

Disruption of TbLpn Expression in *T. brucei* by RNA Interference

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## **Abstract:**

*Trypanosoma brucei*, the causative agent of human African trypanosomiasis, also known as African sleeping sickness, expresses a protein known as TbLpn which plays a critical role in arginine methylation and phospholipid synthesis within *T. brucei*. In order to determine the effect and potential medical benefits of downregulated TbLpn expression in *T. brucei*, RNA interference was used by transfecting *T. brucei* with plasmid DNA through electroporation. After conducting this experimental protocol, *T. brucei* was unable to be grown successfully in media following transfection and the effect of downregulated TbLpn in *T. brucei* requires further investigation in order to determine its potential for developing new drug targets and treatment methods for human African trypanosomiasis. Experimental protocol changes and repetition and verification of the validity of current protocols could lead the way for successful growth of *T. brucei* with downregulated TbLpn.

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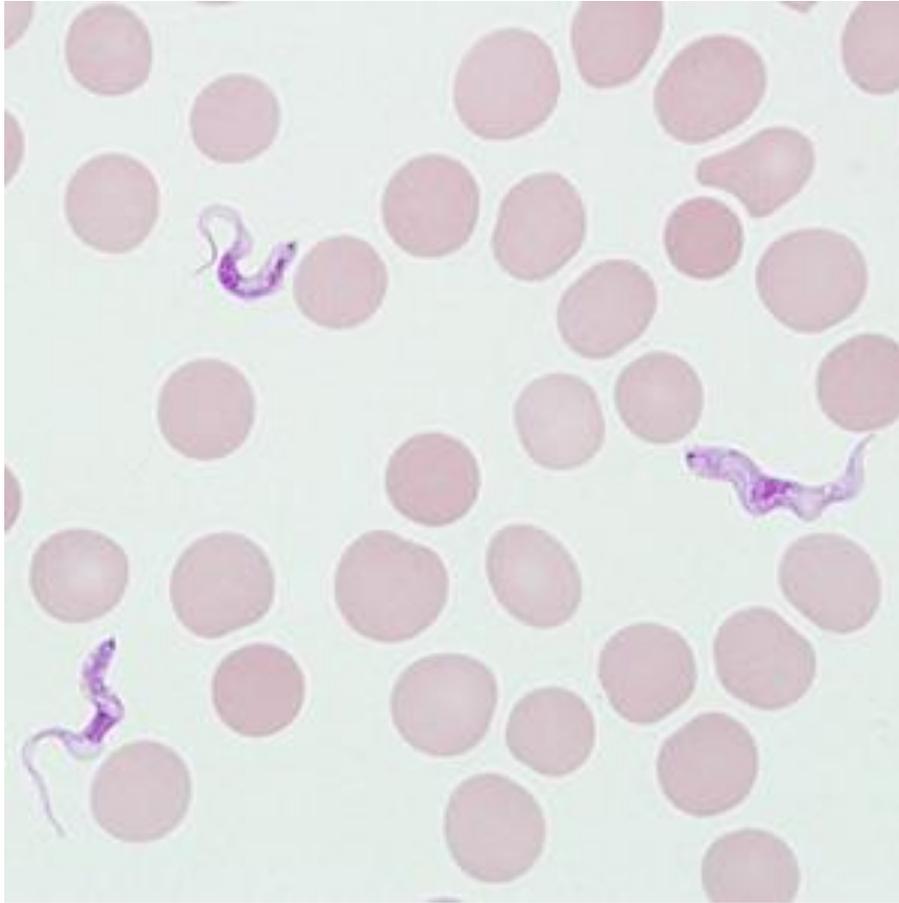
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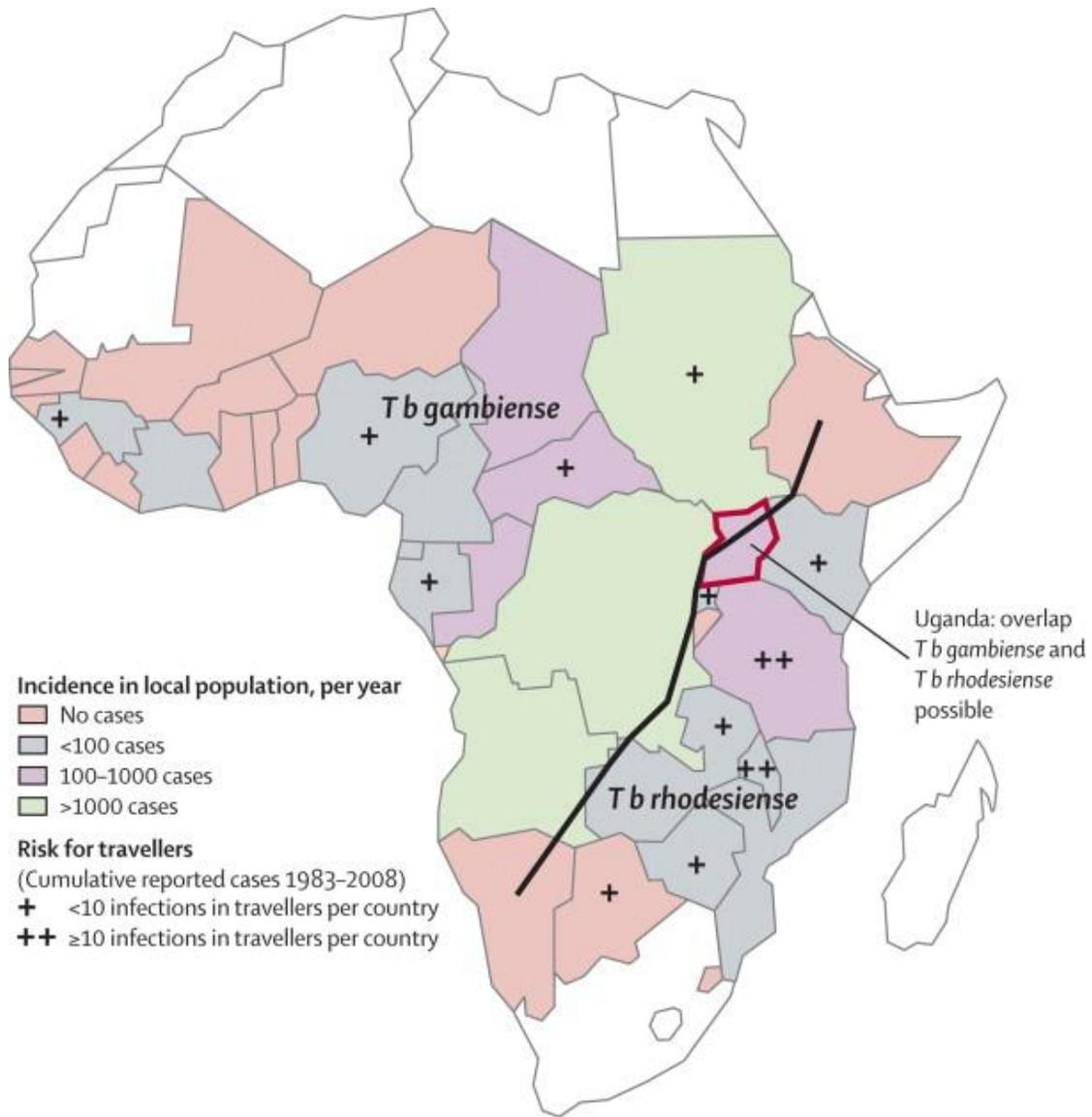
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## Introduction

