EFFECT OF ARGININE METHYLATION ON THE ENZYMATIC ACTIVITY AND FUNCTION OF TbLpn IN *TRYPANOSOMA BRUCEI*

A Senior Honors Thesis

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for Graduation in the Honors College

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I. Abstract

Trypanosoma brucei is a protozoan parasite which causes Human African trypanosomiasis (HAT). *Trypanosoma brucei* is vector borne carrier by the tsetse fly of Sub-Saharan Africa. If left untreated, Human African trypanosomiasis is almost always fatal and represents a very serious health risk. Another version of the disease known as Animal African trypanosomiasis (AAT) also exist and threatens the livestock of farmers. There are two stage of the disease, the first blood steam phase and the second central nervous system phase. In the second phase, *T.brucei* is able to penetrate the blood brain barrier and cause very pronounced disturbances. This phase is where the disease gets its less formal name, "African Sleeping Sickness" due to the sleep disturbances and neurological symptoms prevalent in the second phase. Treatment options and outlooks are better in the first phase and get significantly worse in the second phase. Many of the treatments available are incredibly toxic, have very pronounced side effects, and often involve painful injections. As a result, a more effective means of treating HAT is necessary. The research in this paper seeks to determine the role of protein arginine methylation of specific arginine residues in TbLpn expression and subcellular localization. TbLpn is a lipin protein found in *T.brucei* that is homologous to human and yeast lipins. TbLpn is likely involved in membrane biosynthesis and is regulated post transcriptionally by protein arginine methylation. Utilizing site directed mutagenesis, specific arginine residues on a wild type plew TbLpn plasmid were mutated into lysine residues. These specifically changed arginine residues will then be used in *T.brucei* in order to observe the effects on protein interaction and sub cellular localization.

I. Introduction

T.brucei

Trypanosomes are protozoan parasites prevalent in Africa, and are responsible for both Human African trypanosomiasis (HAT) and animal African trypanosomiasis (1). There are two major subspecies of Trypanosome responsible for human diseases, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* (4). Both are responsible for causing variations of African Trypanosomiasis, *Trypanosoma brucei gambiense* affecting human hosts and *Trypanosoma brucei rhodesiense* targeting animal hosts (4). Human African Trypanosomiasis is fatal if left untreated and is endemic to 36 sub-Saharan Africa countries (5). While treatment is possible, it requires skilled medical personnel and relies on antibiotics and diagnosis techniques that are not always successful (4). This is especially a problem as the people most exposed to the disease are often rural farmers or fishermen who do not necessarily have access to the medical facilities needed for effective treatment (5).

Africa has suffered from several epidemics, the most devastating one taking place between 1896 and 1906 (2). This epidemics which took place in the Congo Basin, Uganda, and Kenya resulted in 300,000 to 500,000 deaths (2). Epidemics have also occurred in the 1920s and, most recently, the 1970s (5). There is still an estimated 65 million people still at risk from the disease (5). While deaths attributed to HAT have decreased over the years, there are an estimated 12 unreported deaths for every one confirmed (4). This is due to the fact that many of the people affected by HAT live in rural areas where reporting is not possible (4). *Trypanosoma* also cause the death of important livestock and animals, economically hurting farmers and their families

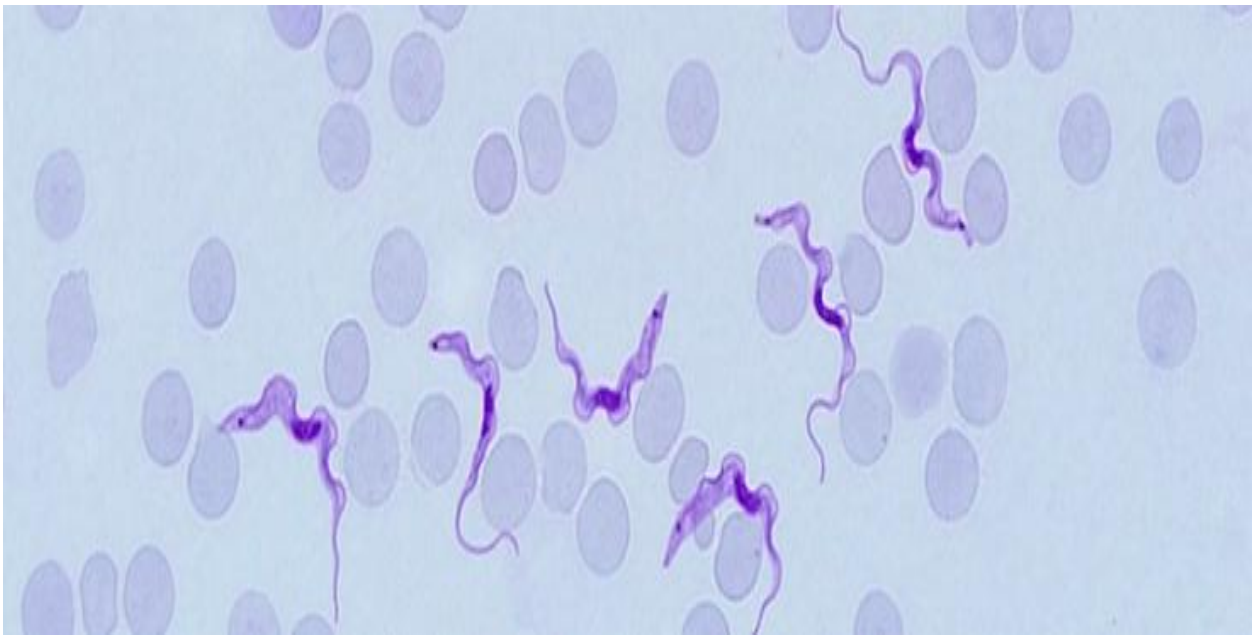


Figure 1: Picture of *Trypanosoma brucei rhodesiense* in a Giemsa-stained blood smear. (6)

