa and *T.brucei rhodesiense*, which is more prevalent in East and Southern Africa as well as east of the Rift Valley (2).

Figure 2: The tsetse fly of the genus *Glossina*. The vector of African Sleeping Sickness which transfers *T. brucei* from mammalian host to mammalian host.

C.) *T. brucei* lifecycle. The lifecycle of *T. brucei* begins when the tsetse fly takes a blood meal from the mammalian host. The larval trypanosome, known as metacyclic trypomastigotes, transforms into bloodstream trypomastigotes once inside the mammalian host. The parasite is carried through the bloodstream to other sites in the human body. The trypomastigotes multiply by binary fission while in the bloodstream. The tsetse fly takes another blood meal, ingesting the bloodstream form trypomastigote. In the fly's midgut, the bloodstream form of the parasite transforms into the procyclic trypanosome and multiplies by binary fission. The procyclic form leaves the midgut, transforming into the epimastigote. The epimastigote multiplies in the salivary glands. At this time, the parasite transforms into the metacyclic trypomastigote. The life cycle repeats after the metacyclic trypomastigote enters another mammalian host (Figure 3) (4).

The lifecycle of *T. brucei* occurs in two stages. During the procyclic stage, the parasite resides in the fly. During the second stage, mammalian stage, the parasite has been transmitted from the fly to the mammalian host.

The procyclic and mammalian forms exhibit three major differences. First, the surface protein expressed between the mammalian and fly *T. brucei* are different. The fly trypanosome expresses the protein procyclin, while the mammalian trypanosome expresses variant surface glycoprotein (VSG). The fly trypanosome is long and slender, while the mammalian form is short and stumpy. Finally, the fly trypanosomes rely on oxidative phosphorylation to produce energy and have highly reactive mitochondria.

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Tsetse fly Stages

Human Stages

Epimastigotes multiply in salivary gland. They transform into metacyclic trypomastigotes.
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1 Tsetse fly takes a blood meal (injects metacyclic trypomastigotes)

Injected metacyclic trypomastigotes transform into bloodstream trypomastigotes, which are carried to other sites.
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7 Procyclic trypomastigotes leave the midgut and transform into epimastigotes.

3 Trypomastigotes multiply by binary fission in various body fluids, e.g., blood, lymph, and spinal fluid.

5 Tsetse fly takes a blood meal (bloodstream trypomastigotes are ingested)

6 Bloodstream trypomastigotes transform into procyclic trypomastigotes in tsetse fly's midgut. Procyclic trypomastigotes multiply by binary fission.

4 Trypomastigotes in blood

△ = Infective Stage
△ = Diagnostic Stage
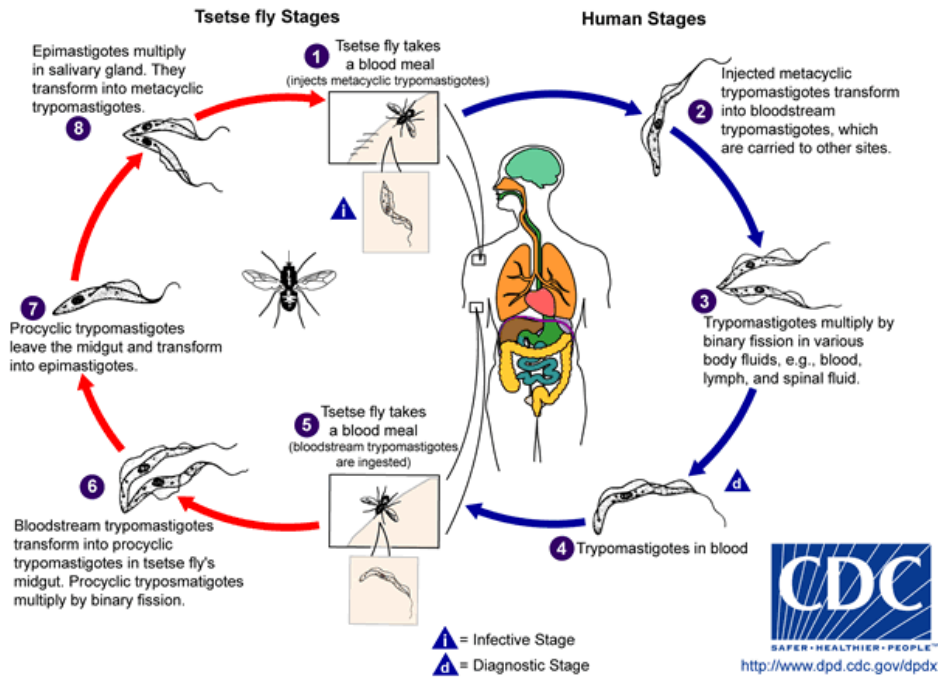
http://www.dpd.cdc.gov/dpdx

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Figure 3: The lifecycle of *T. brucei.*

D.) African Sleeping Sickness. African Sleeping Sickness undergoes two stages. The first stage, known as the bloodstream or hemolymphatic stage, is treatable and symptoms include a hardened chancre at the bite, skin lesions (trypanids) on light skinned patients, tachycardia, fevers, edema, an irregular rash and weight loss. During this stage trypanosomes multiply in subcutaneous tissue, blood and lymph (5). The second stage, CNS stage, is not treatable because the parasite has crossed the blood/brain barrier into the central nervous system of the affected patient. Once in the brain, the disease is almost impossible to treat due to potential damage to the brain and the inability of the drugs to cross the blood/brain barrier. Symptoms include irritability, tremors, speech and sleep disorders, seizures, coma and ultimately death.

E.) Re-emergence of African Sleeping Sickness. African sleeping sickness is a re-emergent disease. In 1999 50,000 cases were reported. The World Health Organization (WHO) estimates about 300,000 to 500,000 new cases each year in Sub-Saharan Countries. Specifically, the WHO reports that the people most exposed live in rural areas dependent on agriculture, fishing, animal husbandry or hunting. Recent reports indicate that the number of cases dropped below 10,000 for the first time in 50 years in 2009 (5). In 2010, the WHO reported a total of 7139 new cases in 36 endemic countries. This is compared to the 9878 cases reported in 2009. This represents a 28% decrease in one year (Figure 4) (5). However, this figure is likely an underreporting due to the progressive nature of the infection and the therapeutic and diagnostic difficulties of the disease. Furthermore, Africa is facing human crises to which most aid organizations and national governments are unable to respond (1).
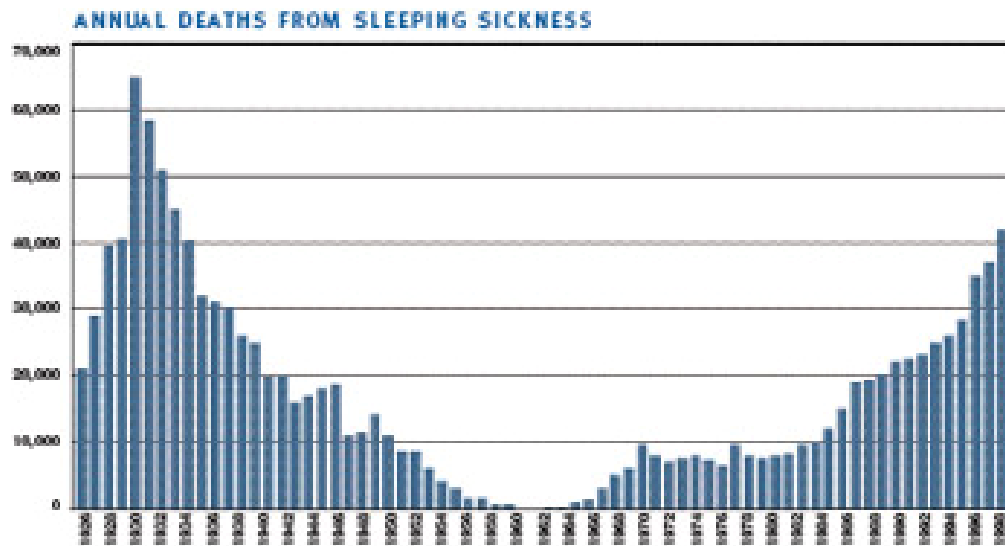
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ANNUAL DEATHS FROM SLEEPING SICKNESS

Figure 4: Chronological Distribution of African Sleeping Sickness. African Sleeping Sickness is a re-emergent disease is Africa. From 1930 to 1960, the rate African Sleeping Sickness infection

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deceased due to the development of treatments. However, soon after, in 1964, African Sleeping Sickness began to increase the developing antigenic variation of the parasite.

Sleeping Sickness was first identified in the closing years of the 19th century. This period was marked by widespread epidemics of the disease in a large majority of Western Africa, including Kenya, Tanzania, Uganda and Nigeria (Figure 5). African Sleeping Sickness was associated with social and environmental disruptions resulting from colonial occupation. African Sleeping Sickness was controlled during the 1960s in much of Africa, but has re-emerged since the 1970s. The re-emergence of African Sleeping Sickness can be attributed to post-independence turbulence, unstable governments, limited public health resources and the re-allocation of domestic and international funding towards those diseases catching more headlines, such as Malaria and HIV/AIDS (2).