Human African trypanosomiasis, also known as African sleeping sickness, is a disease caused by the parasite *Trypanosoma brucei*, a unicellular, flagellated, protozoan (Figure 1). Humans become infected with the disease upon being bit by a tsetse fly that is carrying the parasite. The disease is present mainly in regions of rural Africa and, due to the limitations of resources present in this region, it is very difficult to treat. There are two stages to the disease after infection by *T. brucei*. The first stage of the disease, the hemolymphatic stage, is the easiest to treat as it is only present throughout the blood and lymphatic system. The second stage of the disease, the meningoencephalitic stage, occurs after the disease crosses the blood-brain barrier and invades the central nervous system, cerebrospinal fluid, and the brain (Checchi & Barrett, 2008). Once the disease reaches this stage, the mortality rate is near 100% and drugs can only be administered to aid in the treatment of symptoms but are ineffective at killing the parasite and treating the disease (Checchi & Barrett, 2008). Very few drugs currently exist for the treatment of these symptoms and the drugs that do exist are toxic themselves and patients can often contract serious medical complications or die from the medications (Wenzler et al., 2009). Two subspecies of *T. brucei* are responsible for causing several variations of the disease, known as East African sleeping sickness and West African sleeping sickness, named after the region in which they are most prevalent (CDC, 2012). East African sleeping sickness is caused by *T. brucei rhodesiense* and progresses very quickly, causing death within several months if not treated. West African sleeping sickness progresses much slower, over the course of several years, and is caused by *T. brucei gambiense* (Figure 2) (CDC, 2012).



**Figure 1: *Trypanosoma brucei* in blood smear stained with Giemsa.** *T. brucei* is a unicellular parasite that causes Human African Trypanosomiasis which affects many individuals in sub-Saharan Africa. *T. brucei* ranges in length from 15-35  $\mu\text{m}$ . (Centers for Disease Control and Prevention).



**Figure 2: Geographical distribution of East and West African strains of *T. brucei*.** *T. brucei* has two forms. *T. b. gambiense* is the chronic form and is found in Western and Central Africa and *T. b. rhodesiense* is the acute form found in Southern Africa. These regions, which most of the HAT cases are reported within, is referred to as the “Fly belt. (Centers for Disease Control and Prevention).

Upon being bit by an infected tsetse fly, a tender sore and swelling occurs around the site of the bite. The symptoms of the first stage of human African trypanosomiasis resemble those of the flu and include fever, headache, muscle and joint aches, and swollen lymph nodes. Once the disease has progressed to the central nervous system, more serious symptoms begin to develop. These include an inverted sleep cycle with daytime sleepiness, personality changes, confusion, delusions and hallucinations, partial paralysis, and hormone imbalances (CDC, 2012). These symptoms become progressively worse as the parasite grows and reproduces until eventual and inevitable death occurs.

During the first stage of the disease, one of the drugs used for treatment is pentamidine, a medication that has been around since the 1940s. This drug possesses a wide variety of antiparasitic properties and is also used in the treatment of leishmaniasis and *Pneumocystis* infections (Wenzler et al., 2009). This drug is fairly toxic, and its side effects include burning pains, chest pains, difficulty breathing, difficulty swallowing, skin rashes, and wheezing (Mayo Clinic, 2017). For the late stage of the disease, there are only several drugs that are able to treat any of the symptoms associated with this stage. One drug, melarsoprol, is effective at curing the disease at a rate of 70%. However, about 5% of patients receiving this drug treatment die from encephalopathy, or brain damage that is induced by the drug (Checchi & Barrett, 2008). Another drug used for late stage treatment of the disease is eflornithine, which was developed in the 1990s. It is generally believed that this drug is slightly safer than melarsoprol which is why it is more commonly used for late stage disease treatment and distributed to areas in rural Africa by the World Health Organization (Checchi & Barrett, 2008). However, one of the issues that has been arising with this drug and its continued use in treated human African trypanosomiasis is