Transmission and the tsetse fly

Both *T.gambiense* and *T.rhodesiense* are vector borne parasites (1). They are carried by the blood sucking tsetse fly which lives in sub-saharan Africa (1). Tsetse flies are small insects which live in rural woodlands and thickest spread throughout the African savannah (6). The fly is active and bites its victims during the daytime (6). The fly bites an infected animal, infecting itself with the trypanosome allowing it to grow within the fly itself (1). From inside the fly, *Trypanosoma* first grows in the fly's midgut, undergoing a series of complex biochemical reactions (1). These changes result in an infectious form of *Trypanosoma* which makes it way to the fly's salivary glands infecting the fly for life (1). When the fly goes on to bite a human host, the trypanosome then enters the bloodstream (1). In this early stage, also known as the haemolymphatic stage, the trypanosome spreads to the spleen, heart, liver, eyes, and endocrine organs (1). In a time ranging from weeks to several months, the trypanosome then crosses the blood brain barrier entering the CNS and the late stage of the disease (1).

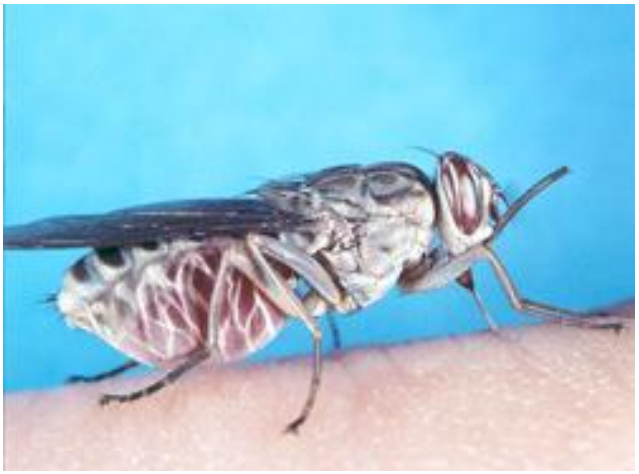


Figure 2: The Tsetse fly of Sub-Saharan Africa. The flies take about 30 days to reach full size from the egg stage. They are the vector of transmission of *T.brucei* and lead to the infection of both humans and livestock. (6)

Life Cycle of the tsetse fly

The tsetse fly has an unusually long life cycle for that of an insect. A female tsetse fly mates just once in its lifetime and produces a single egg in 7-9 days. This single egg then develops into a larva which then burrows into the ground about 9 days later. While in the ground, the larva pupates in the ground and after about 30 days emerges as an adult fly. In about 12 - 14 days, the fly will mature, mate and begin the cycle over again creating more flies (8).

Signs, Symptoms, and Diagnosis

HAT is divided into two stages, early and late. Both stages come with their own accompanying symptoms (1). In some patients, a painful chancre can form at the position of the initial tsetse fly bite (6). The early symptoms include headache, malaise, arthralgia, weight loss, fatigue, and intermittent fever (1). After these initial symptoms, patients can experience enlarged lymph nodes and the enlargement of the spleen and liver (1). There are also several cardiac symptoms associated with the disease including, myocarditis, pericarditis, and congestive cardiac failure. Winterbottoms's sign is a typical feature of *T b. gambiense* and occurs when posterior cervical lymphadenopathy affects the patient (1).

After these initial symptoms, the parasite is able to penetrate the blood brain barrier and enter the CNS (6). This causes the presentation of several neurological symptoms some of which give the disease its common name of African Sleeping Sickness (6). There are mental, motor, and sensory disturbances present in this stage (1). 74% of patients report experiencing sleep abnormalities which include a complete reversal of the normal sleep cycle. Those infected

become nocturnal and a disruption in the stages of sleep (1). Psychiatric disturbances and behavioral changes can also occur at this stage (1).

Diagnosis of HAT is relatively straightforward utilizing laboratory tests (6). Specifically, the parasite must be found within the host's tissues or body fluid (6). Parasite cells of *T. b. rhodesiense* can be found within the bloodstream. *T. b. gambiense* is much harder to spot in the blood requiring a microscopic examination of the patient's lymph node aspirate. Diagnosis requires observation of the parasite in a microscope (6). Additionally, a patient's cerebrospinal fluid must be examined in order to determine which stage of the disease they are in.