In the 20th century, there were three major epidemics of African Sleeping Sickness in Africa. The first epidemic afflicted Uganda and the Congo in 1906. An estimated 300,000-500,000 people died in the Congo basin and the Busoga focus in Uganda and Kenya. The epidemic initiated an investigation of the disease and the development of a cure. In 1902, French physician Charles Louis Alphonse Lavern and French biologist Félix Mesnil reported that sodium arsenite was effective in curing the disease in laboratory animals (6). At the time, this was the only known cure to African sleeping sickness. After further investigation of the drug by German physician Robert Koch, it was discovered that the drug was in fact toxic. Of the 1622 patients treated with the atoxyl, Koch found that 22 patients observed atrophy of the optic nerve with complete blindness. In 1916, the first effective drug was developed to cure African Sleeping Sickness. The compound, known as Bayer 205, is still in use to cure the early stages of *T. b.*

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rhodesiense infection (6).

Countries reporting no cases
Countries reporting under 100 cases
Countries reporting between 100 & 1000 cases
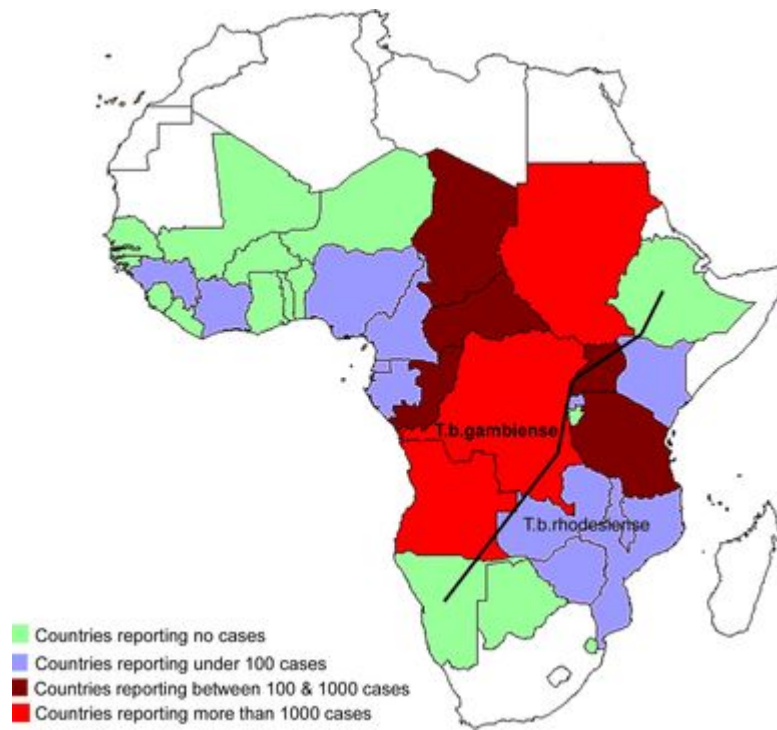Countries reporting more than 1000 cases

Figure 5: Geographic distribution of African Sleeping Sickness. There are two species of *T. brucei*. *T. brucei gambiense* is located in the northwestern portion of the country. Meanwhile, *T. brucei rhodesiense* is located primarily in the southeastern portion of the country. African sleeping sickness is contained primarily within the central region of Africa. The line represents the division between *T.b. gambiense* and *T.b. rhodesiense*.(http://creativecommons.org/licenses/by/3.0/)

Other factors contribute to African Sleeping Sickness's epidemiology besides the lack of an effective, non-toxic treatment. For example, the impoverished socio-economic environment left by colonization. A large amount of the population was displaced by colonial occupation and as a result many people experienced famine, which created ideal conditions for the spread of infection. The mid-1920s brought improvements to nutrition and hygiene (6).

Further factors that contributed to the spread of the disease were conflict, war, and increases in travel and trade. For example, between 1976 and1990, a *T. b. rhodesiense* epidemic coincided with political instability and conflict during and after the rule of Idi Amin (2). The civil war which occurred in Uganda influenced the spread of disease in several ways, including a decline of veterinary and public health services, the collapse of vector control, a re-growth of tsetse fly habitat, and the increasing displacement of humans and animals (2).

The resistance by trypanosome against certain drugs goes beyond drug transport into or outside of the cell. For instance, resistance of Melarsen is associated with a reduction of lipoic acid content (7).

In addition, drug resistance has been shown in a failure to undergo apoptosis. Melarsoprol was shown to induce apoptosis in cancer cells (8). Apoptosis-like cell death was reported in

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procyclic *T. b. rhodesiense* and was suspected in stumpy bloodstream forms (7). Nevertheless, drug resistance in mammalian *T. brucei* is contributed to by high antigenic variation of the parasite, due to the expression of variant surface glycoprotein (VSG).

F.) Treatment. As previously mentioned, African trypanosomes evade the mammalian immune system by antigenic variation of their surface proteins, which is one of the reasons why African sleeping sickness has been considered a re-emerging disease since the late 1990's (7).

There is currently no vaccine against African Sleeping Sickness. However, the treatment has relied on chemotherapy. This poses the problem that drug resistance has been described in every pathogen treated with chemotherapy (7).

Several of the drugs used for African Sleeping Sickness today date back to the first half of the last century. They were developed from synthetic dyes, organic arsenicals and diamidines (7). Many of these drugs are derived from arsenic. Arsenic kills the trypanosome, but is toxic to the patient. It produces many severe side effects, weakening the patients' immune system, and rendering them unable to combat other diseases.

Initiated by Paul Ehrlich, the use of synthetic dyes against African trypanosomes developed into suramin (Figure 6), which is still used to treat the early-stages of African Sleeping Sickness and Nangana in livestock. The problem is that in mammalian cells, suramin was shown to inhibit topoisomerase II. Furthermore, suramin interferes with polyamine salvage and cell signaling (7).

Early treatments of African sleeping sickness had difficulties crossing the blood-brain barrier, as trypanosome can do, initiating the second stage of African Sleeping Sickness. The development of organic arsenical tryparsamide created the first antitrypanosomal drug that penetrated the blood-brain barrier (7). However, extensive use of the drug resulted in tryparsamide–resistant trypanosomes. The problem was solved with the development of Melarsoprol (Figure 7), which inhibits the glycolytic enzymes and phosphogluconate dehydrogenase. This forms a stable complex with trypanothione, MelT, inhibiting trypanothione reductase (7). Melarsoprol is still used for treatment of late stage sleeping sickness, however patients suffer severe side effects such as encephalopathy (9).

suramin

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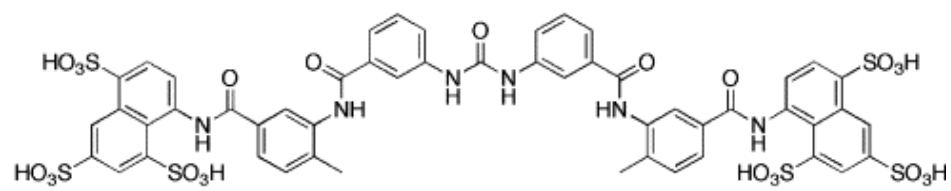
Figure 6: Chemical structure of the drug suramin.
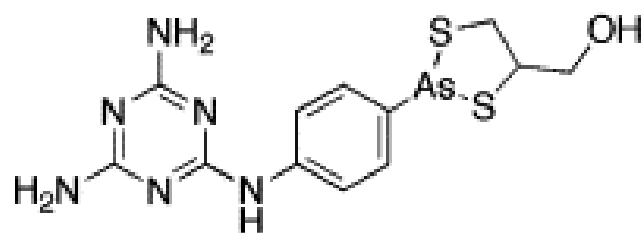
melarsoprol

Figure 7: Chemical representation of Melarsoprol, introduced for the treatment of late stage human African trypanosomaisis.

Another drug which is used in the treatment of African sleeping sickness is Pentamidine (Figure 8). It is used for the treatment of early-stage sleeping sickness. Berenil is one of the main drugs used to treat Nagana. These drugs accumulate in the mitochondria and bind to the minor groove of DNA. They interfere with kinetoplast replication by changing DNA topology and inhibiting topoisomerase. In yeast and mammalian cells, Pentamidine inhibits mitochondrial function (7). Nevertheless, trypanosomes developed ways to escape the host's immune system by switching the surface protein VSG. Due to the expression of different VSG surface protein every cycle, this is the major cause for antigenic variation and the difficulties of producing a vaccine for African trypanosomiasis.

pentamidine

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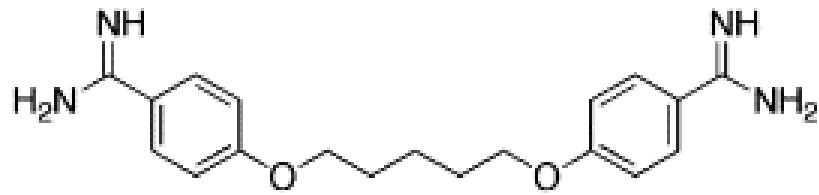
Figure 8: Chemical representation of pentamidine, used for early stage trypanosomiasis.

G.) Variant Surface Glycoprotein (VSG).

Several pathogens evolved strategies for variation of surface proteins which allows a pathogen to establish persistent infection. *Trypanosoma brucei* evade the host's immune system by changing their surface protein VSG (10). African trypanosomes are pathogenic kinetoplastid protozoan which exist in both the tsetse fly and mammalian reservoirs. In each, *T. brucei* contain distinct stage specific glycosol phosphadityl-inositol (GPI) anchored protein (Figure 9). The tsetse fly trypanosomes express the surface proteins procyclin while the mammalian form of *T. brucei* expresses the surface protein VSG (11).