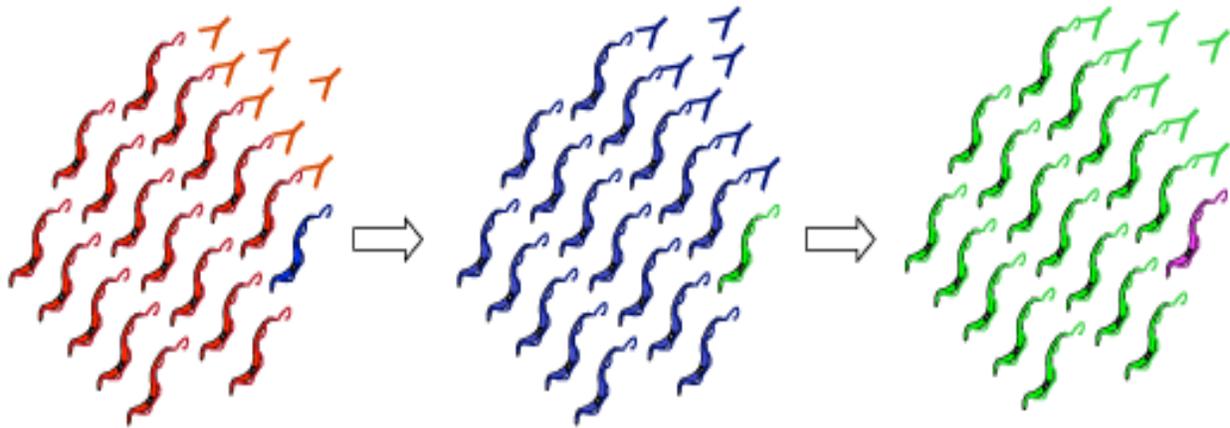
that several strains of *T. brucei* are becoming resistant to the drug which renders them ineffective (Checchi & Barrett, 2008).

Various organizations are taking action to try and stop the spread of African trypanosomiasis, including the World Health Organization. The WHO reports that in 2009 there was less than 10,000 cases of the disease in Africa and that in 2015 there was only 2,804 cases reported. These cases were mainly present in the Democratic Republic of Congo, at around 70% of all confirmed cases of the disease (WHO, 2018). The efforts of the WHO include sending skilled medical staff and drugs to regions of Africa where the disease is present in order to try to combat it. However, due to the limited medical and financial resources of the regions in which the disease is prevalent, the current methods of treatment are not always beneficial to the populations in these areas, as more medical complications can arise from treatment. This is why a push from the scientific community for research in this field is essential. American organizations are also supporting the research of human African trypanosomiasis and *T. brucei* in order to try to help eradicate the disease as well. The National Science Foundation, the National Institutes of Health, and the Centers for Disease Control and Prevention are all supporting research into the subject in order to try to have a potential cure for the disease or new drug target by the World Health Organization's proposed date of 2020.

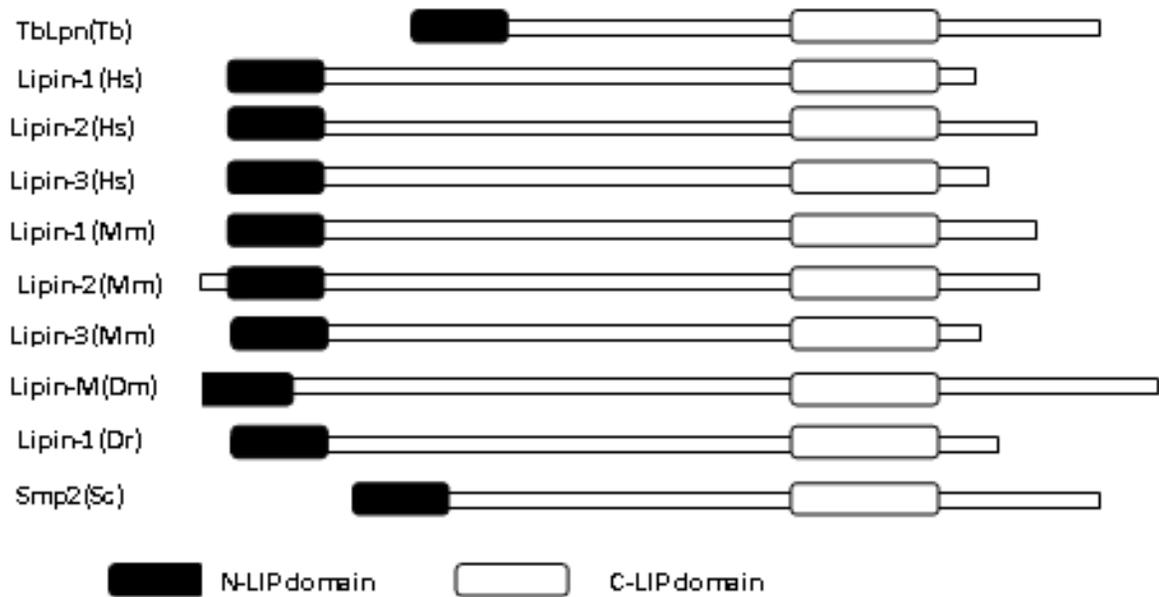
One of the reasons that this disease is so complicated and difficult to treat is because of the variant surface glycoproteins (VSG) on the surface of the parasite. Variant surface glycoproteins are “developmentally regulated genes that mediate immune evasion (Horn, 2014).” The VSG coat is about 15 nanometers thick and is essential for the virulence of the parasite.