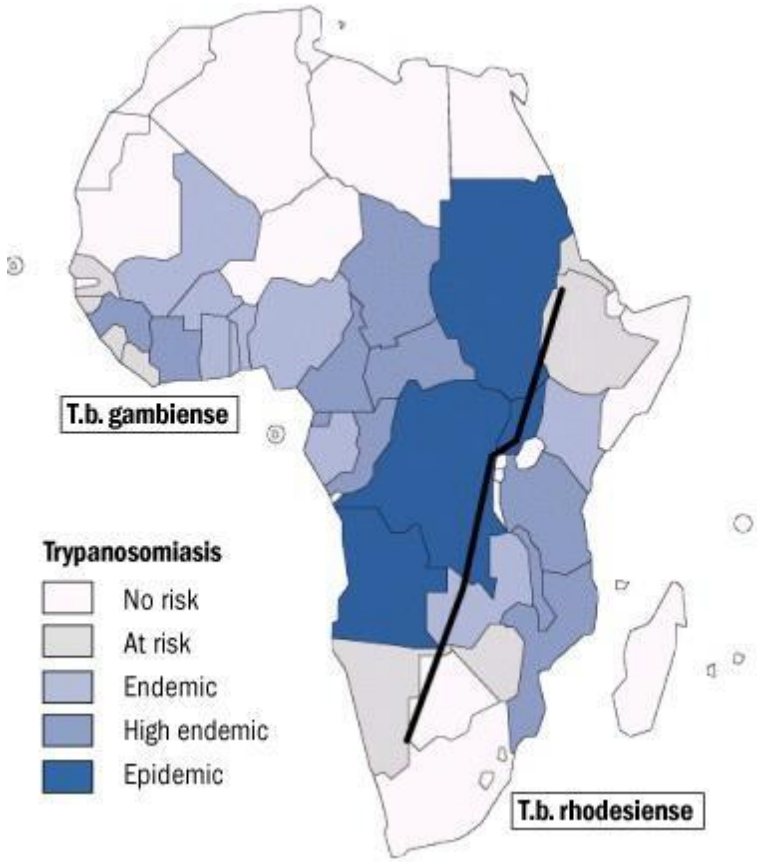


Figure 3: At risk areas for HAT. The most at risk areas include the Democratic Republic of Congo and the Central African Republic. Both nations are located in central Africa, a hot spot for Tsetse fly activity.(5)

Treatment

Treatment of HAT is most effective when a diagnosis can be in the early stage of the disease (1). There are drawbacks, as the majority of drugs used to treat both late and early stage HAT are not available orally and are toxic (1).

The two drugs used to treat the early stages of the infection are Suramin and Pentamidine. Suramin is best used to treat *T.b. rhodesiense* and Pentamidine is used to treat *T.b gambiense*. Pentamidine was invented in 1940 and interferes with the kinetoplast replication of *T.b gambiense* (3). Pentamidine is administered intramuscularly but can be given intravenously (1). There side effects of using the drug including hyper or hypoglycemia, hypotension, and other gastrointestinal disturbances (1). Suramin was invented in the 1920s and works by preventing g-protein signaling with the trypanosome (3). Suramin is administered intravenously and is usually very effective. The drug does come with its own side effects like renal failure, skin lesions, anaphylactic shock, bone marrow toxicity, and neurological complications (1).

For late stage treatment, Melarsoprol, Elfornithine, and Nifurtimox are used. These late stage treatments typically come with more complications as the drugs used are often much more toxic (1). Melarsoprol was created in 1949 and is able to treat the late stages of the disease (3). Melarsoprol is incredibly toxic containing arsenicals and shots of the drug are incredibly painful intravenous injections (1). There are many side effects including reactive encephalopathy in 5% - 10% of patients. This causes 1% - 5% mortality making the drug slightly dangerous to use (3). Unfortunately, there have been recorded instances of drug resistance to Melarsoprol making it a less desirable treatment option (1). Elfornithine was initially developed in the 1970s as an anti

cancer drug (3). It was discovered that Eflornithine can also be used to cure late stage *T.b gambiense* infections irreversibly binding the parasites ornithine decarboxylase receptor (3). *T.b gambiense* has a stable ornithine decarboxylase receptor making it a prime target for Eflornithine (3). The drug is administered intravenously and is less toxic than Melarsoprol (1). Eflornithine works best when used in combination with Nifurtimox. Nifurtimox was developed in the 1960s and is theorized to be related to the generation of reactive oxygen species which damage important cellular components (3). Nifurtimox is able to be taken orally and is used in combination with Eflornithine to reduce costs and increase effectiveness (1). This drug combination only affects *T.b gambiense* leaving Melarsoprol as the only effective treatment.

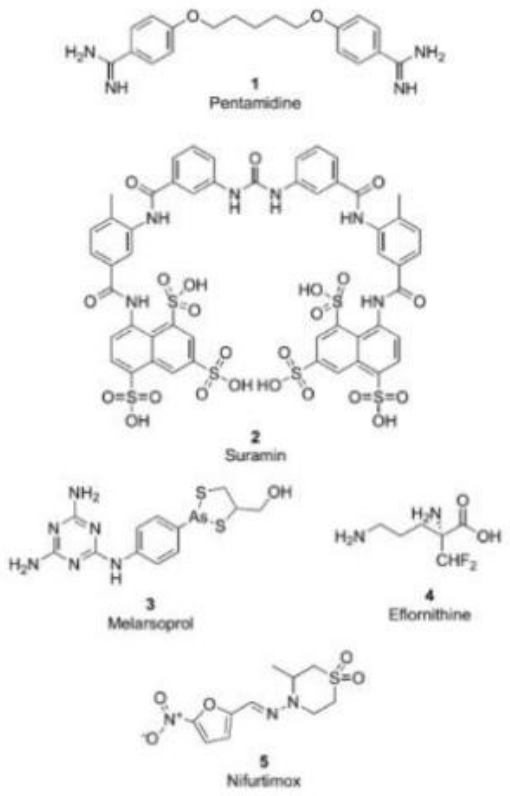


Figure 4: These are the five current drugs which are effective against Trypanosomiasis (3)

VSG Proteins

The surface of *T. brucei* is covered with a dense layer of glycoproteins known as Variant Surface Glycoproteins (VSG). VSG are found at the surface of many parasite cells, but are present in high numbers in trypanosomes (7). These proteins are responsible for the parasite's ability to switch its surface antigens (7). Switching antigens stops the immune system from responding effectively to an infection, it is this ability which prohibits the production of a vaccine for trypanosomes (7). There are over 1,500 different VSG genes in the trypanosome genome, but only one is expressed at a given time. On top of this, only part of the VSG surface can contact the host immune system (7). Because of this, it is only necessary for trypanosomes to change a small portion of their surface proteins in order to avoid the host immune system (7).