Mammalian VSG has multiple forms. VSG is encoded by a polycistronic transcribed telomeric expression site such that each VSG protein expresses one at a time (10). VSG constitutes a dense monolayer surface coat that covers the whole cell. The protein is synthesized in the ER where it is then modified by N-linked glycosolation followed by the addition of GPI (11).

While most VSG genes are not telomeric, many of the genes are present in the telomeres. The expressed VSG is always located in the telomeres (12). VSG expression sites consist of a 45 to 60 kb transcription unit from the promoter to the end of the gene. The variation in length depends on the presence of a 13-kb element at the 5' extremity (12).

After every cycle, VSG is shed and replaced with a new VSG protein. The VSG vaccine for one VSG protein will not affect the other. Central to antigenic variation, recombination of these genes which encode VSG protein allows the parasite genome to encode multiple forms of the
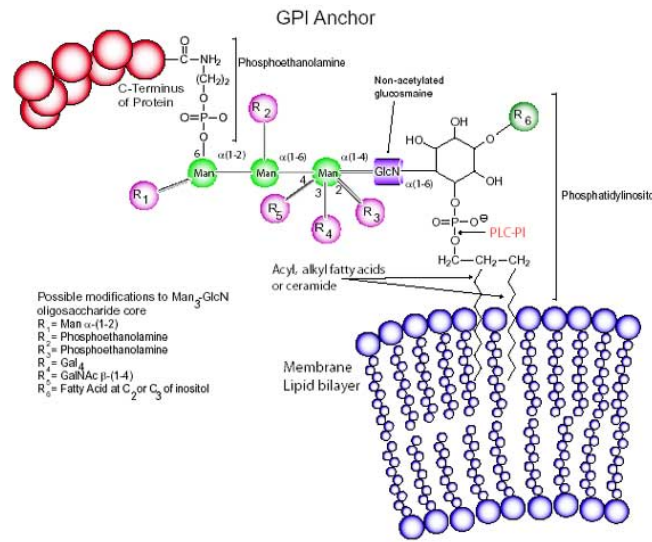
protein (10)

Figure 9: Molecular representation of a generic glycosol phosphadityl-inositol (GPI) anchored protein. The GPI anchor, anchors the surface protein of *T. brucei* to the lipid bilayer of the organism. In mammals, *T. brucei* expresses variant surface protein (VSG) while in the tsetse

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fly, *T. brucei* expresses procyclin. In the mammalian form of the parasite, VSG is expressed one at a time.

In the bloodstream form all but one VSG expression site is repressed. In contrast, in the procyclin form VSG is totally down-regulated (12). During the transition between VSG to procyclin expression, VSG specific mRNA and protein synthesis is repressed and VSG is shed (13). These mechanisms involved in VSG gene silencing and allelic expression are potential targets for chemotherapy (10).

During transcription, only one bloodstream expression site must escape silencing to maintain homogeneity of VSG coat and the ability to rapidly swap the exposed epitopes (10). The mechanism that allows one ES to escape silencing is unknown and the ESB-specific factor has not been identified (10).

Short-range telomeric silencing has been found in regions adjacent to telomeric repeats. The mechanism requires SIR2rp1, which is the only nuclear NAD-dependent histone deacetylase in *T. brucei*. The histone acetyltransferase, HAT1, and histone deaceytlase, DAC1, regulate SIR2rp1. In contrast, long-range VSG ES silencing has been linked with chromatin structure and modification (10).

VSG switching by recombination occurs by gene conversion reactions in which copied silent VSG move into the active ES, ultimately replacing previous VSG. The recombination which operate downstream RAD51-mediated strand exchange are not fully understood, however RAD51-mediated homologous recombination has shown to be important in antigenic variation (10).

The lifecycle of *T. brucei* cycles between the bloodstream form and the fly form. The transition between these two forms include changes in morphology, metabolism and RNA and protein expression. Therefore, because the genome of *T. brucei* is transcribed constitutively, regulation of gene expression occurs exclusively by post-transcriptional mechanisms (14). These mechanisms include mRNA processing, mRNA degradation, translational efficiency, protein processing and modification (14).

H.) Gene Expression in *T. brucei.* Transcriptional regulation is an important contributor to the control of gene expression in both prokaryotes and eukaryotes. Given the abundance of transcriptional regulation in most organisms, the fact that small eukaryotic organisms, such as *T. brucei*, evolved to rarely use it. This has been found as a consequence of genome organization (15). For example, protein coding genes are arranged in long head to tail tandem arrays of 10-100 genes. In addition, processing into mature monocistronic mRNAs occurs by trans-splicing the spliced leader of SL-RNA to the 5' end of each mRNA (15).

The regulation of gene expression occurs at the post-transcriptional level with one exception. The genes that code for *T. brucei* cell surface proteins procyclin and VSG (15). Kinetoplastids are exposed to extracellular stimuli and stresses similar to other organisms and their ability to adapt their gene expression to new environments is crucial (15). The parasite responds by initiating a complex program of developmental transmissions that allow adaptation to the new host (15).

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The parasitic cycle of *T. brucei* are also characterized by a succession of different forms adapted to different environments they encounter, and the alternation of growing and non-growing stages (12). In the mammalian form of *T. brucei* the cells are actively dividing and mitochondrial function is repressed. The parasite expresses the surface protein VSG. In contrast, the procyclic form of *T. brucei* has fully active mitochondria in which oxidative phosphorylation can be utilized (12).

Transcriptional regulation is essentially absent in *T. brucei*. Therefore, gene regulation is primarily controlled at the post-transcriptional level. Post-transcriptional mechanisms of regulation influence splicing, transport, stability, localization and translation (14). Post-transcriptional regulation is mediated by *trans*-acting factors that recognize *cis*-acting sequences. The kinetoplastids rely on post-transcriptional mechanisms with post-transcriptional regulons, the candidates for gene regulation (14).

The initial discoveries of splice leader (SL) *trans*-splicing were made in *T. brucei,* and it has remained the preferred organism for spliceosomal studies (16). Expression of VSG has been a target for research because VSG mRNAs contain a 5'-splice leader region not encoded in the VSG gene. In addition, the discovery of a Y structure intermediate that corresponded to the *cis*-splicing lariat indicates that SL transfer functions are analogous to intron removal (16). This SL *trans*-splicing reaction is not restricted to VSG mRNA but is also used in the maturation step of trypanosomatid mRNAs. The genomes of trypanosomatids tandemly arrange in polygenic clusters which transcribe in a polycistronic fashion. *Trans*-splicing and polyadenylation lead to cleavage upstream and downstream of the coding regions. Although the sequences or mechanisms

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responsible have not been fully described, the richness in pyrimidine of the 5' environment of each gene may account for differences in *trans*-splicing, influencing the final mRNA (12).

Alternative splicing is important to achieving tissue specific gene expression and regulating expression during development. Kinetoplastids are unusual in that their genomes have lost their introns. This rules out exon skipping or intron retention as a mechanism to produce distinct proteins (15). Each mRNA in *T. brucei* exists in several isoforms, each one differing in the lengths of their UTRs. This suggests an evolutionary shift toward *trans*-splicing to avoid the production of frame-shift proteins. However, some examples of alternative splicing result in 5' truncation of the open reading frame and different intracellular localization of these proteins (15).

VSG mRNA represents the most abundant polyadenylated transcript (12). Polyadenylation of mRNA represents an important post-transcriptional modification. Polyadenylation has various effects on RNA stability. *In vitro* studies with *T. brucei* mitochondria suggest polyadenylation plays a role in regulating RNA stability (17). Protein factors that are involved in such pathways, such as poly(A) polymerases (PAPs) play a key role in understanding how polyadenylation regulates mRNA stability. Several of these enzymes have been described to catalyze polyadenylation reactions. These alternations in the polyA-tail length exerted positive or negative effects on steady-state levels of mitochondrial mRNA (17).

Although gene regulation is a post-transcriptional event, the result of gene regulation in *T. brucei* produces RNA binding proteins. These proteins might regulate gene expression that are part of the regulon (14). Several RNA binding proteins serve as substrates for a group of enzymes

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known as protein arginine methyltransferases (PRMTs).

I.) Protein Arginine Methylation. Arginine methylation is a post-translational modification which adds CH_3 to the N-terminus of arginine (Figure 10). Arginine is a positively charged amino acid known to mediate hydrogen bonding and amino aromatic interactions. Methyl groups are added to the guanidino nitrogens on arginine. Two major types of PRMTs have been described. First, both type I and II PRMTs catalyze the transfer of a methyl group from S-adenosylmethione (SAM) to an arginine residue, resulting in monomethylarginine (MMA). Second, type I PRMT adds a second $–CH_3$ to the same nitrogen, forming di-methylarginine (DMA). Finally, the second arginine is added on different nitrogen by type II PRMTs.