There are an extremely large number of VSG genes in *T. brucei* (more than 1,500) however, only one can be expressed at a time (Horn, 2014). The main reason that *T. brucei* is able to evade immune responses within their host is because once the immune system develops a response that coordinates with the current VSG that is being expressed by the parasite, *T. brucei* switches from one VSG to a different VSG which renders all of the immune cells formed to combat the infection useless (Figure 3). This is why a vaccine is unable to be developed to irradiate African trypanosomiasis since all VSGs cannot be targeted through one vaccine and would be impossible to develop over a thousand vaccines to target every VSG that is expressed by *T. brucei*.

Another protein that has been studied and could possibly play a role in *T. brucei* survival, growth, and reproduction is one that is called TbLpn, which stands for *T. brucei* lipin homologue (Figure 4). In many organisms, lipin proteins carry out dephosphorylation of phosphatidic acid (PA), generating diacylglycerol (DAG), which is then used to synthesize phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Pelletier et al., 2013). PC and PE are the major phospholipids present in trypanosomes and are essential for both life cycle forms of *T. brucei* to grow (Pelletier et al., 2013). It has been shown that TbLpn has phosphatidate phosphatase activity, which suggests that TbLpn may play a role in phospholipid synthesis (Pelletier et al., 2013). TbLpn is a homologue of the lipin proteins found in other organisms. Lipin proteins are present in organisms across a wide evolutionary spectrum; these include protozoa, yeast, fish, and mammals (Pelletier et al., 2013). Members of the lipin protein family serve many necessary cellular functions, but their main functions involve biosynthesis of phospholipids and triacylglycerols, acting as a transcriptional cofactor in regulating lipid metabolism genes. Additionally, lipin homologues have been shown to play an important role in



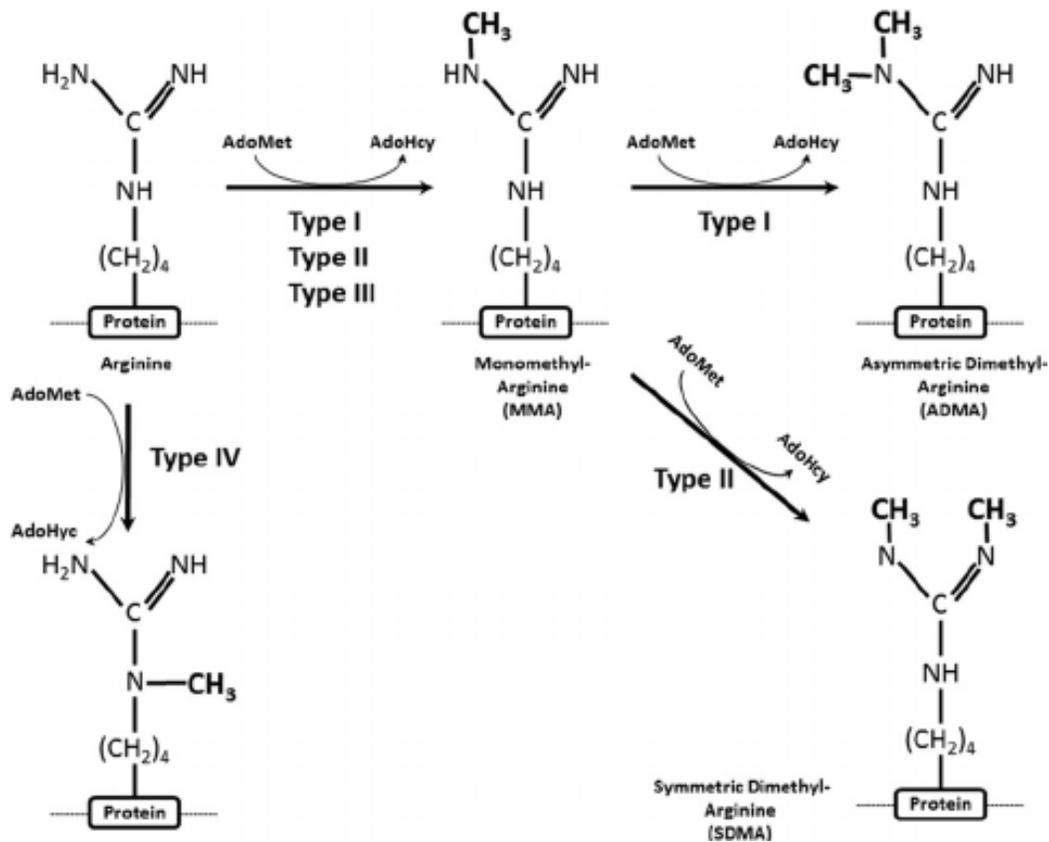
**Figure 3: Antigenic Variation in *T. brucei*.** VSGs can switch from one to another or express a clone-specific singular VSG. One switch occurs every 100,000 cells per generation. This mechanism of switching is called antigenic variation and allows the parasite to evade the human immune system and drug treatments.