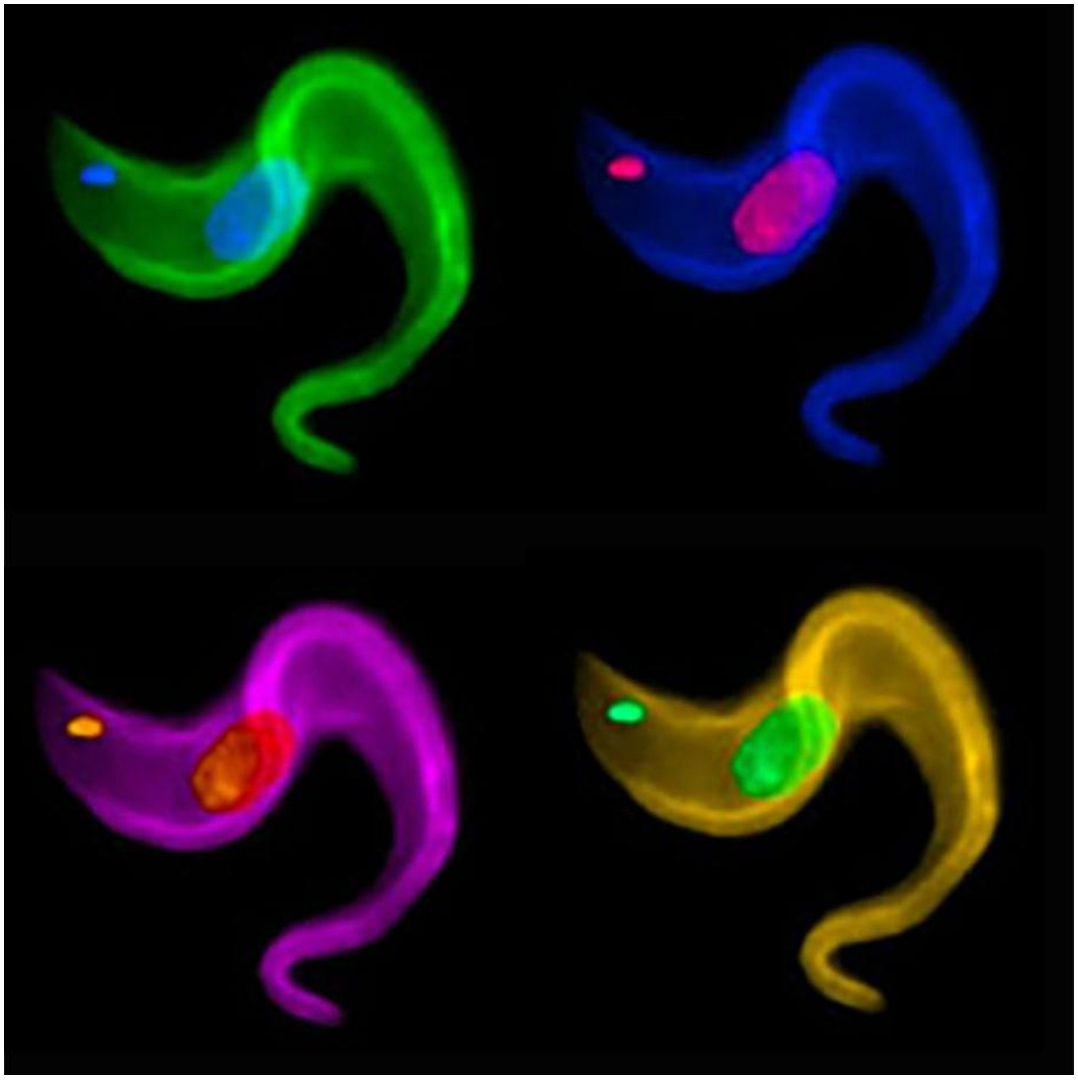


Figure 5: Antigenic variations is the ability to switch surface antigens. *T. brucei* achieves this through the use of VSG proteins which allow the parasite to avoid and confuse the host immune system. (11)

Morphology

African Trypanosomes have a very unique structure which helps the parasite survive and infect other hosts. Trypanosomes have a singular flagellum and carry an important compact disc of mitochondrial DNA known as the kinetoplast. The kinetoplast is theorized to assist the trypanosome in infecting its host the tsetse fly. Trypanosomes outside of the tsetse fly partially or completely lose their kinetoplast remaining locked within the bloodstream of the host organism.

T. brucei has a unique strong yet flexible microtubule cage which shapes the cell. This cage limits access to cells so much that molecular exchange is only possible at a spot at the base of the flagellum known as the flagellar pocket. This pocket not only allows for the transfer of cell materials but also is coated in VSG proteins which fool the immune system of the host.