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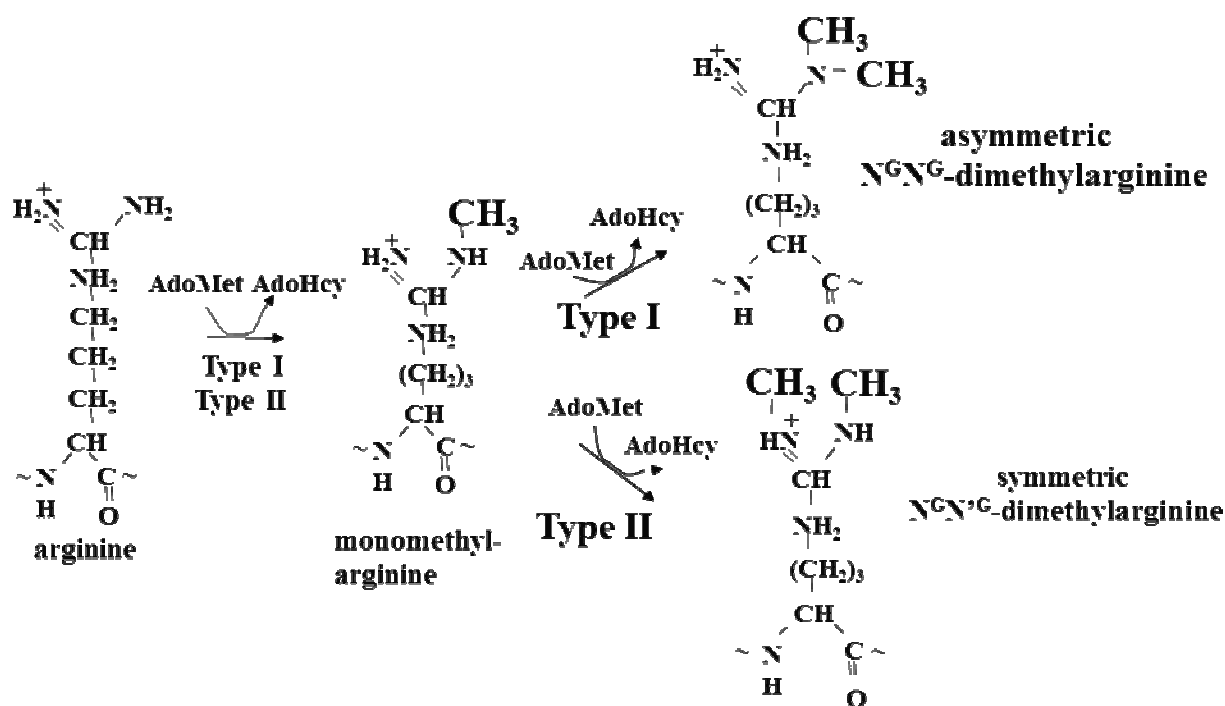
Figure 10: Arginine Methylation Reaction. Arginine methylation is a post-translational modification catalyzed by protein arginine methyltransferase (PRMT). Both type I and type II PRMT add a methyl group onto arginine, resulting in monomethylarginine. In addition, type I PRMT add a second methyl group onto the same terminal nitrogen as the first reaction, resulting in an asymmetric dimethylarginine. Type II PRMT adds the second methyl group onto the opposite N-terminus, resulting in a symmetric dimethylarginine molecule.

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It is undetermined how arginine methylation changes protein function. Although methylation should not affect the overall charge of an arginine residue, it is expected to add bulk and hydrophobicity that can promote or inhibit intra- or intermolecular interactions. These interactions change the shape and the function or stability of the methylated protein. These changes serve to facilitate or interfere with intermolecular interactions or enzymatic activities that play important roles in specific signaling pathways. (18).

Cellular processes regulated by arginine methylation include RNA processing, transcriptional regulation, signal transduction and DNA repair. Arginine methylation has the potential to control multiple aspects of gene expression through regulation of both protein:protein and protein:RNA interactions. Although, the impact of arginine methylation on nuclear and cytoplasmic processes has been shown, the role of argnine methylation in regulating mitochondrial gene expression is not well understood (19).

Several PRMTs are found in multiple organisms. PRMTs are present in yeast and mammals. TbPRMT1 was the first PRMT described in protozoan. TbPRMT1 is the major type I PRMT found in *T. brucei*.

PRMT1 methylates substrates with regions containing Arg-Gly-Gly repeats. PRMT-1 is an enzyme with long, meandering but continuous grooves providing multiple sites that bud substrate peptides (18). The structure of PRMT-1 reveals a dimer ring characterized by an acidic cavity at the dimer interface. This acidic cleft forms the binding surface which targets arginine residues of

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positively charged arginine-rich regions of substrate proteins for methylation (20). TbPRMT1 displays protein methyltransferase activity. It has been shown that arginine methylation by TbPRMT-1 modulates the ability of several proteins to interact with different RNA and ribonucleoproteins.

As mentioned, arginine methylation regulates several cellular processes. One of these processes is signal transduction which is governed by post-translational modifications that alter protein function by altering protein:protein interactions. Several pathways utilize arginine methylation for signal transduction downstream of the interferon receptor, T-cell receptor, cytokine receptor and nerve growth factor (NGF) receptor (22).

Transcription regulation is another cell process that is regulated by arginine methylation. Arginine methyltransferases modify proteins that function at many steps in cellular regulation such as cytoplasmic and nuclear signal transduction, nuclear-cytoplasmic shuttling and transcriptional activation (18). Specifically, PRMT1 has been confirmed to be an arginine-specific histone methyltransferase. PRMT1 methylates H4 at Arg-3. The methylation reaction of H4 by PRMT1 is stimulated by the acetylation of this histone by p300 (18).

In *T. brucei,* TbPRMT1 appears to play a role in the lifecycle. A screen for gene function indicates growth impairment and nuclear defects in the bloodstream form after depletion of PRMT1 expression via RNA interference. This growth defect is not seen, however, in the procyclic form. It appears that this stage-specific growth defect which is explained by the role of PRMT1 in *T. brucei* mitochondrial gene expression (20).

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In order to elucidate TbPRMT1 functions, a yeast-two hybrid analysis was used to identify proteins interacting with TbPRMT1 in *T. brucei*. Among the proteins shown to interact with TbPRMT1 were TbMiz1, a protein containing a zinc finger domain and TbADP, a protein containing an acidic domain. In addition, a homolog of yeast and mammalian lipin proteins was also identified. This protein, known as Tblpn, has 2 conserved domains characteristic of lipin proteins with 2 conserved aspartic acid residues in *T. brucei*.

J.) Lipin. Lipin plays an important role in adipocyte development. Adipocytes are the source of secreted factors such as leptin and adiponectin which have important roles in metabolism in the brain and peripheral tissues. Adiponectin increases glucose uptake in skeletal muscle and reduce hepatic glucose production and inflammation (21). Lipodystrophy and obesity promote lipid accumulation in skeletal muscles and pancreatic beta cells, leading to impaired function of these tissues and impaired metabolic homeostasis (21).

Lipin-1, the founding member of the lipin protein family, was identified by cloning of the mutant gene underlying lipodystrophy in mice with fatty liver dystrophy (21). Lipin-1 deficiency has shown that lipin is required for adipocyte differentiation, which occurs through an ordered cascade of gene expression changes (21). Deficient cells fail to induce expression of two key transcription factors, PPARγ and C/EBPα along with their target genes (21).

Mutations of the lipin protein decreases adipocyte development associated with the disease fatty liver dystrophy (fld) in mice. The mutation converts evolutionary conserved glycine to

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arginine. This results in a phenotype similar to the null mutation. The *fld* mouse lacks normal tissue deposits throughout the body (21).

In contrast, an over expression of lipin causes obesity in mice (Figure 11). A study which characterized lipin proteins revealed that lipin-1 transgenic mice had normal adipocytes, but had increased fat cell triglyceride content and increased expression of lipogenic genes, including fatty acid synthase, acyl-CoA carboxylase and DGAT. It is suspected that tissues are efficently trapping fatty acid in the adipose tissue, reducing lipid deposit in the muscle, liver and other tissues (21).

Figure 11: An obese mouse as a result of lipin over expression.

Lipin plays an important role in lipid synthesis and signaling in yeast (Figure 12). Studies of lipin-1 revealed important physiological roles in metabolic homeostasis. Lipin-1 has been shown to serve as an enzyme for triacylgerol and phospholipid biosysnthesis. In addition, lipin-1 has been shown to act as a transcriptional coactivator that regulates expression of fatty acid utilization and lipid synthetic genes (21).

Lipin plays a role in the synthesis of TAG, which is a principal constituent of lipid droplet cores. Moreover, phospholipids are components of the lipid droplets, which includes phosphaditylcholine (PC) which requires DAG (23). Triglyceride cores synthesize through acylation of glycerol-3-phosphate. Diacylglycerol, a precusor to TAG formation, also required for the synthesis of phospholipids, including phosphotidylcholine (23). In most mammalian cell types, TAG is synthesized via the glycerol phosphate pathway (21).

The process is a Mg^{2+}-dependent dephosphorylation of phosphatidic acid (PA) to form diacylglycerol (DAG) releasing inorganic phosphate. DAG reduces to triacylglycerol (TAG), producing fatty acids. The process catalyzed by the enzyme PA phosphatase (PAP), encoded by lipin (23). DAG and PA together play a key role in cell signaling, in both growth of the nuclear/ER membrane and the regulation of phospholipids synthesis genes.