**Figure 4. Schematic representation of TbLpn amino acid sequence aligned with members of the lipin family.** TbLpn [T. brucei (Tb), accession number AAX78871], Lipin-1 [Human (Hs), AAH30537], Lipin-2 [Human (Hs), AAI52449], Lipin-3 [Human (Hs), CAI42978], Lipin-1 [Mouse (Mm), NP\_766538], Lipin-2 [Mouse (Mm), AAH39698], Lipin-3 [Mouse (Mm), EDL06298], Lipin-M [Drosophila melanogaster (Dm), NP\_001188884], Lipin-1 [Danio rerio (Dr), AAX19945], Smp2 [Saccharomyces cerevisiae (Sc), BAA00880] [62]

nuclear membrane biogenesis in yeast (Pelletier et al., 2013). TbLpn was shown to have two conserved domains that play a role in “adipocyte development and phospholipid biosynthesis in mammalian and yeast cells (Pelletier *et al.*, 2013). Additionally, Pelletier et al. found that TbLpn was present in the cytosol and the nucleus of *T. brucei*, along with greatly modified versions of TbLpn due to “post-transcriptional modifications.” The study by Pelletier *et al.* further concluded that, as a result of their findings, TbLpn could be responsible for phospholipid synthesis in *T. brucei* and that TbLpn is the “only lipin or phosphatidic acid phosphatase to exhibit post-transcriptional modifications that include methylated arginine residues *in vivo* (Pelletier et al., 2013).”

Arginine methylation is responsible for various cellular processes including “signal transduction, RNA transport and processing, protein localization, and transcription (Pelletier et al., 2013).” Protein Arginine Methyltransferases (PRMTs) are enzymes that transfer methyl groups from AdoMet to the arginine residues in proteins. There are four different types of PRMTs, each classified by the way the terminal arginine is modified (Figure 5). The first step in arginine methylation is to transfer a single methyl group from AdoMet to the terminal nitrogen group on the arginine, creating monomethylarginine (MMA). This step can be completed by types I-III PRMTs, however, type III can only catalyze MMA whereas types I and II can also catalyze further reactions. Type I PRMTs transfer another methyl group to the same terminal nitrogen creating an asymmetric di-methylarginine (ADMA). Type II PRMTs catalyze the transfer of a methyl group to the terminal nitrogen group located next to the original terminal nitrogen group creating a symmetric di-methylarginine (SDMA). The type IV PRMTs transfer a methyl group to the internal nitrogen of the arginine.



**Figure 5: The four types of PRMTs.** Arginine methylation is a post-translational modification that transfers methyl groups to the arginine residue and helps to regulate protein function. There are four types PRMTs, enzymes that transfer the methyl groups. The four different types of PRMTs are each classified by the way the terminal arginine is modified. Type I-III PRMTs transfer a single methyl group to the terminal nitrogen group on the arginine residue creating monomethylarginine (MMA). Type III can only catalyze this reaction.

In *T. brucei* there are five PRMT enzymes suggesting that arginine methylation has distinct contributions during the life cycle of the parasite. The observation that TbLpn has methylated arginine residues suggests that it could affect its enzymatic activity or interactions with other proteins.

In order to try to down regulate the expression of the TbLpn protein in *T. brucei*, RNA interference is one technique that can be implemented to accomplish this. RNAi is “the mechanism through which gene-specific, double-stranded RNA triggers degradation of homologous transcripts (Ullu et al., 2004). RNAi is a natural cellular mechanism that can be induced in the laboratory environment for experimental use. RNAi functions by targeting cellular mRNA which is then degraded by short-interfering RNA and double-stranded RNA. Double-stranded RNA is cut into smaller fragments by a dicer enzyme which creates short-interfering RNA fragments. These siRNA fragments bind to special proteins and remove one of the double-stranded RNA (Ullu et al., 2004). These siRNA can be engineered to match virtually any gene across a variety of organisms. The remaining strand of RNA then binds to mRNA according to standard Watson-Crick base pairing. After this point, the mRNA can either be destroyed or marked for further regulation (Ullu et al., 2004). Therefore, RNAi is an ideal way to try and affect the expression of TbLpn in *T. brucei* since it can be performed experimentally, while also being a natural cell mechanism, and it can be targeted to highly specific genetic regions for a specific purpose instead of having a widespread affect across the cell’s genetic material.