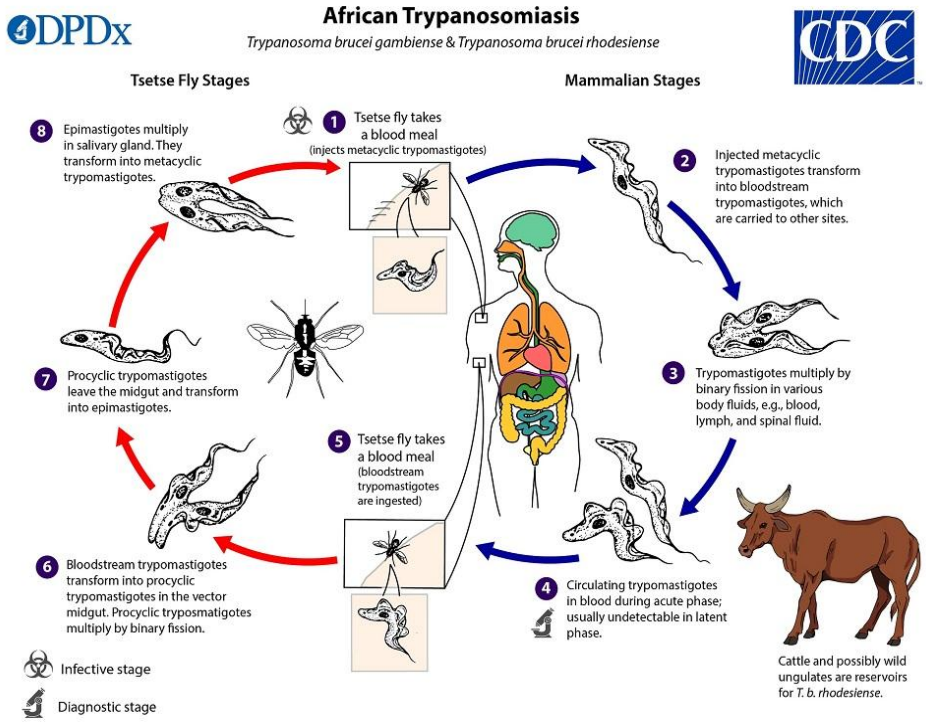


Figure 6: The full life cycle of the Tsetse fly and the *T.brucei* parasite (6)

Protein Arginine Methylation

Protein arginine methylation is a form of post transcriptional modification that regulates several cellular processes. In *T. brucei* there is little to no known transcriptional regulation, the majority of regulation is done post transcriptional. One way that this post transcriptional regulation is achieved is through protein arginine methylation. This process involves the transfer of methyl groups from AdoMet to arginine residues by a group of proteins known as protein arginine methyltransferases or PRMTs. There are a variety of different PRMTs which correspond to different steps in the post transcriptional process. Protein arginine methylation and the corresponding PMRTs are incredibly common in yeasts and humans. The process alters protein function generally speaking by altering protein-protein interactions. The additional methyl group added to the arginine does nothing to the overall charge, however the side chain shape of the residue is altered. This increases the residue's hydrophobicity and steric hindrance while also eliminating a potential hydrogen bonding donor group. As a result, protein arginine methylation alters arginine's ability to bind other proteins and nucleic acids. There is also evidence that protein arginine methylation can have an effect on the subcellular localization of the modified proteins (9).

As mentioned previously, protein arginine methylation plays a role in post transcriptional regulation in *T. brucei*. Phospholipid biosynthesis plays a huge role in the survival of *T. brucei* and allows the parasite to change its forms and morphology. *T. brucei* expresses a protein that is homologous to lipin proteins found in both humans and yeast. Due to their enzymatic activity, these proteins are also referred to as phosphatidate phosphatase or PAP. PAPs are involved with many important functions including membrane biogenesis, energy metabolism, and adipose

tissue development. In *T. brucei*, the lipin homologue is known as TbLpn. TbLpn is unique because it contains methylated arginine residues meaning protein arginine methylation is used by *T. brucei* for the regulation of TbLpn. Previous evidence have suggested that TbLpn is likely methylated by TbPRMT1 and TbPRMT7, two major trypanosome PRMTs.