The importance of TbLpn in trypanosomes is strongly suggested by the fact that phosphatidylcholine and phosphatidylethanolamine constitute the majority of cellular phospholipids in *T. brucei.* In addition, as *T. brucei* synthesizes PC and PE *de novo*, the pathways leading to the generation of PC and PE are essential in *T. brucei*. Finally, as mentioned earlier, PE

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is a precursor of GPI synthesis and regulation of its synthesis is likely to play an essential role in
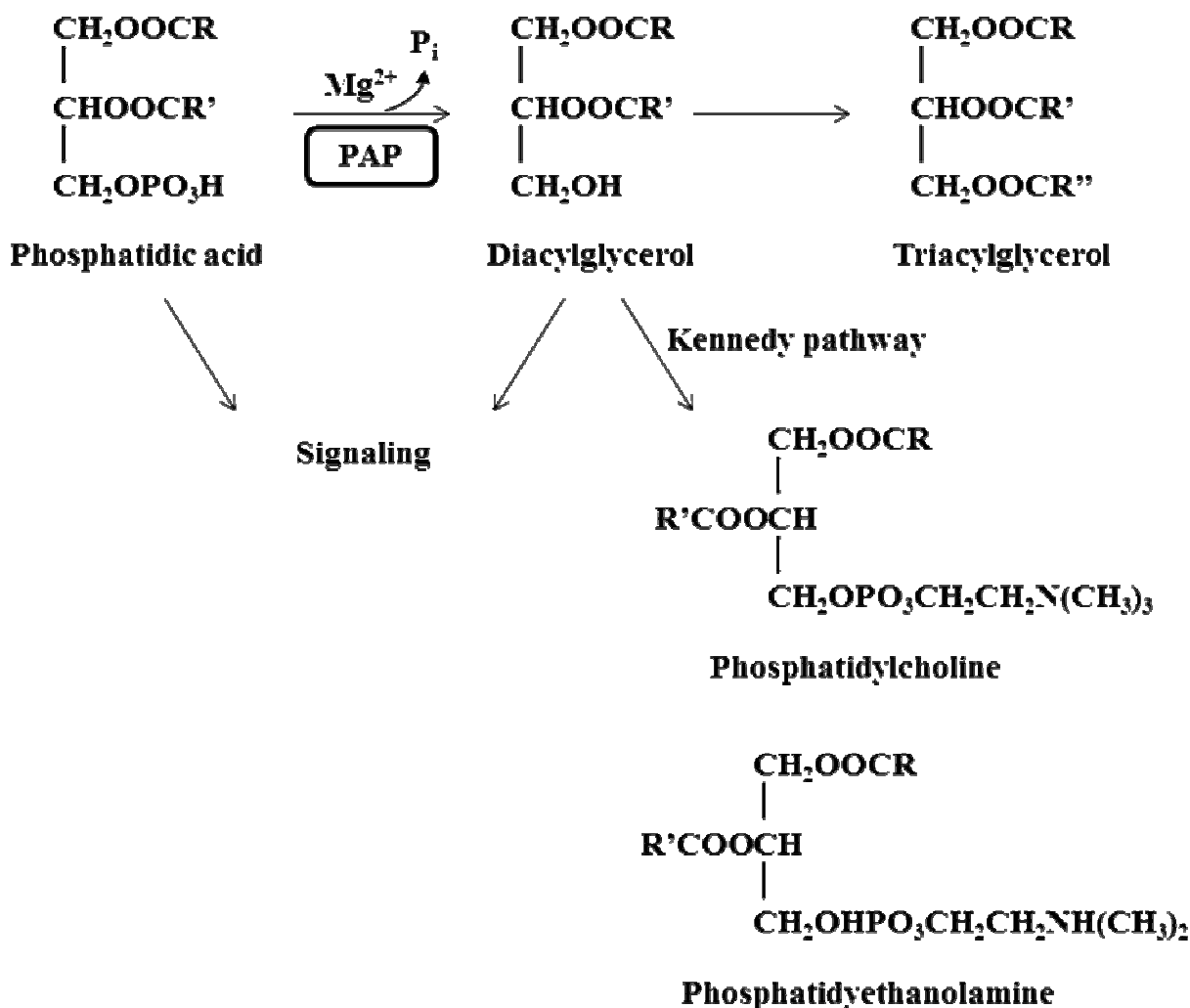
VSG expression.

Figure 12: Role of fatty acid signaling in yeast. Phosphatidic acid (PA) is dephosphorylated into a diacylglycerol (DAG) molecule by a Mg^{2+}-dependent reaction. This reaction is catalyzed by phosphatidic acid phosphatase (PAP) enzyme, present in yeast. DAG can further be reduced into triacylglycerol (TAG), the precursor to fatty acids. In addition, DAG is the precursor to the phospholipids, phosphatidylcholine and phosphatidylethanolamine and in conjunction with PA, can be used in signaling.

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K.) Objectives.

       1.) Determine cellular localization of TbLpn. The purpose of this study is to determine where the protein TbLpn is located in the cell. This will be done by first extracting cytosolic and nuclear extract from trypanosome cells and then assessed by a Western Blot using anti-TbLpn antibodies.

       2.) Determine whether TbLpn interacts with TbPRMT1 *in vivo*. Due to the results of the yeast-two hybrid analysis, we want to determine if there is an interaction between TbPRMT1 and TbLpn *in vivo*. In a western hybridization, an interaction between TbLpn and TbPRMT1 will be present if TbPRMT1 is pulled down with TbLpn during an immunoprecipitation.

       3.) Determine whether TbLpn is methylated *in vivo*. This study will determine if TbLpn is methylated *in vivo*. An immunoprecipitation, will purify TbLpn from cellular extract. The presence of methyl-arginine will be assessed by a Western Blot using antibodies specific in recognizing methyl-arginine.

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III. Materials and Methods

A.) Preparation of Cytosolic and Nuclear Extract. Buffer A (150 mM sucrose, 20 mM of KCl, 3 mM of $MgCl_2$ and 20 mM Hepes (pH 7.9)) is required for the protocol. To prepare the cells, first spin down cells at 6,090 X g for 10 minutes. Wash twice in 25 ml Buffer A with 25 μl of 1M DTT. Centrifuge cells at 6,090 X g for 10 minutes. Resuspend cells at 5 x 10^8 cells/ml in Buffer A and add NP-40 to 0.2%. Pass through a 26-gauge needle three times carefully. This is the whole cell extract. Centrifuge at 13,000 X RPM for 10 minutes. Recover the supernatant. This is the cytosolic extract. Resuspend the pellet in 1 ml Buffer A. Pass through a 26-gauge needle 15 times carefully. Centrifuge at 13,000 X RPM for 10 minutes. Discard the supernatant and rinse the pellet with 0.5 ml of Buffer A. Centrifuge at 13,000 RPM for 10 minutes. Discard the supernatant. Resuspend the pellet in 0.5 ml of Buffer A. This is the nuclear extract.

B.) Immunoprecitation of TbLpn. In a 1.5ml tube, mix cytosolic extract (50 μl) with 10 μl of purified polyclonal anti-TbLpn antibodies or 10 μl IP buffer (20 mM Hepes (pH 7.9), 150 mM sucrose, 150 mM KCl, 3 mM $MgCl_2$, 0.5 % NP-40, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 mM PMSF). Add 240 μl of IP buffer. Incubate at 4 °C for 2 hours or overnight. Then, add 10 μl of Protein A-Sepharose. Incubate at 4 °C for 1 hour. Centrifuge at 3,000 X g for 30 seconds. Carefully remove and keep the supernatant. This is the unbound fraction. Wash the pellet 5 times at 5 minutes each with 1 ml of IP buffer, making sure to spin at 3000 X g for 30 seconds between each wash. Resuspend the pellet in 40 μl of water. This is the bound fraction. Take 20μl of sample and add 5 μl of 5X SDS-PAGE buffer (50 % glycerol, 10 % SDS, 0.25 M DTT, 4X stacking gel

buffer (pH 6.8), trace of bromophenol blue). Boil at 100°C for 5 minutes.

C.) Gel Preparation and Gel Electrophoresis. Samples will be run on an SDS-PAGE gel. Prepare a 7.5 % separating and a 5 % stacking gel. For a 7.5 % separating gel, mix 938 μl of 40% acrylamide, 1.25 ml of 4X separating gel buffer (1.5 M Tris (pH 8.8), 0.4 % SDS), 2.8 ml of ddH$_2$O, 5 μl of TEMED and 75 μl of 10 % ammonium persulfate (APS). Pour into gel mold and let polymerize for 5-10 minutes. Next, prepare a 5 % stacking gel, mix 312 μl of 40 % acrylamide, 670 μl of 4X stacking gel buffer (0.5 M Tris (pH 6.8), 0.4 % SDS)), 1.48 ml of ddH$_2$O, 5 μl of TEMED and 30 μl of 10% APS. Pour into gel mold until its about to overflow, add well comb, let polymerize for 5-10 minutes. Mix 100 ml of 10 X running buffer (0.25 M Tris, 1.92 M glycine, 1% SDS) with 900 ml of ddH$_2$O to make a 1X Running buffer. Put the gel and a buffer plate in to the holder. Fill BIORAD Mini Protean® tetra cell and gel holder, with half of the 1 X running buffer. Load the samples, starting with 5 μl of MW markers, skipping a well between each sample. Put on cover, and run at 150 V for about 1 hour.

D.) Transfer. Prepare a transfer buffer by mixing 100 ml of methanol with 50 ml of 20X CAPS buffer (0.2 M CAPS (pH 11.0)). Fill with dH$_2$0 to 1 L. Pour 1/3 of transfer buffer into a small tub. Cut two pieces of gel blotting paper. Cut one piece of Immobilon membrane. Cut the left corner of membrane. Assemble the sandwich. Place the clear side in the Methanol/20X CAPS bath. Next, place a sponge, followed by a piece of blotting paper. Soak the membrane in methanol for 30 seconds before placing it on the blotting paper. Remove the gel from the mold carefully by lifting the front plate, making sure not to disturb the gel. Remove the stacking gel. Cut the corner of the gel on the side with the markers. Carefully remove the gel from the base plate and place onto the membrane. Cover with the second piece of blotting paper, sponge and close. Place in the transfer

<center>36</center>

apparatus, with the black side facing away. Pour the Methanol/20X CAPS into the BIORAD Mini Protean tetra cell, add an ice pack and run at 150V for 1 hour.