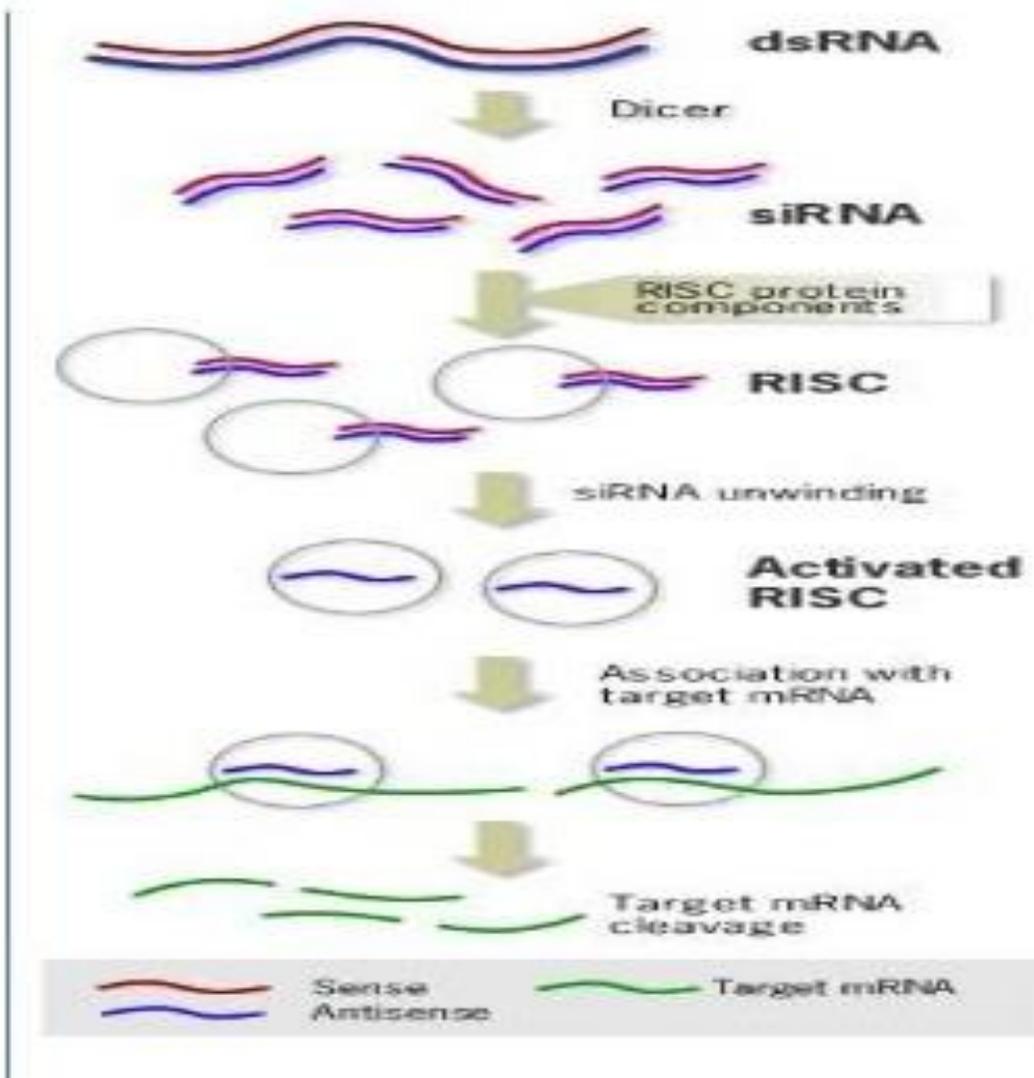


Figure 6. Down regulation of gene expression by the RNAi machinery.

## Materials & Methods

In order to perform a transfection of *T. brucei* PF 29-13 cells, a very strict protocol must be followed in which several experimental techniques are implemented. Each technique must be done correctly, otherwise, the subsequent steps will not be able to be conducted successfully and the transfection will be ineffective. These procedures include amplification of the 3' untranslated region (UTR) of TbLpn which is then cloned into the p2T7-177 plasmid in order to be transformed into *Escherichia coli* cells, selecting for the cells that have taken up the plasmid with ampicillin and then growing them in large quantities, transfecting *T. brucei* PF 29-13 cells with the plasmid using an electroporation machine, and then selecting for the transfected cells using phleomycin. This extremely sensitive set of protocols was performed various times in order to try to successfully grow *T. brucei* with the modified DNA in media and sustain them as they grow and reproduce.

**Cell culture.** First, *T. brucei* PF 29-13 cells were grown in SM media in order to obtain a concentration of around  $4.6 \times 10^6$  cells/ml. This concentration of *T. brucei* is required in order to perform a successful transfection since around 90% of the cells die following the electroporation step. The SM medium that was used to grow *T. brucei* contained 7.4 g of SM powder, 150 mL of water, and 625  $\mu$ l of MEM-vitamins. The pH of the medium was then adjusted by adding NaOH until the pH increased from around 4.5 to 7.5. Following this step, water was added to the medium mixture in order to obtain a final volume of 225 ml. The medium was then sterilized through a filter with 0.22  $\mu$ m pore size. Following filtration, 25 mL of heat-inactivated fetal bovine serum and 2.5 mL of 100x penicillin/streptomycin mixture was added to the medium,

also in a sterile environment, and the medium was then distributed to several tubes after the addition of a starter culture of *T. brucei* PF 29-13. The cells were then placed in a rotating incubator at 27°C and allowed to grow until the desired concentration of cells was reached, while changing the medium every several days with fresh SM media in order to ensure that the increasing acidity of the medium as the cells grow and reproduce does not kill them. The increasing acidity can be detected in the medium by the color change from a deep, dark red to a more brown/dark yellow color.

**Plasmid preparation.** In preparation for the transfection, the 3' UTR of TbLpn was amplified in order for use in later experimental procedures. The 3' UTR of TbLpn was used to target TbLpn because it allows for RNAi-directed degradation of TbLpn mRNA and subsequent complementation of these TbLpn-RNAi cells with plasmids encoding mutated versions of TbLpn without the mutated mRNAs being targeted by the RNAi machinery. TbLpn 3'UTR was amplified by 40 cycles of PCR from procyclic form *T. brucei* genomic DNA using primers TbLpn-3UTR-5' (5'-CGGGATCCTGGCGTGGGTGTGGTGATT-3') and TbLpn-3UTR-3' (5'-CCCAAGCTTGGGGAGTTGTGAGGAAAAGGTGG-3'), which allowed introduction of *Bam*HI and *Hind*III restriction sites respectively (underlined). The PCR product was then cloned into the *Bam*HI and *Hind*III sites of the p2T7-177 plasmid for later use in the transfection of *T. brucei*. Chemically competent *E. coli* DH5α cells were transformed using published methods. In order to select for *E. coli* cells that have taken up the p2T7-177 plasmid, LB plates containing ampicillin were used, since this particular plasmid contains an ampicillin resistance gene. Therefore, only cells that have taken up the plasmid DNA will survive in the presence of ampicillin.