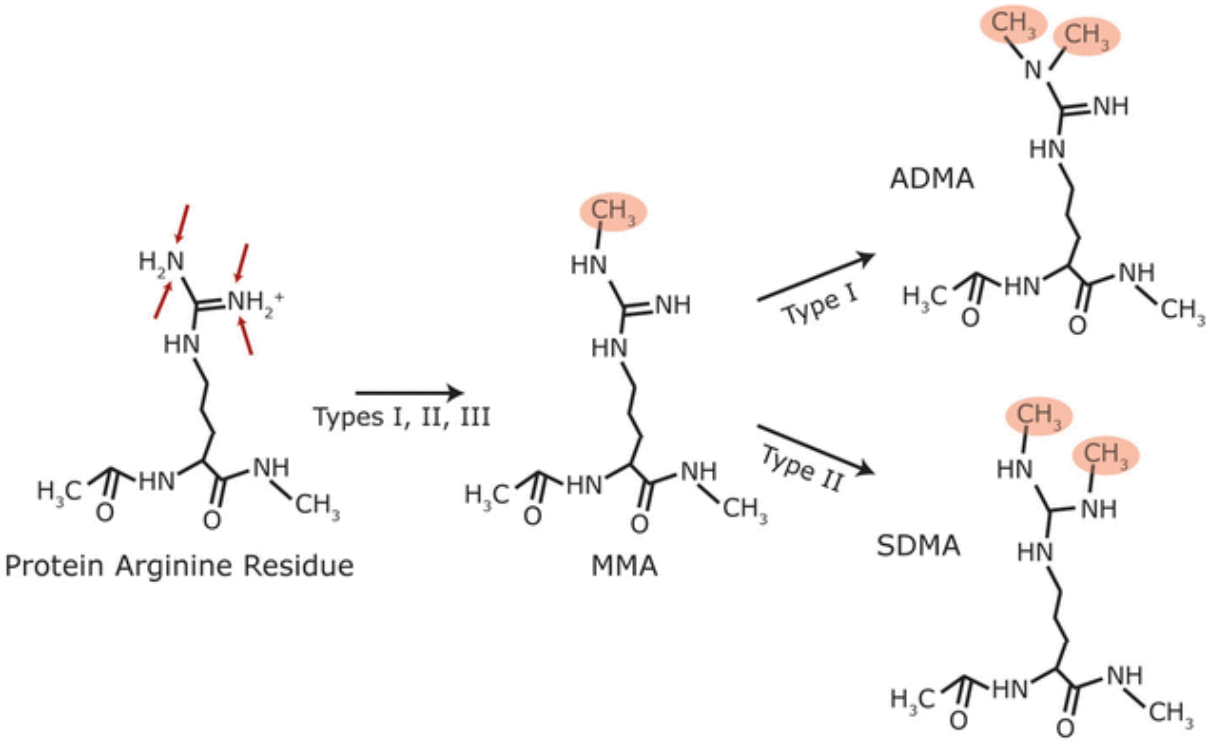


Figure 7: An example of the types of protein arginine methylation that can occur utilizing ADMA and SDMA(12)

Objective

The overall objective is to determine the role of protein arginine methylation of specific arginine residues in TbLpn expression and subcellular localization. This will be done using a technique known as site-directed mutagenesis. Specific arginine residues will be converted to lysine residues within a wild TbLpn protein in the plew plasmid. The results of these mutations will then be visualized utilizing gel electrophoresis and western hybridization. This will confirm that the intended mutations in TbLpn have occurred. These mutated TbLpn plasmids can then later be inserted into *T.brucei* and the effects can be observed.

II. Materials and Methods

Plasmid isolation

To begin plasmid isolation of the plew-TbLpn plasmid containing the wild type TbLpn, a 500-ml culture of *Escherichia coli* DH5α containing the plew-TbLpn plasmid were grown in LB broth containing 100 µg/ml ampicillin. Cells were spun at 4000 rpm for 10 minutes at 4°C. The pellet was then resuspended in 10 ml of solution 1 [50 mM glucose, 25 mM, Tris (pH 8.0), 10 mM EDTA], and transferred into 2 small centrifuge tubes. 5.6 ml of freshly made solution 2 (0.33 N NaOH, 1% SDS) was then added to each tube. The tubes were then both incubated on ice for 5 minutes. Next, 9.5 ml of solution 3 (5 M Potassium acetate/acetic acid) was added to each tube. The tubes were then incubated on ice for 10 minutes. The tubes were then centrifuged at 14,000 X g for 15 minutes at 4°C, and the supernatant transferred into a new tube. 9.5 ml of isopropanol was added to each tube which were then incubated at room temperature for 2 minutes. The tubes were centrifuge again at 14,000 X g for 2 minutes and the supernatant was again discarded. The pellets were air dried for 20 minutes. The pellets were resuspended in 6.25 ml of TE buffer (pH 7.6-8.0) and 12.5ul of RNAse. Next, the pellets were incubated at 37°C for 15 minutes. Following incubation, the pellets were again centrifuged at 14,000X g for 15 minutes and the supernatant was transferred to a new tube. The plasmid DNA was precipitated utilizing 6.6ml of 5M ammonium acetate and 16.7ml of isopropanol. The tubes were centrifuged at 14,000 X g for 20 minutes and the supernatant was discarded again. The pellets were next resuspended in 18.75 ml of 70% ethanol and then centrifuged again at 14,000 X g for 5min. The supernatant was likewise discarded. The pellets were then air dried and resuspended in 250 ul of TE.

Site-directed Mutagenesis

Mutated versions of TbLpn, in which arginine residues were changed to lysine residues were generated by PCR amplification from plew79-TbLpn using the QuikChange II XL™ Site-Directed Mutagenesis Kit (Agilent Technologies). The PCR reaction is consisted of 5 ul of 10X reaction buffer, 0.2 ul of plew79-TbLpn, 1.5 ul of 5' mutagenic primer (3 µM final concentration) 1.5 ul of 3' mutagenic primer (3 µM final concentration), 1 ul of dNTP mix, 3 ul of Quik Solution, 37.8 ul of deionized H_2O, and 1 ul of PfuUltra HF DNA polymerase (2.5 U/µl). For mutagenesis of Arg-32 to Lys-32, the following mutagenic primers were used: R32K- (5'-TCACCCTGATGGTAAGTTACATAGCACC-3') and R32K-3' 5-CGGTGCTATGTAACTTACCATCAGGGTG-3'). For mutagenesis of Arg-123 to Lys-123, the following mutagenic primers were used: R123K-5' (5'-CCGTAAAATCCCTAAAGCACCGATTATTTGGC -3') and R123K-3' (5'-GCCAAATAACTTGTGCCGTAGGGATTTTACGGG -3'). The samples were amplified using the following cycling: 95°C for 5 min, followed by 18 cycles of 95°C/50 s, 60°C/50 s, 68°C/8 min, ending with one cycle at 68°C for 7 min. Next, 1 ul of DpnI restriction enzyme was added to the samples and incubated at 37°C for 1 hour prior to the transformation technique.