E.) Western Blot. Immediately following transfer, soak membrane in 1X TBS for 1minutes. Incubate in 10 % dry milk in 1X TBS for 1 hour at room temperature or overnight at 4 °C. After incubation, rinse 2 X 5 minutes in 1X TBS-T. Incubate in 2 % dry milk in 1X TBS-T containing the primary antibody (1:1000 dilution) for 2 hours at room temperature or overnight at 4 °C. Rinse 2 X 15 minutes in 1X TBS-T. Incubate in 2 % dry milk in 1X TBS-T containing the secondary antibody for 1 hour or 4 °C overnight (GAR-HRP, 1:10,000 dilution). Rinse 2 X 15 minutes in 1X TBS-T. Incubate in developing solution for 5 minutes at room temperature. Remove excess solution on extra blotting paper and place in Saran Wrap. Expose to a film.

F.) Stripping. Stripping buffer consists of 7.5 g of glycine mixed with 250 μl Tween-20. Add 400 ml of ddH$_2$O. Adjust pH to 2.5 then complete to 500 ml. Soak blot in stripping buffer for 1 hour at 80 °C. Soak the blot 2 X 15 minutes in 1X TBS. Incubate 5 minutes in developing solution and expose to film. Film should be blank. The purpose is to check if blot was completely stripped. If the blot is blank, continue to a western analysis. If the blot is not blank, soak in stripping buffer for an additional 1 hour.

G.) Coomassie. Immediately following electrophoresis, put gel directly into Coomassie stain (0.5 % in 10% methanol/15% acetic acid). Incubate in stain for about 30 minutes. Prepare destaining solution. Mix 100 ml of methanol with 150 ml of acetic acid. Fill to 500ml with dH2O. Once gel has soaked in coomassie for 30 minutes, discard coomassie and begin destaining. Begin by

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pouring destain onto the purple gel and rotate for 5-10 minutes. Repeat until bands form. If bands are not present on gel after complete destain, continue to a silver stain.

H.) Silver Stain. Incubate gel in 50 % methanol and 12 % acetic acid for 10 minutes. Discard solution then incubate gel in a 10 % ethanol, 5 % acetic acid solution for 10 minutes. Discard and mix a 0.06 % potassium permanganate solution and add to gel. Rotate 5 minutes. Discard and add a 0.1 % potassium carbonate solution for 5 minutes. Wash with 50 ml of dH_2O, 6 X 5 minutes. Add a 0.1 % silver nitrate solution for 10 minutes. Discard and add 50 ml dH_2O for 2 minutes. Prepare a 200 ml solution of 2 % potassium carbonate with 81 µl formaldehyde. Add 50 ml of solution for 1 min, then an additional 50 ml for 1 min until bands form. Discard solution between each wash.
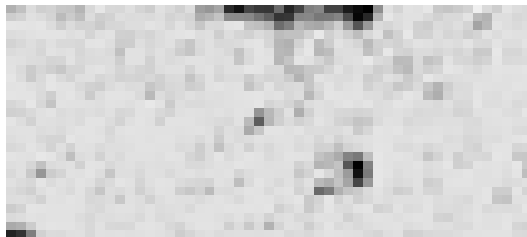
IV. Results

A.) Intercellular localization of TbLpn. In order to determine whether TbLpn is methylated, it is important to determine where TbLpn is located within the cell. We took cells from *T. brucei* and broke them down into nuclear and cytosolic extracts.

Cytosolic and nuclear extracts were run on an SDS Page gel using a 5% stacking gel and a 7.5% separating gel. We used EZ-Run Protein Marker for a protein ladder. Following electrophoresis, the gel was transferred onto a PVDF membrane to perform a western blot using anti-TbLpn antibodies. Using this antibody we can determine whether TbLpn is located in the cytosol or nucleus. By using TbLpn, the antibody would bind to any TbLpn protein brought down in each extract. The results from this western blot show that TbLpn is located in the cytosol (Figure 13). Although we can conclude that TbLpn is located in the cytosol, that is not to say that TbLpn is not also in the nucleus. The results of the western blot can only suggest that TbLpn could be in the nucleus but at undetectable amounts.

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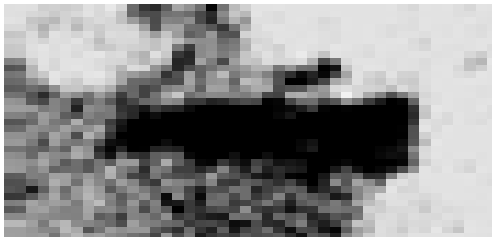
Figure 13: TbLpn located in the cytosol. The results of the western hybridization showing that TbLpn is present in the cytosol.

B.) Interaction between TbLpn and TbPRMT-1. The next step was to confirm the *in vivo* interaction between TbLpn and TbPRMT1, an interaction that was originally determined by yeast-two-hybrid analysis. To start, we took the cytosolic extract containing TbLpn, and preformed an immunoprecipitation of TbLpn. This procedure purifies the protein from any other proteins contained in the extract. Next, we performed a SDS Page gel using a 5% concentration stacking gel and a 7.5% concentration separating gel. We then transferred to a PVDF membrane for a western blot. For this western blot we used anti-TbPRMT1 antibodies

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The result shows a positive interaction between TbLpn and TbPRMT1 (Figure 14). Based on these results, when TbLpn was pulled down in the immunoprecipitation various proteins that were attached to TbLpn were pulled down with it. In this case, because TbPRMT1 interacts with TbLpn this means that TbPRMT1 was also brought down. This also suggests that TbPRMT1 could be one of several PRMTs which methylate the protein TbLpn. Indeed, PRMTs tend to associate in a relatively stable manner with their substrates, and several mammalian methylproteins have been identified through protein-protein interaction screens with PRMTs.

To be sure of these results, a reverse immunoprecipitation using anti-TbPRMT1 antibodies was performed, followed by a western hybridization with anti-TbLpn. The results of this procedure revealed a band at the size of TbLpn confirming that TbLpn and TbPRMT1 interact *in vivo* (not shown).
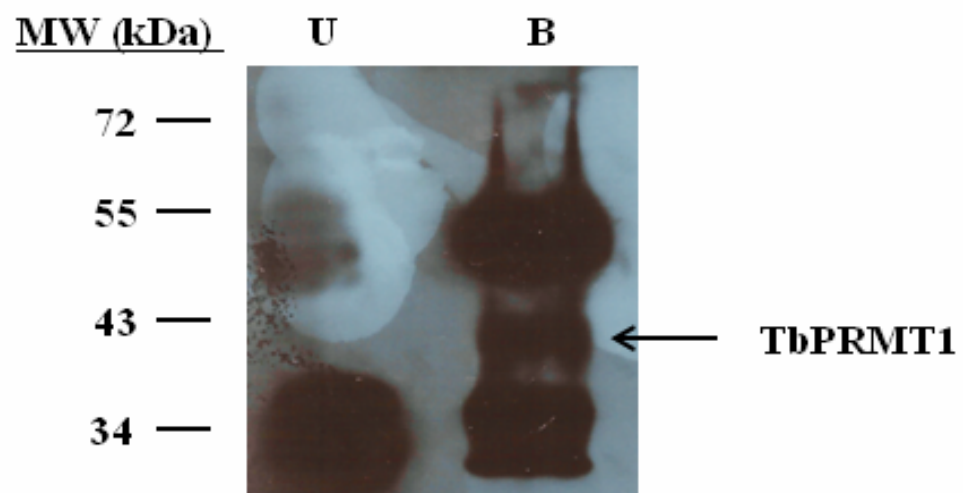
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Figure 14: Interaction between TbLpn and TbPRMT1. The western blot showing the

interaction between TbLpn and TbPRMT1. The results show that when TbLpn was pulled down during the immunoprecipitation, TbPRMT1 was also pulled down as it was attached to TbLpn. **C.) TbLpn is methylated *in vivo*.** An interaction between TbPRMT1 and TbLpn suggests that TbLpn could be methylated *in vivo* because, as mentioned, TbPRMT1 is an enzyme which catalyzes the reaction which adds methyl groups onto arginine. To determine whether TbLpn is actually methylated we used anti-MERG antibodies. These antibodies were raised against a peptide containing 7 asymmetric dimethylarginine residues alternating with 8 glycine residues. methylated arginines. This motif is found most prevalently among verified dimethylarginine-containing proteins. The antibodies have been shown to specifically recognize methylated arginine residues (24).

An immunoprecipitation for TbLpn purifies the protein from the cytosolic extract. The western hybridization used the anti-MERG for the primary antibody. According to the results, a definite band of approximately 82-85 kDa clearly indicates methylation of TbLpn (Figure 15). We confirm this because, like the previous blot, we observe a band at the size of TbLpn, revealing that TbLpn is methylated.