Next, the plasmid was purified in order to be used during the transfection of *T. brucei*. In order to do this, one liter of *E. coli* DH5 $\alpha$  cells containing the p2T7-177-3'UTR plasmid grown overnight at 37°C in the presence of 100  $\mu$ g/ml ampicillin. The next morning, the cells were spun at 4,000 RPM for 10 minutes at 4°C in an accuSpin 3R centrifuge (Fisher Scientific). The supernatant was then discarded, and the cell pellet resuspended in 10 ml of GTE buffer (50 mM of glucose, 25 mM of Tris [pH 8.0], and 10 mM of EDTA). Following gentle mixing, 11.2 ml of lysis solution (0.33 M NaOH, 1% SDS) was added to the mixture and the solution was incubated on ice for 5 minutes. Then, 19 ml of neutralization solution (3 M potassium acetate, 2 M acetic acid) was added to the mixture, and the solution was once again incubated on ice for 10 minutes. The mixture was then centrifuged at 14,000 X g for 15 minutes at 4°C. The supernatant was transferred to a clean tube, and 19 ml of isopropanol was added, and the mixture was once again centrifuged at 14,000 X g for 2 minutes. The supernatant was discarded and the pellet remaining was allowed to air dry for 20 minutes. The pellet was then resuspended in 12.5 ml of TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA), and RNase A was added at a concentration of 0.1 mg/ml. The solution was incubated at 37°C for 15 minutes, centrifuged again at 14,000 X g for 15 minutes, the supernatant transferred to a new tube, and the plasmid DNA was precipitated with 3.3 ml of 10 M ammonium acetate and 16.7 ml of isopropanol. Once again, the mixture was centrifuged at 14,000 X g for 20 minutes, the supernatant discarded, and the pellet remaining was resuspended in 18.75 ml of 70% ethanol. This solution was finally centrifuged one more time at 14,000 X g for 5 minutes, the supernatant discarded, and the pellet was allowed to air dry before it was resuspended in 250  $\mu$ l of TE buffer. The resulting 250  $\mu$ l solution was the purified P2T7-177-3'UTR plasmid.

***T. brucei* transfection.** Once the cells are ready to be transfected using the purified p2T7-177-3'UTR plasmid, the plasmid was digested with the restriction enzyme *NotI*, which recognizes a specific guanine-cytosine sequence for DNA digestion. This linearizes the plasmid DNA in preparation for transfection. In order to perform the digestion, 200 µl of the purified plasmid (approximately 200 µg) was mixed with 25 µl of 10x green digestion buffer (Fermentas) along with 5 µl of *NotI* restriction enzyme (Fermentas) and 20 µl of deionized or ultraviolet-light sterilized water. Digestion was allowed to proceed for 60 minutes at 37°C. In order to ensure that the digestion was done properly, a 1% agarose gel containing 0.5 µg/ml ethidium bromide was prepared and allowed to solidify. Then, 5 µl of 1 kb DNA ladder along with a 10 µl solution containing 2 µl of undigested p2T7-177-3'UTR plasmid, 1 µl of 10x green digestion buffer, and 7 µl of water was added to two of the wells of the solidified agarose gel. In addition, 10 µl of the digested plasmid was added to a third well in the gel and the gel was run in TAE buffer (40 mM Tris [pH 8.0], 20 mM acetic acid, and 1 mM EDTA) in an electrophoresis device at 125 volts for 40 min.

After confirmation that the plasmid was successfully digested, 50 µg of the digested plasmid was used to transfect  $4 \times 10^7$  PF *T. brucei* 29-13 cells. In order to perform the transfection, a Neon® transfection system from ThermoFisher Scientific was used along with the accompanying Neon® transfection buffers and solutions. First, 100 ml of exponential growth *T. brucei* culture in SM medium was centrifuged at 1,300 X g for 10 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 25 ml of sterile PBS buffer without calcium or magnesium. This mixture was then centrifuged again at 1,300 X g for 10 minutes at room temperature. Following centrifugation, the supernatant was once again

discarded, and the cell pellet was resuspended in Resuspension buffer R (ThermoFisher Scientific) to give the mixture a cellular concentration of  $4 \times 10^8$  cells/ml. The Neon® electroporation tube was then filled with 3 mL of Electrolyte buffer E2 (ThermoFisher Scientific), and the tube was inserted into the electroporation machine. Then, 50 µg of NotI-linearized plasmid was transferred into a sterile 1.5-ml Eppendorf tube. One hundred µl of the *T. brucei* PF 29-13 cells were also added to the Eppendorf tube and the solution was gently mixed. The Neon® pipette was then used to extract a pre-measured volume of the cell/DNA mixture and the pipette was inserted into the electroporation tube, being cautious to avoid obtaining air bubbles in the pipette tip while performing the extraction. These cells were then electroporated to incorporate the plasmid DNA into them by exposing them to 3 electrical pulses of 10 milliseconds each at 1,325 volts in the Neon® electroporation system. Following this step, the pipette was removed, and the mixture was transferred to 10 ml of fresh SM medium which also contained the antibiotics G418 at a concentration of 15 µg/ml and Hygromycin B at a concentration of 50 µg/ml. The extraction of the cell/DNA mixture and electroporation was repeated 10 times, placing each set of electroporated cells into a separate tube. The tubes were then incubated at 27°C overnight in the presence of 5% CO<sub>2</sub>. The next day, phleomycin was added to each tube at a concentration of 2.5 µg/ml. The phleomycin works as a selective antibiotic that is active against many cells by disrupting the DNA double-helix. Cells resistant to phleomycin have special proteins that are able to bind phleomycin and prevent it from destroying the DNA. The *T. brucei* PF 29-13 cells have a resistance to phleomycin upon successful electroporation which is why it is used as a selective antibiotic.