In order to obtain maximum transformation efficiency, the DpnI-digested PCR (50 µl) was transferred to a 1.5-ml tube and concentrated as follow: 25 µl of 10M ammonium acetate was added and mixed well. One hundred and fifty µl of ice-cold 100% ethanol was then added, mixed well, and the mixture centrifuged at 13,000 RPM for 15 min at 4°C. The supernatant was removed, and the pellet resuspended with 500 µl of 70% ethanol. Following centrifugation at 13,000 RPM for 5 min at 4°C, the supernatant was removed, and the pellet dried in a warm

plate at 37°C (tubes should be opened) for 10-15 min. The dried pellet was finally resuspended with 5 µl of TE buffer.

Bacterial Transformation

Bacterial transformation was performed by first warming an NZY+ broth to 42°C. In addition, a 14 ml snap cap tube was pre chilled on ice. Two LB-ampicilin plates (100 µg/ml ampicillin) for each transformation were warmed up to room temperature. A vial of *E.coli* XL-gold cells were also thawed on ice. One hundred ul of the *E.coli* cells were transferred into the 14ml snap cap tube. Four ul of β-mercaptoethanol was also added to the snap-cap tube and was swirled gently. Then, 5 ul of concentrated PCR was pipetted into the tube and was mixed by stirring gently with the pipette tip. The tube was then incubated on ice for 30 minutes. Next, 900 ul of the NZY+ broth was added to the tube and the tube was incubated at 37°C for 1 hour at 225rpm. After incubating, 200 ul of the cells was spread on each LB plate, and the plates were incubated overnight at 37°C.

Isolation of plasmid using the Mini prep protocol

The next day, typically 6-10 colonies were used to inoculate 3 ml of LB broth containing 100 µg/ ml ampicillin, and the cultures grown overnight at 37°C at 225 rpm. The next morning, plasmids from these cultures were purified using the GeneJet Plasmid Miniprep kit (Thermo Scientific). First, the cultures were centrifuged at 13, 000 rpm for 30sec, and the supernatant discarded. The pellet was then resuspended in 250 ul of resuspension solution. The solution was transferred to a microcentrifuge micro centrifuge tube which was then vortexed to ensure complete resuspension. Two hundred and fifty ul of the lysis solution was then added and mixed

by inverting the tube 4-6 times. The solution became slightly clear and viscous. The tube was then incubated for 3 mins at room temperature. The solution cannot be incubated longer for 3 minutes, or the supercoiled plasmid DNA may begin to denature. After the lysis solution, 350 ul of neutralization solution was added and mixed thoroughly by again inverting the tube 4-6 times. The microcentrifuge tube was then centrifuged for 5 minutes at 13,000 rpm in order to pellet the cell debris and chromosomal DNA. The supernatant fluid was then transferred to a Genejet spin column utilizing a pipette. It is important to not transfer any of the present white precipitate, as that is the previously mentioned cell debris. The spin column was then centrifuged at 13, 000 rpm for 1 minute and the flow through was discarded. Five hundred ul of wash solution was then added to the spin column, which was centrifuged again for another minute at 13,000 rpm. The flow through was discarded and the same exact wash procedure was repeated. After repeating the wash, the flow through was discarded again and the spin column centrifuged at 13,000 rpm for an additional minute to ensure removal of all excess solution. This step ensured there was no residual ethanol present in the plasmid preps. The spin column was then transferred into a fresh 1.5 ml microcentrifuge5ml micro centrifuge tube. Fifty µl of elution buffer was added to the spin column which was then incubated at room temperature for 2 minutes. The column was then centrifuged at 13, 000 rpm for 2mins. After centrifuging, the column was discarded and the purified DNA was stored at -20°C.

Digestion

The prepared plasmids were digested and ran on gels in order to visualize the results. To digest the plasmids, a master mix was prepared. This mix consisted of Green Buffer (Fermentas) (1.5 X number of tubes), BamHI (0.5 X number of tubes), XhoI (0.5 X number of tubes), and

water (2.5X number of tubes). Five ul of the master mix was distributed into each plasmid meant to be run on the gel. The tubes were then incubated at 37°C for 30 minutes. The tube was then incubated on ice for 10 minutes, every 2 minutes the tube was again swirled. After the 30 minutes, the tube was then heat shocked in a water bath at 42°C for 30 seconds. After heat shocking, the tube was placed back on ice for 2 minutes.

Gel Electrophoresis

In order to determine whether the isolated plasmids contained the TbLpn gene, the digested plasmids were run onto an agarose gel for Agarose gel electrophoresis. This gel was composed of 0.7 g Agarose, 70 mL 1X TAE and 3.5 μL EtBr. The gel was microwaved for 30 seconds and left to solidify for 20 minutes. Five μL of blue marker was dispensed into well one and 15 μL of the digested plasmids were dispensed into the corresponding well. The gel was set to run at 125 V for 35 minutes.