Similar to the previous experiment, we performed a reverse immunoprecipitation using anti-MERG antibodies followed by a western blot using anti-TbLpn antibodies. The results of this experiment again revealed a band at the side of TbLpn. This means that when MERG was pulled down in the immunoprecipiation, TbLpn was also pulled down (not shown).
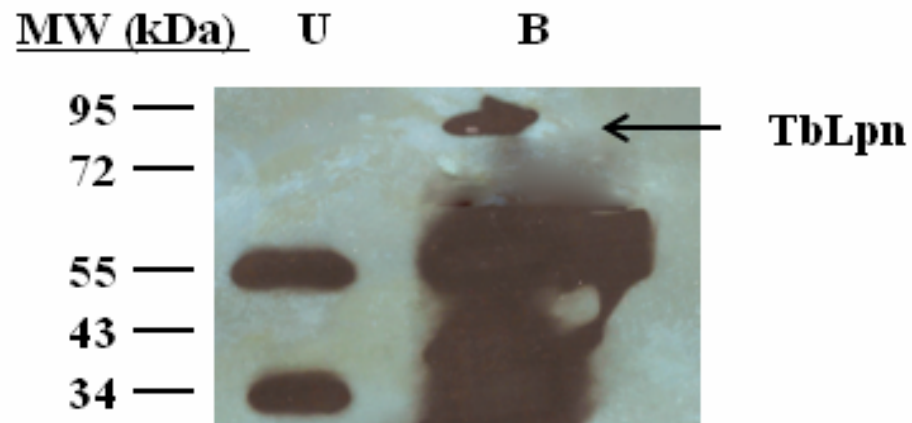
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Figure 15: TbLpn is methylated *in vivo*. TbLpn was immunoprecipitated from cytosolic extract, and analyzed by SDS-PAGE. The presence of methylated arginines onto TbLpn was assessed by western hybridization using anti-MERG antibodies. The results show that TbLpn is methylated *in vivo* by an interaction with MERG.

V. Discussion

The purpose of this project was to characterize TbLpn in *T. brucei*. The protein lipin plays a role in adipocyte development in mice. Furthermore, lipin plays an important role in lipid synthesis and signaling in yeast. Specifically, lipin plays an important role in the production of certain phospholipids and the production of fatty acids. In particular, phosphatidylcholine (PC) and phosphaditylethanolamine (PE) which are products of the Kennedy pathway for lipid biosynthesis. These particular phospholipids constitute not just the composition of the cell membrane in *T. brucei.* In addition, PE is a precusor of glycosolphosphatidylinositol (GPI) synthesis. In yeast, lipin is utilized in the initial reaction by dephosphorylating phosphatidate. The product of this would be used in the expression of surface protein, which changes between fly form and mammalian form *trypanosome* and also contributes to *T. brucei* high antigenic variation.

The first step in our investigation was to localize TbLpn *in vivo.* As our results show, TbLpn is localized in the cytosol and not the nucleus. The presence of TbLpn in the cytosol allows us to determine more specifically the function of TbLpn *in vivo*. Proteins found in the nucleus display different functions than proteins found in the cytosol. Specifically, nuclear enzymes primarily function to modify and replicate DNA. Nuclear enzymes also initiate protein synthesis. In contrast, cytosol enzymes carry out the majority of cellular reactions, which include lipid synthesis. In this case, because TbLpn has been shown to be a homolog to the protein lipin in mice and yeast, TbLpn in *T. brucei* should play a role in lipid synthesis. The importance of TbLpn role within *T. brucei* relates to the synthesis of the GPI anchor. The precursor of the GPI anchor is phosphaditylethanolamine (PE), which is a product of the CDP-ethanolamine branch of the Kennedy pathway in eukaryotes (25). The significance of PE is that it represents the second major

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glycerophospholipid class in eukaryotes. PE has been shown to affect protein folding and promote membrane fusion and fission. In addition, it represents the donor of the ethanolamine for GPI synthesis (25).

The formation of PE, as mentioned, can be synthesized by the CDP-ethanolamine branch of the Kennedy pathway. It is carried out by either a head group exchange with phosphaditylserine (PS) or an acetylation of *lyso*-PE (25).

The first reaction of the CDP-ethanolamine branch is catalyzed by ethanolamine kinase, resulting in the formation of phosphoethanolamine, activated using CTP by CTP:phosphoethanolamine cytidylyltransferase (ET) to form CDP-ethanolamine (25). This reaction is followed by the transfer of the ethanolamine moiety to diradylglycerol, which is catalyzed by CEPT which has dual specificity (25). It has been shown that in *T. brucei*, the PE branch of the Kennedy pathway is essential for cell growth. The inhibition of this pathway has been shown to block *de novo* synthesis of GPI anchors and further prevented ethanolamine phosphoglycerol addition to eukaryotic elongation factor 1A. Furthermore, ablation of ET activity resulted in disruption of mitochondrial morphology and ultrastructure, demonstrating a direct effect which reduced PE levels on mitochondrial integrity (25).

A second phospholipid that is important to *T. brucei* is the synthesis of phosphatidylcholine (PC). PC represents the most abundant glycerophospholipid class. PC is generated by the CDP-choline pathway of the Kennedy pathway (25). This pathway involves the sequential action of three enzymes to generate PC from its precursors. Although this pathway is

responsible for the production of PC, human liver cells synthesize approximately one-third of PC by methylation of PE. This reaction is catalyzed by PE N-methyltransferase. Both pathways are involved in the regulation of lipoprotein metabolism (25).

In *T. brucei*, phospholipids are synthesized *de novo*, meaning they synthesize phospholipids from scratch which can be an advantage or disadvantage as we saw in this study. The advantage being that it ensures the integrity of the molecule as well as it gives the parasite the freedom to modify the molecule, resulting in some of the environmental advantages such as antigenic variation. The disadvantage is that inhibition of the pathway blocks synthesis of other molecules down the line.

The data also shows that TbLpn is not present in the nucleus, but that does not mean the protein is not there. TbLpn could still be in the nucleus but in non-detectable amounts. By making some key observation based on TbLpn's mammalian homolog, lipin, clues into the function of this protein can probably rule out any possible nuclear localization. Recall that studies of lipin-1 revealed the important physiological roles in metabolic homeostasis. Nevertheless, cellular functions of lipin have been identified as serving as an enzyme for triacylglyerol (TAG) and phospholipid biosynthesis. In addition, lipin acts as a transcriptional coactivator that regulates the expression of fatty acid utilization and lipid synthetic genes (21). However, for this project we are only concerned with characterizing the protein by determining certain qualities of TbLpn. Specifically, whether TbLpn is methylated *in vivo*.

In *T. brucei,* gene regulation is controlled at the post-transcriptional level. As a result, gene

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regulation produces RNA-binding proteins which are substrates for the enzyme protein arginine methyltransferase (PRMT). Arginine methylation plays an important role in gene expression in *T. brucei.* Arginine methylation is a post-translational modification catalyzed by the protein arginine methyltransferases (PRMTs).

The next step in our investigation was to determine an interaction between TbPRMT1, an enzyme that adds methyl groups onto arginine residues, and TbLpn. There are two major types of PRMTs. Both type I and II PRMTs catalyze the transfer of a methyl group from S-adenosylmethione (SAM) to an arginine residue, resulting in monomethylarginine (MMA). Type I PRMT adds a second $-CH_3$ to the same nitrogen, forming di-methylarginine (DMA). Finally, the second arginine is added on different nitrogen by type II PRMTs.

PRMT1 played a significant role in providing the initial clue into whether TbLpn was methylated. The data suggested an interaction between TbLpn and TbPRMT1 which allowed our investigation to proceed with MERG, which recognized methylated arginine groups. The interaction between TbLpn was found because an initial immunoprecipitation for TbLpn pulled down the protein from cytosolic extract along with all the associated proteins on TbLpn. The transfer imprinted the gel picture onto a membrane. During the initial western, antibodies specific for TbLpn bound to the protein, which made the protein visible on film. Next, the blot was stripped of all the antibodies for TbLpn. A western hybridization used TbPRMT1 and bound to only PRMT1 brought down along with TbLpn during the immunoprecipitation. Because this experiment showed an interaction between TbLpn and TbPRMT-1, it means that the pellet of the bound fraction during the immunoprecipitation contained both TbLpn and TbPRMT-1 which was

associated with our target protein.

Arginine methylation regulates cellular processes. These include RNA processing, DNA repair, transcriptional regulation and signaling transduction. Arginine methylation modulates protein-protein interaction due to the altered state of arginine residues.

Arginine methyltransferases modify proteins that function at different steps of cell regulation. For example, cytoplasmic and nuclear signaling transduction, nuclear-cytoplasmic shuttling, transcriptional activation and multiple post-transcriptional steps in gene expression (18).

PRMTs implicated in signaling pathways regulate transcription. PRMT binds to the cytoplasmic domain of interferon α/β receptors and cells resistant to growth and inhibition by interferon (18).

In *T. brucei,* TbPRMT1 appears to play an important role in the lifecycle. Specifically, gene function in *T. brucei* suggests growth impairment and nuclear defects in the bloodstream form of the parasite following depletion of TbPRMT1 expression by RNA interference. This finding is unlikely due to the role of TbPRMT1 in *T. brucei* mitochondrial gene expression as mitochondrial activity is decreased in the bloodstream form (20).