Following the electroporation protocol, the cells are maintained in a 27°C, 5% CO<sub>2</sub> environment, while taking periodic analyses of the cells to determine their growth. After electroporation, it takes several weeks for cells that have not died to recover from the intense electrical current. Succeeding this time period, growth was determined by analyzing a sample from each tube under a microscope, along with observing color changes in the SM media resulting from acid production from *T. brucei* as they continue to grow and reproduce. In tubes that had growth, the SM media was changed with fresh media in order to ensure that the increasing acidity of the media did not kill the cells. The cells that should have been in the media at this point, in theory, would be *T. brucei* that had successfully taken up the plasmid DNA following electroporation. This protocol was repeated several times in order to try to obtain the intended outcome.

## Results

Unfortunately, *T. brucei* cells were unable to be grown in significant quantities following electroporation. There are many things that can go awry when trying to maintain the *T. brucei* cells in the post-transfection stage. Assuming that the cells did not die from electroporation, several complications occurred throughout the various trials conducted of *T. brucei* transfection. In one trial of transfections, the media was contaminated with yeast, most likely *Candida albicans*, and the trypanosomes were unable to be grown in media successfully. In another trial, there was an issue with the carbon dioxide incubator and the 5% CO<sub>2</sub> environment was unable to be maintained and the cells died before the issue was detected. In other trials, growth was simply not detected following transfection, indicating that the cells most likely did not survive electroporation. No matter how many times the protocol was repeated, an experimental issue arose and *T. brucei* cells were unable to be grown containing any modified DNA that could potentially downregulate the expression of TbLpn.

The difficulty of growing *T. brucei* following these experimental protocols was clearly indicated by the lack of results and inability to grow *T. brucei* in media successfully. Upon several protocol modifications and repeat of current experimental protocols, the intended results may be able to be produced and then its subsequent implications analyzed.

## Discussion

In order to try to obtain successful results after transfection, various changes could be made to increase the probability of a successful outcome. One thing that needs to be reviewed is the sterile environment that the experimental protocol was conducted in. During one trial, the media was contaminated with yeast, which indicates that the experimental environment was not sterile. In order to try to fix this issue in the future, the ultraviolet light that was used for sterilization of the environment could be left on longer in order to ensure that all microorganisms are killed prior to the preparation of the SM media. Also, the negative pressure hood that was used as an experimental workplace should be checked to ensure it was used properly and that there are no mechanical issues with it. Additionally, the antibiotics used throughout the various experimental protocols should be checked to ensure their effectiveness and their integrity. Other antibiotics and selective antibiotics could also be reviewed to use instead of, or in addition to, the antibiotics already used in the experimental protocols. The selective antibiotic used in this experiment, phleomycin, functions once a plasmid is incorporated into *T. brucei* through electroporation (Jefferies et al., 1993). The antibiotic resistant genetic information is “incorporated into the tubulin locus by homologous recombination and originates at the upstream tubulin promoter (Jefferies et al., 1993). This was done prior to our experimentation, as the *T. brucei* PF 29-13 already had this antibiotic resistance incorporated into its genetic material. Use of this selective antibiotic helps ensure that the media does not get contaminated and *T. brucei* would be able to grow without any hindrance from other microorganisms. Other selective antibiotics might also aid in the selection for *T. brucei* that has taken up plasmid DNA. These antibiotics should also be resistant by *T. brucei*, however, the resistant information should be

targeted to a genetic location other than the tubulin locus in order to determine if they are more useful than phleomycin or can be used in combination with phleomycin to allow for better growth of *T. brucei* following the transfection protocol.

Due to the previous studies conducted on TbLpn and its subsequent role in arginine methylation and phospholipid synthesis, it can be assumed that TbLpn plays some type of essential role in *T. brucei*. Being able to affect its production *in vivo* could have significant medical implications and lead to the replacement of drugs used to treat human African trypanosomiasis and possible new treatment methods that work on specifically targeting *T. brucei*, either in the blood stream or lymphatic system or cerebrospinal fluid, instead of broad spectrum anti-parasitic techniques. More specialized forms of treatment are crucial for dealing with *T. brucei* infection due to some of the unique properties it possess, along with other forms of trypanosomes. Since there is currently an extensive amount of research being conducted on *T. brucei*, the World Health Organization is predicting that new drug targets and potential infection treatments will be discovered within the next 10 years. This is crucial due to the increasing resistance *T. brucei* has developed to current drugs in use for human African trypanosomiasis treatment. The drug, melarsoprol, which is used for late stage disease treatment, is taken up by *T. brucei* by the P2 aminopurine transporter. The gene *TbAT1* has been shown to play a central role in loss of P2 transporter function, causing subsequent *T. brucei* resistance to melarsoprol (Matovu et al., 2003). Therefore, this gene, along with several others, should be considered when determining potential drug targets so that resistance can be avoided in newly developed drugs for disease treatment. Thus, that is why TbLpn knockdown using RNAi is a promising area of

investigation since it could potentially lead to influence of arginine methylation and phospholipid synthesis in *T. brucei* which could alter the virulence of the parasite.

Finally, the protocol needs to be repeated over again, as many times as required, until it can be followed all the way through without any experimental or environmental errors. Growing *T. brucei* following electroporation is a tedious and sensitive process. The process can be disrupted by the slightest miscalculation of media ingredient concentrations, temperature changes, or environmental gases present. Only repeating this protocol error-free can ensure that nothing hinders the growth of *T. brucei*. Once able to grow *T. brucei* in significant quantities following electroporation, the effects of downregulated TbLpn can be fully studied and its implications analyzed. This could lead the way for potential drug targets to combat human infection of *T. brucei* and subsequent contraction of African sleeping sickness.

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