III. Results

Several oligonucleotide primers were used in an attempt to mutate the wild type TbLpn gene present in the plew-TbLpn plasmid at specific arginine residues. Site-directed mutagenesis was performed by PCR for R123K and R32K. Following site-directed mutagenesis as outlined in the Methodology section, the plasmids thought to contain the R32K and R123K mutations were digested with the restriction enzymes BamHI and XhoI. The digested plasmids were then dispensed onto an agarose gel, and the size of the DNA fragments produced by digestion were determined by comparison with the GeneRuler 1 kb DNA Ladder. Both the R32K and R123K lanes show a band of the expected size (2, 429 base pairs). As shown on figure x, both lanes resulted in a positive presence of TbLpn.

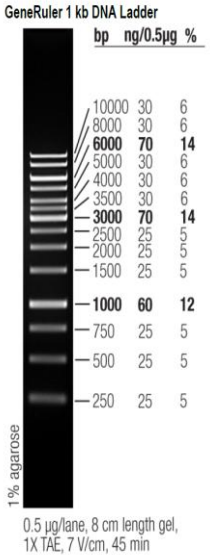
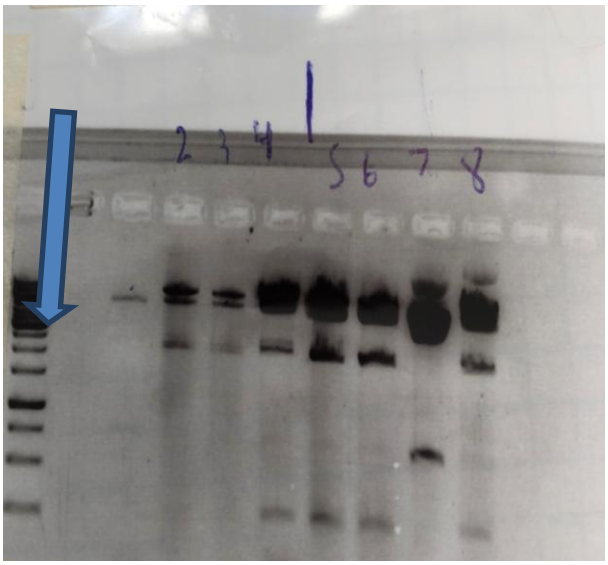


Figure 8: Gel run picturing desired band at 2500 base pairs, 1-4 being R123k and 5- 8 being R32k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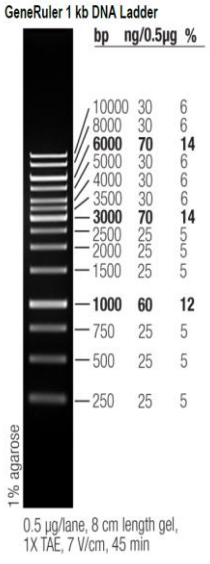
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4000 30 6
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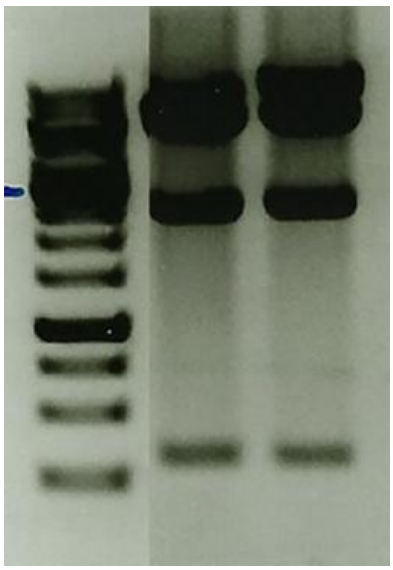

Figure 9: Gel run picturing desired band at 2500 base pairs for R123K and R32k. Zoomed in for better view.

IV. Discussion

The lipin protein found in *T.brucei* plays a vital role in phospholipid biosynthesis. This lipin is unique in that it is regulated post transcriptionally by protein arginine methylation which could have an effect on subcellular localization and protein interactions. The primary goal of this research is to determine the effects of specific arginine residues by converting them to lysine using site-directed mutagenesis. These mutated TbLpn can later be transformed into *T.brucei* cells which will allow for the observation of the effects. Several mutated TbLpn plasmids were created in this experiment containing the oligonucleotide primers R123K and R32K.

Disruption of the lipin protein in *T.brucei* can have drastic effects on the parasite. TbLpn specifically plays a role in dephosphorylation of phosphatidic phosphate (10). This is important as this step creates what is known as diacylglycerol or DAG (10). DAG is used in the synthesis of triacylglycerol, known as TAG, along with phosphatidylcholine (PE) and phosphatidylethanolamine (PC) These phospholipids are important and represent the majority of phospholipids present in *T.brucei* (10). Phospholipids are incredibly vital for cellular membranes and are specifically linked to the growth of *T.brucei into* its different forms (10). Disruption of TbLpn could lead to disruption of the cell's growth and its ability to infect its hosts (10). Phosphatidylcholine is also linked to the synthesis of glycosylphosphatidylinositol or GPI (10). The infamous VSG proteins on the coat of *T.brucei* which allow for antigenic variation are reliant on GPI. GPI is responsible for anchoring the VSG proteins to the cell membrane meaning without GPI *T.brucei* loses one of its most effective virulence factors (10).

The next step is to see what effect the TbLpn mutations have on subcellular localization and protein interaction. It is clear that a disruption in TbLpn could be catastrophic for *T.brucei* and provide a solution for the problems caused by it. Future research could provide further insight and vital information.

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