In yeast and humans, protein arginine methylation modulates multiple cellular pathways. Research has primarily focused on transcriptional regulation and the effect of arginine methylation on the exposed tails of histones H3 and H4. Methylation of arginine residues in histones has been

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linked to transcriptional activation or indirect repression of transcription by the activation of lysine methylation at adjacent sites (26). It is reported that several nonhistone substrates of arginine methylation modulate transcription. These substrates are present either as transcriptional coactivators with histones or as DNA binding transcription factors. It is reported that the largest family of nonhistone proteins that are arginine methylated are RNA binding proteins (RBPs) (26). Recall, several RNA binding proteins serve as substrates to PRMTs. The reason for arginine methylated RBPs is the occurrence of glycine-arginine rich domains. Arginine methylation of RBPs affects multiple pathways including pre-mRNA splicing, RNA stability and translation (26).

In addition, protein arginine methylation alters protein function. This includes certain protein functions influenced by protein:protein interactions, protein:nucleic acid interactions as well as protein trafficking. Adding methyl groups to arginine alters the shape of arginine's side chain. This increases the hydrophobicity and steric hindrance by removing a potential hydrogen donor. This alters the ability of arginine methyl proteins to bind with other proteins or nucleic acids (26).

PRMT1 has been localized in the cytoplasm and nucleus of yeast and human cells. The pathway in which PRMT1 is imported into the nucleus needs to be determined. It is suggested that PRMT1 is imported by indirect interactions with unmethylated substrates (20). This idea is supported by assays which demonstrate nuclear accumulation of PRMT1 upon the inhibition of cellular methylation (20).

In relation to *T. brucei*, TbPRMT1 has been shown to play an important role in the lifecycle

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of *T. brucei*. Screens of TbPRMT1 suggest functions to impair growth as well as nuclear defects in the bloodstream form of *T. brucei* (20). Regulation of protein function through arginine methylation may be important during the lifecycle transitions that *T. brucei* undergoes as they move from the environment, to the tsetse fly and their mammalian host. The lifecycle of *T. brucei* requires changes in gene regulation and morphology (26).

Although we determined that TbPRMT1 interacts with TbLpn, there are other PRMTs present in *T. brucei* that can methylate TbLpn. In *T. brucei* there are 4 additional TbPRMTs, TbPRMT3, TbPRMT5, TbPRMT6 and TbPRMT7. As we move on in our investigation of the protein TbLpn, we want to determine the specific TbPRMTs that methylate TbLpn. Although through this investigation we showed that TbPRMT1 interacts with TbLpn, it may not be the major TbPRMT that methylates TbLpn.

PRMT3 is a cytosolic protein containing a C_2H_2-type zinc finger domain of the amino-terminal part of the protein (20). Truncation of the amino-terminal region of PRMT3 results in the enzyme that methylates arginine residues of a recombinant substrate but not from cell extract. These results concluded that the zinc finger domain is a key feature which allows for substrate selectivity (20).

The biological role of PRMT3 was concluded by studying the fission of yeast. In *S. pombe*, the first substrate revealed for PRMT3 was the 40S ribosomal protein S2 (rpS2) (20). This protein contains a conserved methyltransferase core domain which was first obtained from rpS2. This suggests a significant role for PRMT3 in ribosome function among eukaryotes (20). It was seen, by

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a deletion of PRMT3, that production of an unmethylated rpS2 substrate in addition to yielding an imbalance in the 40S/60S ribosomal subunit ratios. However, significant changes at the levels of monosomes and polysomes are not shown. This is consistent with the absence of growth defects in PRMT null cells (20). A study based on genome wide biological response of PRMT3 suggested that PRMT3 null cells respond to a small ribosomal subunit deficit by upregulating the translational efficiency of the 40S ribosomal protein mRNAs (20).

Ribosomal proteins are subject to posttranslational modifications including methylation (20). Arginine methylation of ribosomal subunits is conserved evolutionarily among eukaryotes. In addition, ribosomal subunits are modified by lysine methylation and methyltransferases that catalyze these modifications (20). In conclusion, although through this investigation we revealed a positive interaction between TbLpn and TbPRMT1, this data on PRMT3 reveals another PRMT that could methylate TbLpn.

Another candidate that could interact with TbLpn is TbPRMT5. The evolutionary conservation of PRMT5 reveals the overall functional role of this enzyme. However, according to several reports, deciphering the role of PRMT5 has been difficult. Using several different models including yeast, *Drosophila* and *Xenopus*, function remains elusive (20). Using the yeast homolog for PRMT5, histone synthetic lethal 7 (Hsl7) and Shk1-binding protein 1 (Skb1) indicated that only Hsl7 catalyzes the formation of monomethylarginine *in vitro* (20). In contrast, the mammalian model has revealed PRMT5-dependent formation of symmetrical dimethylarginine (20). However, key aspects of this enzyme are still unknown so there cannot be an assumption either way as to whether it can be considered an additional PRMT that methylates TbLpn.

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The presence of different sets of PRMT encoding genes in parasites suggest that protein arginine methylation make distinct contributions during the lifecycle (26). In *T. brucei*, three of the five PRMTs present remain unchanged at the RNA level during development. In addition, TbPRMT3 and TbPRMT7 show a decrease in RNA levels during the transition from log growth to stationary growth (26). Also, TbPRMT7 RNA increases during differentiation from the mammalian form to the fly form (26). A study using RNAi mediated knockdown observed the affect on function of different PRMTs. The most significant observation recorded was based on the depletion of TbPRMT6. This was observed in both bloodstream and fly forms of *T. brucei* (26). These cells observed slow growth under normal conditions in addition to low serum stress in the fly form. Also, TbPRMT6 knockdown cells exhibited defects in cytokinesis. However, it was observed that TbPRMT6 methylates *in vivo* (26).

Our final objective in this study was to determine whether TbLpn is methylated. The significance of methylation is that it modulates protein-protein interaction. There are few to no reports on arginine methylation having effects on enzymatic activity. However, if we look at other proteins that are methylated, the methyl group affects such characteristics of proteins like their ability to bind to other substances as well as there ability to catalyze certain reactions. Methylation of an amino acid has the potential to change certain characteristics of the molecule which can affect overall protein folding as well as interacts with water.

To draw an overall conclusion, we must look at the possible role that arginine methylation has on the protein lipin. Moreover, methylation of arginine residues may promote or inhibit TbLpn role in the pathway. Cell signaling, by arginine methylation, may activate or deactivate the

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pathway. This is similar to the process of phosphorylation which activates several pathways. For example in the JAK-STAT pathway, tyrosine residues are phosphorylated by tryosine kinases which promote binding of the STAT protein.

In order to understand the role of arginine methylation on lipin protein, we must understand the role of lipin-1 on fatty acid synthesis. The importance of the protein may suggest how arginine methylation effects the pathway of which lipin is used.

Not understood is the mechanism which increases expression results in enhanced insulin sensitivity in adipose tissue. In contrast, lipin-1 expression leads to enhanced expression of glucose transporter 4 (GLUT4) and increased adipocyte glucose uptake (21). It can be hypothesized that these mechanisms could be regulated by arginine methylation. Lipin plays an important role in lipid signaling and signaling. Even though the primary step dephosphorylates phosphatidate (PA) into diacylglycerol (DAG), when used in conjunction, both molecules are involved in signaling, which could be regulated by arginine methylation.

The membrane of the endoplasmic reticulum, consisting of DAG, probably derived by the dephosphorylation of PA by lipin, has been suggested to enhance the recruitment of perilipin proteins to lipin. These proteins that coat the lipid droplets serve as targets of the peroxisome proliferator-activated receptor (PPAR) transcription factors. These proteins are regulated by lipin (23). It can be hypothesized that methylated TbLpn in *T. brucei*, may play a key role in either the overproduction or mutation of the protein. In the case of overproduction, a methylated TbLpn

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could act as a permanent on switch, signaling for the continuous production of fatty acids. In contrast, the addition of the methyl group may produce a mutant protein.

TbLpn can also function to facilitate as a cytoplasmic-nuclear shuttle, similar to Npl3p. The protein, Np13p is localized to the nucleus in a distinct non-nuclear pattern similar to hnRNP shuttling between the nucleus and the cytoplasm (27). Np13p export depends on the ongoing RNA polymerase II transcription suggesting the association between the export of poly(A)$^+$RNA and Npl3p (27).

It is suggested that hnRNPs play key regulatory roles during RNA processing, mediated by post-translational modifications of the hnRNPs. RNA processing is one of the cellular processes regulated by arginine methylation. hnRNP modifications include phosphorylation and methylation. Mammalian hnRNPs contain about 65% methylated arginines in the nucleus suggesting that methylation has an important effect on function (27). Arginine methylation modulates protein-protein interactions which supports the idea that methylation of hnRNPs effect function of these proteins. Similar effects could result by methylation of TbLpn.

To move on with our investigation of the protein TbLpn, as mentioned, we would first like to identify the specific TbPRMTs which interact with TbLpn. In this discussion, we have outlined some of the characteristics of the PRMTs found in *T. bruce,i* however much of data found on these PRMTs remain inconclusive. Nevertheless, with the mere fact that we know that each exist, we can start experimenting with each TbPRMT to determine which enzyme methylates TbLpn.

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In addition, we would like to measure the effect of methylation on the enzymatic activity of TbLpn. This would be done by first identifying the specific arginine residues that are methylated and then comparing the wild-type TbLpn molecules to a mutant TbLpn. Mutant TbLpn would contain arginines that were exchanged for a lysine. By gaining insight of the characteristics that define our protein, we can further learn about its importance within *T. brucei* and eventually produce a comparable vaccine for African sleeping sickness.

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